Eya: a dual function nuclear factor crucial for regulation of developmental gene expression and prevention of apoptosis in response to genotoxic stress

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Publication Date
2009

Peer reviewed|Thesis/dissertation
Eya: A Dual Function Nuclear Factor Crucial for Regulation of Developmental Gene Expression and Prevention of Apoptosis in Response to Genotoxic Stress

A dissertation submitted in partial satisfaction for the requirements for the degree of Doctor of Philosophy

in

Biology

by

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2009
DEDICATION

In recognition of their constant support and unconditional love, this thesis is dedicated to my grandparents, Claude and Patricia Munsell. Their lives have been an inspiration to me and I can only hope that my work in the past and future will continue to inspire their pride.
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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Michael G. Rosenfeld for his support and enthusiasm over the past six years. Without his guidance and encouragement this thesis would not have been possible.

I would additionally like to acknowledge my collaborators within the Rosenfeld lab, Bong-Gun Ju, Francesca Telese, and Tina Wang. Their work helped to bring this thesis project to a successful conclusion. In particular, I would like to thank Bong-Gun Ju, who assisted with key biochemistry experiments in our study of Eya in the DNA damage response. Working with Bong-Gun not only allowed the project to proceed but has made me a significantly more proficient biochemist.

I would finally like to thank Jessica Tollkuhn, Loren Olson, and Gratien Prefontaine, my personal friends from the Ronsenfeld lab. Their friendship and support, particularly in my first few years in the lab, brought crucial levity and perspective to my time as a graduate student.

A revised form of Chapter 3.2 of this thesis has been submitted for publication in the journal Nature under the title “Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions”. The dissertation author was the primary investigator and author of this paper.
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BOOK CHAPTERS


ABSTRACT PRESENTATIONS


UCSD All-Grad Research Symposium 2008.  *Cook P.J.* and Rosenfeld, M.G.  Regulation of FoxG1 Expression by Wnt Signaling in Neural Progenitor Cells.  Oral Presentation

Eya: A Dual Function Nuclear Factor Crucial for Regulation of Developmental Gene Expression and Prevention of Apoptosis in Response to Genotoxic Stress

by

Peter Joseph Cook

Doctor of Philosophy in Biological Science
University of California, San Diego, 2009

Professor Michael G. Rosenfeld, Chair
Professor Cornelis Murre, Co-chair

The developmentally regulated nuclear factors of the Eya family function in transcriptional regulation of developmental genes and have been recently characterized as tyrosine phosphatases. Eya is a component of the retinal determination gene network, which is crucial for the development of multiple organ systems in mammals, with loss of Eya leading to increased apoptosis and organ agenesis. While phosphatase activity is important for proper organ development, lack of confirmed substrates for Eya phosphatase activity has prevented a deeper understanding of Eya’s function. We sought to identify phosphatase substrates for Eya using multiple strategies including biochemical purification, transcriptional assays, and candidate testing. Contrary to initial
assumptions, the phosphatase activity of Eya was dispensable for transcriptional activation of the Eya target gene Sall1 and transcriptional activation was wholly mediated by the N-terminal transactivation domain. We found that the phosphatase activity of Eya functions in a non-transcriptional context, by preventing apoptotic death under conditions of genotoxic stress. Under DNA damage conditions, Eya relocates within the nucleus to sites of DNA double strand breaks, where it interacts with the histone variant H2AX. H2AX is phosphorylated under basal conditions on tyrosine 142, a mark that is directly removed by Eya as part of the DNA damage response. H2AX is known to become phosphorylated on serine 139 early in the DNA damage response, and this phosphoserine mark serves as a binding surface for various DNA repair proteins, including MDC1. Persistent phosphorylation of tyrosine 142 in the absence of Eya leads to increased cellular apoptosis in response to genotoxic stress due to differential binding of apoptotic versus repair complexes to the phosphorylated tail of H2AX. Thus, in the context of the DNA damage response, Eya possesses a novel anti-apoptotic function that is dependent on phosphatase activity. We propose that in-vivo Eya exists as a dual-function nuclear factor, activating a transcriptional program as a component of the retinal determination network in basal conditions and dephosphorylating H2AX on tyrosine 142 as a component of the DNA damage response under conditions of genotoxic stress.
Chapter 1

Eya and the Retinal Determination Gene Network
At the intersection of the emerging fields of genomics and stem cell biology lies the study of developmentally regulated changes in genome-wide transcriptional programs. How does a cell manage to select the gene transcription program that corresponds to the proper differentiation pathway for that cell’s specific temporal and spatial criteria? How does a specific genetic program direct the functional and morphological cellular changes that accompany differentiation? These questions touch the core biological mechanisms of how multicellular organisms are formed and how they function, and have profound implications for human health.

The events of embryogenesis provide insight to the multitude of regulatory strategies by which metazoans control transcriptional and cellular processes. Changes in transcriptional repertoire have to be carefully controlled by developmentally regulated transcription factors, many of which have been identified as key mediators of cell differentiation and lineage commitment as well as maintenance of the stem cell state. Increasingly, developmentally regulated transcription factors are being grouped into families based on function toward a specific cell or tissue fate. For example, basic helix-loop-helix factors of the MyoD family direct muscle cells differentiation [1] while Neurogenin factors drive neuronal differentiation [2]. Homeodomain factors of the Six family are key players in multiple organ systems and play a major role in specification of the eye in *Drosophila* and mammals. The so-called retinal determination (RD) pathway includes Six in conjunction with a key co-factor, Eya and is crucial for the development of several mammalian organ systems, including the kidney, as demonstrated by targeted homozygous deletion of the mouse genes Six1
and Eya1 [3, 4]. Significantly, the human homologues of these genes, SIX1 and EYA1 have been implicated the genetic disease BOR syndrome, characterized by deficiencies in kidney and ear development. Eya functions as a transcriptional co-activator, binding to Six factors that target specific promoters based on the DNA-binding homeodomain. Eya has been demonstrated to be crucial for activation of Six target genes based on an N-terminal transactivation domain. Thus, this co-factor plays an important role in positively regulating a transcriptional program that is crucial for proper development that has been evolutionarily conserved from Drosophila to humans. However, the function of Eya has turned out to be somewhat more complicated than its transcriptional role initially suggested. In 2003, several labs independently identified a functional phosphatase enzymatic domain within the C-terminus of all known Eya homologues [3, 5, 6]. Eya was shown to be a functional phosphatase enzyme in-vitro with specificity for phosphotyrosine residues. Experiments in Drosophila showed that, while transgenic expression of a wild-type Eya construct in an Eya mutant background would rescue the eye development phenotype associated with Eya loss of function, an enzymatically-dead mutant Eya transgene failed to rescue [6]. This indicated that phosphatase activity was crucial for Eya’s function in development, at least in the context of the Drosophila eye. However, a deeper understanding of the mechanism by which the enzymatic domain of Eya functions to promote proper organogenesis awaited identification of bona-fide in-vivo substrates for phosphatase activity.

In this chapter we summarize the biological role of the retinal determination gene network in general and Eya in particular in the context of Drosophila and
vertebrate development. Additionally, we will describe the functional domains of Eya and the characterization of Eya phosphatase activity.

1.1 Biological Functions of Eya: *Drosophila*

The *Drosophila* compound eye is morphologically highly distinct from its mammalian eye counterparts, yet it shares with them a surprising degree of conservation in terms of the genes controlling its early development. The compound eye is composed of roughly 750 to 800 repeated units known as ommatidia [7]. Each ommatidium is an independent optic unit consisting of eight neuronal photoreceptor cells and a set of non-neuronal accessory cells including lens-secreting cone cells, pigment cells, and interommatidial bristles. The eye first forms in the developing fly embryo during the third larval instar from a region dubbed the eye imaginal disc. The eye imaginal disc is comprised of epithelial eye progenitor cells which remain undifferentiated until the third instar, at which point a depression forms along the posterior edge of the eye disc. This depression, known as the morphogenic furrow (MF), sweeps along the eye disc from the posterior to the anterior end, leaving in its wake clusters of differentiating cells which go on to form individual ommatidia. The *decapentaplegic* (dpp) and *hedgehog* (Hh) signaling pathways are known to be the key secreted signals required for MF initiation and progression. Hh is initially expressed at the posterior margin of the eye disc, where it functions in MF initiation. As differentiations progresses, Hh is secreted by the newly formed neurons posterior to the MF, helping to push the furrow toward the anterior end. Dpp signaling is required
for transcriptional activation of multiple eye-specification genes within the eye disc prior to initiation of the MF. However, dpp and hh are also involved in the development of multiple fly organs besides the eye, and thus other factors must confer eye specificity to the cells of the eye imaginal disc. Mutagenesis screens identified a set of genes, the loss of which led to failure of eye formation and the misexpression of which induced the formation of ectopic eye tissue. These genes were eventually grouped into the retinal determination (RD) network which currently has seven identified members, eyeless (ey), twin of eyeless (toy), sine oculis (so), eyes absent (eya), dachshund (dac), eye gone (eyg), and optix [8, 9].

The RD network gene first proposed to be a master regulator of eye fate was eyeless (ey), a homoedomain DNA-binding transcription factor homologous to the mammalian gene Pax6 [10-12]. Ey is expressed early in the imaginal disc, prior to MF formation, and as the MF progresses anteriorly, expression of ey drops behind it. Toy and eyg are also Pax6 homologues and the three genes are thought to act at the top of a transcriptional hierarchy controlling eye development. While ey mutants show complete failure of eye formation and ey transgenes are strong inducers of ectopic eye expression, ey itself is not specific to the eye imaginal disc. Specificity is thought to be provided by genes at the second tier of the RD network. The nuclear factors dachshund (dac), sine oculis (so), and eyes absent (eya) are all more specific to the eye imaginal disc in their expression pattern and are thought to act downstream of ey and toy to induce eye development. So is a DNA-binding homeodomain-class transcription factor homologous to human SIX genes. Eya represents a novel class of transcriptional co-factors that lack a DNA-binding domain but possess a strong
transactivation domain. *Dac*, a putative co-repressor with homology to Ski/Sno factors, is expressed throughout the eye field prior to and during propagation of the morphogenic furrow. Genetic studies have suggested that *dac* function is specific to MF initiation, not propagation [13]. *So* and *eya* are expressed early in the eye disc and expression is maintained throughout differentiation. Both genes are thought to be
crucial for both MF initiation and propagation. Early studies by Bonini, *et al.* indicated that *eya* mutant flies did not show a defect in cell proliferation within the eye disc but instead demonstrated massive cell death of eye progenitors prior to initiation of the MF, most likely via apoptosis [14]. Flies with mutations in *so* showed an almost indistinguishable phenotype, with differentiation blocked and massive cell death of progenitors induced [15]. Interestingly, a subsequent study by Pignoni, *et al.* showed that when loss of *so* or *eya* function was induced in patches of cells within the eye disc rather than the whole disc, a distinct overproliferation phenotype was seen within the mutant clones [16]. The cells maintained their epithelial state and proliferated rapidly before ultimately dying. These studies suggested that *so* and *eya* primarily function directly to control cell proliferation, rather than cell death. In *so* and *eya* mutant eyes, expression of *ey* is maintained, but *dac* expression is lost, supporting the idea of a hierarchy of transcriptional control with *ey* at the top followed by *so* and *eya*, with *dac* ultimately downstream. These factors were shown to act in a combinatorial fashion via transgenic studies where it was shown that the expression of *eya* with *so* or *eya* with *dac* had a much stronger phenotype in terms of induction of ectopic eyes that any of these factors alone [16, 17]. Furthermore, biochemical experiments demonstrated that drosophila *eya* protein can physically interact with either *so* or *dac*.

While the primary role for *eya* in *Drosophila* development seems to be in directing eye development, *eya* may also function in gonad development in flies, possibly in conjunction with *dac*. The *Drosophila* embryonic gonad is comprised of a set of somatic cells and a set of germ cells [18]. The mesodermally-derived somatic
cells are known as somatic gonadal precursors and go on to form the somatic component of the mature gonad, which plays a supportive role in gametogenesis. 

*Dac* mutant *Drosophila* embryos of both sexes show genital structural defects and *Eya* has been shown to be expressed in the somatic gonadal precursor cells. During development, the somatic gonadal precursors help to guide the germ cells into the gonad. *Eya* seems to function in cell fate decision in these cells; in *eya* mutants, gonadal precursor cells are specified, but fail to maintain this cell fate. Ultimately, *eya* mutation results in failure of gonad formation, with germ cells scattered throughout the posterior end of the *Drosophila* embryo [18]. The function of *eya* in eye development versus gonad development seems to be distinct, with *eya* regulating cell survival in one context and cell fate in the other. This difference may be related to differing sets of transcriptional targets, which may relate to different transcription factor complexes that recruit eya activity. The cell fate function in gonad development does not appear to be conserved in mammals, as there is no reported gonadal phenotype for *eya* mutation in either mice or humans.

*Eyes absent* is a key transcription factor in *Drosophila* development and is a member of a transcriptional hierarchy with Pax6 homologues at the top activating Eya as well as Six-family homeodomain genes. The genetic power of the *drosophila* system has allowed for the identification of many of the members of this transcriptional network, as well as the various signaling pathways that regulate their expression and activity. However, a better understanding of how this genetic network functions in mammalian systems has required the generation and characterization of a series of mouse mutants, homozygous-negative for different RD-network members.
1.2 Biological Functions of Eya: Mouse

The retinal determination network has been conserved from *Drosophila* to mammals (Table 1). While *eyes absent* exists as a single gene in *Drosophila*, in vertebrates it has been expanded to a gene family with at least three members. *Xenopus* studies have identified three homologues, *Xeya1-3* [19], while in Zebrafish, mice and humans a fourth gene, Eya4 has also been found. All vertebrate Eya homologues show strong conservation of the C-terminal Eya Domain, with lower conservation of the N-terminal transactivation domain. Preliminary expression profiling of the different Eya homologues in developing mouse embryos suggested significant overlap of expression for Eya1 and Eya2 [20, 21]. Strong expression was detected in cranial placodes, including the lens and nasal placode, as well as in the somites, myogenic and connective tissue of the limb buds, and the developing kidney. Eya3 expression was strong in the anterior region of the embryonic head, but was more specific to the head mesenchyme, branchial arches, and neural tissue, including the brain [21]. Eya3 seemed to be excluded from the cranial placodes that expressed Eya1/2, and instead was present in the areas surrounding those structures. The more recently discovered Eya4 displayed an expression pattern similar to that of Eya1/2, with expression in the craniofacial mesenchyme, limb bud and skeletal muscle [22].

The phenotype for embryonic deletion of Eya1 in the mouse was first detailed in work from the laboratory of Richard Maas in 1999 [4]. Homozygous mutant embryos died postnatally with major defects in multiple tissues including ears, kidney and skeletal
muscle. At birth, homozygous Eya1 mutant embryos were seen to have significant craniofacial defects, including open eyes, lack of ears, and cleft palate. Heterozygous mice survived to adulthood but showed significant hearing loss in one or both ears, attributed at least in part to mechano-sensory defects relating to the transmission of sound through the ossicles and the conduction of sound through the middle ear. These phenotypes were suggested to be similar to the human genetic disorder BOR syndrome which is caused by haploinsufficiency for EYA1, and suggest a conserved role for Eya1 in mammalian ear development.

Close analysis of Eya1 mutant mice showed otic abnormalities in the outer, middle and inner ear. The outer ear frequently displayed malformed or absent auricles and preauricular pits, both of which are common features of BOR syndrome.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>The Retinal Determination Network Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>The seven genes that comprise the RD network in Drosophila are listed in conjunction with the mammalian homologues in mouse and human. When available, the relevant phenotypic data from loss of function studies is included. In most cases the mammalian homologue continues to function in eye development, but several have expanded roles in the development of kidney, ear and other tissues.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drosophila</th>
<th>Phenotype</th>
<th>Mouse</th>
<th>Phenotype</th>
<th>Human</th>
<th>Loss of Function Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>eyeless (ey)</td>
<td>embryonic lethality, loss of head structures</td>
<td>Pan6</td>
<td>Eye, CNS defects</td>
<td>EYA1</td>
<td>Congenital absence of all but the root of the iris</td>
</tr>
<tr>
<td>ase (a)</td>
<td>embryonic lethality, loss of head structures</td>
<td>Six1</td>
<td>Lung, kidney, Ear defects</td>
<td>Six1</td>
<td>Complete absence of tissues of the eyes, BOR syndrome</td>
</tr>
<tr>
<td>eyes abscend (ey)</td>
<td>loss of eye tissue, sterility</td>
<td>Eyel-4</td>
<td>Lung, kidney, Ear defects</td>
<td>DACH-1</td>
<td></td>
</tr>
<tr>
<td>dachshund (dach)</td>
<td>lack of eye tissue, leg defects</td>
<td>Dach-2</td>
<td>early postnatal lethality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eye gone (eng)</td>
<td>optic</td>
<td>Pan6</td>
<td>Eye, CNS defects</td>
<td>Pan6</td>
<td>Congenital absence of all but the root of the iris</td>
</tr>
<tr>
<td>otopic</td>
<td></td>
<td>Six3</td>
<td>Cranio-facial, CNS defects</td>
<td>Six3</td>
<td>Complete absence of tissues of the eyes</td>
</tr>
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</table>

The external ear canal was also seen to be completely absent in some mutants. The middle ear showed malformation of the ossicles and absence of the tympanic cavity.
Most severe were the aberrations of the inner ear, where most structures, including key sensory neurons that make up various ganglia that serve the ear, were completely missing. The inner ear develops from the otic placode, a thickening of the embryonic head ectoderm which invaginates at embryonic day 9 to form the otic cup [23]. The otic cup subsequently pinches off to form the otic vesicle. In Eya1-/- embryos the otic vesicle forms, but fails to develop further and significant apoptotic cell death is seen within the vesicle by embryonic day 10.5. Genetic control of these progressive steps in ear development is controlled in part by homologues of the Drosophila retinal determination network. Pax2 and Pax8 are both expressed in the otic ectoderm and both seem to lie upstream of Eya1 as their expression is unaffected in the Eya1 knockout [24]. In contrast, expression of Six1 was greatly reduced, suggesting that the Pax-Eya-Six hierarchy is evolutionarily conserved. Additionally, Eya seems to play an important role in sensory neuron development in the context of the inner ear. Significant work has focused on the loss of sensory neurons derived from the otic placode seen in Eya1-/- embryos [25]. The fate of these neurons is determined at an early step in development of the otic placode by expression of Neruogenins 1 and 2, basic helix-loop-helix transcription factors that subsequently activate a set of proneural factors including NeuroD and Math3. Studies were performed using a naturally occurring Eya1 mutant allele dubbed Eya1\textsuperscript{BOR} generated by insertion of an AIP transposon into intron 7, which results in an approximately 50% decrease in Eya1 expression in homozygous mice. These mice displayed severe inner-ear phenotypes with cochlear dysmorphogenesis and loss of relevant neurons. Neuronal fate was seen
to be specified in mutant embryos as seen by expression of Neurogenin, however these neuronal precursors are not maintained and are lost via apoptosis by e10.5 [25].

Eya also seems to play a major role in skeletal muscle development. One of the primary phenotypes in mice homozygous null for the Eya interacting protein Six1 is severe muscle hypoplasia contributing to death at birth [26]. Similarly, the double knockout of Eya1 and Eya2 displays an almost complete loss of limb muscle [27]. Six and Eya genes are expressed early at e9.5 in the somites which give rise to skeletal muscle precursors and seem to function in specification of this lineage [28]. Myoblast specification and differentiation is controlled by the homeodomain gene Pax3 and a set of muscle-specific basic helix-loop-helix transcription factors termed muscle regulatory factors (MRF’s) [1]. These include MyoD, Myf-5, Myogenin, and MRF4. It was demonstrated that Six1 could bind to a consensus target sequence in the promoter of myogenin called a MEF site and could synergize with Eya factors to directly activate myogenin expression [29]. MEF sites have subsequently been identified in the promoter of Pax3 as well as other MRF family members. Thus Six proteins, in conjunction with Eya, regulate the specification and differentiation of muscle precursor cells. However, the Pax-Eya-Six hierarchy here seems to be inverted such that Six and Eya lie upstream of Pax3. It is possible that the Pax/Eya/Six network has evolved independently in different organs, and thus represents a particularly ancient transcription factor system.

One of the most obvious phenotypes seen in the Eya1−/− embryos upon initial analysis was the lack of kidneys. Eya1 plays a crucial role in renal development, in conjunction with Six1, which also shows a loss of kidney structure in knockout
studies[30]. The functional mammalian kidney derives from two embryonic structures, the metanephric mesenchyme and the nephric duct [31, 32]. The metanephric mesenchyme originates from the intermediate mesoderm and first appears in the mouse embryo around e10.5, adjacent to the caudal end of the nephric duct. Subsequently, signals from the metanephric mesenchyme result in the formation of the uteretic bud, a tube which branches off from the nephric duct and extends toward the metanephric mesenchyme. As the uteretic bud grows, it penetrates the metanephric mesenchyme and begins extensive branching. Signals from the branched uteretic bud cause the mesenchymal cells to aggregate and condense around the branched ends. Mesenchymal cells subsequently undergo mesenchymal to epithelial transition and from renal epithelial cells, which go on to form the renal tubules and finally the functional nephrons. Reciprocal signals from these two structures thus give rise to the mature adult kidney. One of the key signals is thought to be the secreted factor Gdnf, which is expressed in the metanephric mesenchyme, while its receptor c-Ret is expressed in the nephric duct. Loss of expression of Gdnf or c-Ret results in the failure of uteretic bud formation [33].

The establishment of the metanephric mesenchyme and the resulting signaling events are controlled by a set of transcription factors, the function of which has been elucidated using mouse genetic models. The metanephric mesenchyme is defined by a now familiar genetic circuit which includes the homeodomain factors Pax2 and Six1, as well as the co-activator Eya1 [34]. In this case, the retinal determination network controls a transcriptional program that defines the mesenchyme as being functional for the production of inductive signals to the uteretic bud as well as competency to respond
to uterine bud invasion. Mouse knockouts of both Eya1 and Pax2 have shown loss of Gdnf expression and failure of the uterine bud to form [35], while molecular studies have provided strong evidence for Gdnf as a direct transcriptional target for Eya1, Six1, and Pax2 [3]. These factors also positively regulate each other in complicated feedback loops. Six1 and Pax2 are both downregulated in metanephric mesenchyme of Eya1 mutant mice, while Pax2 expression is reduced in Six1 mutant mice. In addition to the role in fate specification of the metanephric mesenchyme, the Six and Eya transcriptional program seems to govern a more general proliferation and survival response, with transcriptional targets such as c-Myc potentially functioning to promote cell proliferation and survival in multiple tissues. Supporting this conclusion, BrdU labeling experiments in Six1–/– embryos demonstrated a clear cell proliferation defect in mutant versus wild type littermates [3]. Loss of proliferation was accompanied by an increase in apoptotic cell death in the metanephric mesenchyme of Eya1–/– mice at e10.5, similar to the increased apoptosis seen the Eya1 mutant inner ear [4].

Functional analysis for the vertebrate Eya homologues other than Eya1 is less complete. Homozygous deletion of Eya2 in mice resulted in viable animals with no obvious phenotype, suggesting broad redundancy between Eya2 and Eya1. Recent work in Xenopus demonstrated that morpholino-knockdown of Xeya3 expression in early stage Xenopus embryos resulted in tadpoles with acute hypoplasia of the anterior neuroectoderm, accompanied by increased apoptosis [19]. Similarly, overexpression of Xeya3 by direct injection of the mRNA resulted in overproliferation and expansion of this region. These finding have not been confirmed in mice, but suggest that Eya3 continues to function in controlling the survival and proliferation of progenitor cell
populations. Morpholino analysis of Eya4 function in Zebrafish suggested a role in heart development, with loss of Eya4 function resulting in dilated cardiomyopathy [36]. Eya4 is also involved in mammalian ear development, as Eya4 mutations are associated with late-onset deafness in humans[36, 37].

Mouse genetic studies have demonstrated that the Pax/Six/Eya network originally characterized with regard to Drosophila eye development is of fundamental importance in the development of multiple mammalian organ systems. This transcription factor system regulates cell proliferation, cell fate decisions or both depending on the tissue context. Increased apoptosis has emerged as a hallmark of the loss of Eya1 in target tissues and suggests a strong role for Eya1 and potentially other Eya homologues in regulating survival and proliferation pathways, possibly via transcriptional regulation of anti-apoptotic target genes. However, apoptotic death can be a downstream result of many different cellular pathways, including failure to properly initiate differentiation pathways, and the mechanism by which Eya blocks apoptosis is still unclear.

1.3 BOR syndrome

Branchial-oto-renal syndrome (BOR syndrome, also known as Melnick-Fraser syndrome) is a human genetic disease with an incidence of approximately one in forty-thousand and which is thought to be responsible for up to 2% of all cases of profound deafness in infants [38]. The disease is characterized by a widely varying set of phenotypes and can be more accurately thought of as a heterogeneous group of diseases with a common genetic linkage. The most common symptoms include
hearing loss (93% of patients), auricular malformations including preauricular pits or tags (82% of patients), branchial fistulae (49% of patients), and renal abnormalities ranging from mild to complete renal agenesis (67% of patients). The closely related disease branchial-oto syndrome (BO syndrome) lacks renal phenotypes but maintains branchial and otic abnormalities. BOR syndrome also has been proposed to be allelic to the more severe disease, oto-facio-cervical (OFC) syndrome which features hypoplasia of the cervical musculature resulting in pronounced sloping shoulders as well as variable mental retardation in addition to hearing loss [39]. BOR syndrome and related diseases are inherited in an autosomal dominant fashion and genetic mapping initially placed the disease locus within chromosome 8q12-22 [40]. This locus contains the human EYA1 gene and subsequent studies have revealed that EYA1 is mutated in approximately 70% of all BOR cases [41]. A second disease locus for BOR and BO syndromes has been mapped to chromosome 14q23.1 [42]. The disease gene within this locus was revealed to be SIX1, the EYA1 binding partner. In the initial study identifying SIX1 as a BOR disease gene, three different amino acid mutations were identified, one in the Six Domain and two in the DNA-binding homeodomain. All three of these mutations were shown to decrease the physical interaction between SIX1 and EYA1, and thus are predicted to abolish the composite transcription factor consisting of the DNA-binding factor SIX1 and transactivating co-factor EYA1[43]. Genetic studies in mice have shown both Six1 and Eya1 to be involved in development of ear and kidney tissues, with broadly overlapping phenotypes, strongly supporting a combinatorial function for the two genes and a causative role for these mutations in human disease.
Genetic screens identified multiple mutations within EYA1 from BOR patients. An early analysis of 20 BOR patients from unrelated families identified 14 mutations within the EYA1 coding region including frameshifts, deletions, and amino acid substitutions [44]. All of the mutations were located within or immediately adjacent to the EYA Domain, suggesting a crucial role for this domain in EYA1 function in the context of BOR disease. Experiments focusing on five of these missense mutations within the EYA Domain showed that the majority of them prevented direct binding of EYA1 with SIX1 by GST pull-down assays and decreased nuclear EYA1/SIX1 complex formation by co-immunoprecipitation [45]. Of note, no defect was seen in subcellular localization of any of the Eya1 mutants, despite defects in Six1 interaction and binding to heterotrimeric-G-proteins (see Chapter 1-4). Using drosophila eye development as a readout for Eya function, Mutsuddi et. al. demonstrated that EYA1 mutations identified in BOR patients compromised the ability of EYA1 to induce ectopic eyes in transgenic flies [46]. BOR-associated mutations were also seen to abrogate Eya phosphatase activity in in-vitro enzyme assays using the artificial substrate pNPP as well as a phospho-tyrosine peptide substrate (see Chapter 1-5). In addition to these results, transcriptional assays using an EYA-SO responsive reporter in drosophila S2 cells demonstrated reduced transactivation potential for all BOR-associated Eya mutants tested. Thus mutations of EYA1 that have been identified in BOR patients significantly effect Eya function, most likely through a combination of disrupting protein-protein interactions with key EYA1 binding partners such as SIX1 and by blocking phosphatase enzymatic activity.
In contrast to the phenotype in drosophila for Eya haploinsufficiency, BOR syndrome does not typically include defects in eye development. However, a study by Azuma, et. al. found that in a group of patients with congenital cataracts and ocular anterior segment anomalies contained novel EYA1 missense mutations in the EYA Domain [47]. Three mutations were characterized based on three unrelated patients, one of whom also displayed symptoms more typical of BOR syndrome. Subsequent functional studies of these ocular defect-associated mutations showed that they were able to effectively induce ectopic eye formation in *Drosophila* [46] and two out of the three mutations showed measurable phosphatase enzymatic activity, in contrast to BOR-associated mutations. Thus it seems that EYA1 does play a limited role in the development of human eye structures, particularly in the anterior region. However, functional studies of EYA1 mutants associated with ocular defects indicate that the molecular function of EYA1 in the context of eye development may be distinct from that in other tissues such as kidney and ear, as mutations associated with ocular defects and oto/renal defects have divergent readouts on Eya activity assays.

Eya has four homologues in humans, designated EYA1-4. While Eya1 has the broadest expression pattern and the most well-characterized function in mammalian development based on mouse genetic models, it is not the only Eya gene associated with human disease. A form of late-onset autosomal dominant progressive sensorineural hearing loss was mapped to chromosome 6q23-24 [48], a region that includes EYA4. Subsequently, three different mutation of EYA4 were identified in three different families displaying late-onset deafness [37]. All three result in premature stop codons that delete all or part of the EYA Domain. EYA4 in humans
thus seems to have an important function in inner ear maintenance and development. Eya4 is expressed in the inner ear during mammalian development (e14.5 in rat by in situ hybridization) and is maintained in adult inner ear tissue. The late onset phenotype may reflect functional redundancy between Eya4 and Eya1 during embryonic inner ear development which does not exist in adult tissue where Eya1 expression is much lower. The most severe of the EYA4 deletion mutations was found in a patient that also displayed dilated cardiomyopathy [36]. Zebrafish morpholino experiments to test the effect of this mutation of Eya4 on embryonic heart development showed swelling of the heart and defects in heart function [36].

Eya family genes play important roles in mammalian development, as reflected by the multiple human diseases associated with mutations in Eya genes. While no human diseases have been associated to date with EYA2 or EYA3, potentially reflecting functional redundancy with EYA1, differing phenotypes between mutations within the EYA Domains of EYA1 and EYA4 suggest non-redundant roles for different Eya family members. EYA1 is associated with several developmental syndromes which do not always have overlapping phenotypes. The observation that EYA1 mutants associated with ocular defects function differently in molecular activity assays that mutants associated with BOR syndrome suggest that differing functions for EYA1 at the molecular level may reflect functions for EYA1 in different developmental contexts. The discovery that Eya proteins are functional tyrosine phosphatases may provide insight into why different disease phenotypes are linked to different Eya activity defects.
1.4 Domain Structure of Eya

The *Drosophila* factor Eyes Absent (Eya) is critical for proper embryonic development of metazoans. Eya is defined by the C-terminal Eya Domain (ED) which spans from amino acids 486 to 760 in *drosophila* [49] (Fig. 2). This domain is highly conserved between species as well as within the four Eya homologues in mammals. The Eya Domain contains binding sites for the key Eya co-factors in *drosophila*, *so* and *dac*, which are key for Eya function in development. The N-terminus of Eya is notably more divergent between species and between family members in mammals. However, close analysis from the lab of Ilaria Rebay revealed a small tyrosine-rich region within the N-terminus with higher degree of conservation which they dubbed Eya Domain 2 (ED2) [50]. ED2 lies within a larger proline/serine/threonine-rich region, and reporter assays using Gal4-Eya fusion proteins showed that this region was crucial for transactivation activity. A UAS reporter was modestly activated by full-length Eya-Gal4DBD fusion protein, but highly activated by N-terminus alone, suggesting that the N-terminus can act independently as a potent transactivator, and that the C-terminal ED may possess auto-inhibitory function. Deletions within the
P/S/T region, particularly deletion of ED2, significantly reduced activation of the reporter, demonstrating the importance of this region for transactivation. Eya lacks any defined DNA binding domain and has not been shown to possess intrinsic DNA binding activity. The classic model involves Eya being recruited to target genes via binding to the homeobox DNA-binding transcription factor Six1 and subsequently activating transcription via the N-terminal transactivation domain. Thus only Eya and Six together are able form a functional transcriptional activator. In agreement with this model, the mammalian Six family members which cannot bind Eya protein (Six3, Six6) act as constitutive repressors [51, 52].

The N-terminal domain of *Drosophila* Eya was found to contain two consensus MAPK phosphorylation sites [53]. These sites are conserved in the mammalian Eya homologues. In-vitro kinase assays showed that the downstream
effecter MAPK ERK was able to effectively phosphorylate wild type Eya but not a phosphorylation site mutant. Other MAPK-family kinases, including JNK, showed no activity toward Eya. Ectopic eye assays in drosophila using a dpp-Gal4 strain to drive overexpression of Eya transgenes in eye tissue showed that mutating both phosphorylation sites to alanine strongly suppressed the ectopic eye formation phenotype in comparison to the wild-type Eya transgene, while mutating the sites to aspartic or glutamic acid to mimic phosphorylation increased ectopic eye induction [53]. Similarly, activation of MAPK signaling by co-expression of activated Ras with Eya to promote phosphorylation of these sites increased activity of an Eya-dependent reporter beyond what was seen with Eya alone. Mutation of the fly homologue of the receptor tyrosine kinase EGFR, known to be an upstream activator of MAPK signaling and ERK was also seen to suppress ectopic eye formation. Thus, Eya seems to be positively regulated by the MAPK pathway via phosphorylation of two conserved serine residues in the N-terminal transactivation domain, possibly in response to RTK signaling, although how MAPK phosphorylation affects Eya function is unknown.

Despite established nuclear functions for Eya, the mammalian homologues of Eya lack a defined nuclear localization signal. Instead, Eya is thought to be shuttled into the nucleus by its binding partner Six in a manner dependent upon the Eya domain. Cell fractionation and immunostaining experiments have demonstrated that mouse Eya homologues 1, 2 and 3 are primarily localized to the cytoplasm when expressed alone in transient transfection assays, while co-expression with Six2, 4, or 5 resulted in Eya relocalizing to the nucleus [54]. Significantly, Six3, which does not interact with Eya failed to promote nuclear translocation. Deletion of the C-terminal
Eya domain, required for Six protein binding, abolished the translocation effect. Six proteins may function as shuttles for Eya, directly binding to Eya protein in the cytoplasm and transporting it into the nucleus, and thus regulating the activity of the composite transcription factor at the level of subcellular localization. Whether or not this function of Six proteins is regulated by specific signaling pathways and/or post-translational modification is currently unknown.

Independent yeast 2-hybrid assays by two groups identified a strong interaction between Eya2 and G-alpha-i family small G proteins, specifically G-alpha-z and G-alpha-i2 [55, 56]. These factors are associated with the cell membrane as part of a heterotrimeric G-protein complex and interaction with Eya2 was seen to be dependent on G-protein activation (binding to GTP). G-alpha-z is unique to the G_i family in that it is insensitive to inhibition by pertussis toxin, and displays a much slower rate of GTP hydrolysis suggesting a more prolonged signal upon GPCR stimulation than other G_i family members [57]. G-alpha-z also has a specific expression pattern, primarily being expressed in developing brain and nervous system. Expression of G-alpha-z decreases after birth, suggesting a potential developmental role for this small G protein. G-alpha-z bound the EYA Domain of Eya2 and this interaction blocked binding of Eya2 to Six proteins and subsequent translocation to the nucleus. Thus, Eya may be sequestered in the cytoplasm at the cell membrane via binding to activated G-proteins as a result of signaling through GPCRs. This possibility adds another level of complexity to the signaling mechanisms that control Six-Eya function, potentially placing GPCR signaling and RTK signaling in contradictory roles in regards to Eya.
activation, and providing a potential cellular mechanism for fine-tuning Eya activity based on subcellular localization.

While the N-terminal transactivation domain is divergent between members of the mammalian Eya family, the C-terminal Eya Domain is highly conserved. Studies of the Eya Domain have previously revealed a highly conserved motif with significant homology to the enzymatic domain of the haloacid dehalogenase family of hydrolases, which includes phosphatase enzymes.

1.5 Eya as a Protein Tyrosine Phosphatase

The presence of the C-terminal Eya Domain (ED) is the defining characteristic of Eya family proteins. The domain is highly conserved both between species as well as between the four mammalian Eya homologues. The Eya Domain is known to contain the binding site for Six-family proteins, which play a crucial role in Eya function in embryonic development. In order to identify novel motifs that may also be important for Eya function, analysis of the primary amino acid sequence of the Eya Domain was undertaken by several groups [3, 5, 6]. It was discovered that all known Eya proteins contain the signature motifs of a family of enzymes known as the haloacid dehalogenases. This diverse group of enzymes includes dehalogenases, ATPases, phosphonatases, phosphomutases, epoxy hydrolases, and a set of phosphotases that rely on metal-ion binding for catalytic activity [58, 59]. The primary catalytic motif is DXXX(T/V), with an aspartate typically occupying the third residue in phosphatases, a threonine in P-type ATPases, and a tyrosine in L-2-haloacid
dehalogenases. A second conserved motif consists simply of a serine or threonine residue ((S/T)XX) and is proposed to have a role in substrate binding, while the third domain is defined as (G/S)DXX(N/T)D and has been shown to function in coordinating the activity of the metal ion in catalysis. The best characterized phosphatases in this family are the RNAP2 CTD phosphatases FCP1 [60] and SCP1 [61, 62] and the phospho-L-serine phosphatase PSP which functions in serine biosynthesis. Protein database homology searches have identified several additional phosphatases in the HAD family, including TIMM50, a mitochondrial protein thought to function in transport of proteins across the mitochondrial inner membrane, and Dullard, a nuclear membrane protein shown to be important in neural tube development in *Xenopus* [63].

In an effort to understand the catalytic mechanism for HAD-type phosphatases, the crystal structures of PSP and SCP1 were resolved. For PSP a series of crystallographic “snapshots” were produced detailing various steps in the catalytic reaction [64]. The overall structure of the catalytic domain features an alpha/beta hydrolase fold which is in an open conformation in the apo-enzyme, but adopts a closed conformation upon substrate binding. In the closed conformation the three HAD signature motifs are brought into proximity, forming a catalytic pocket. The first aspartate within the DXDX(T/V) domain initiates nucleophilic attack on the phosphate group of the substrate leading to formation of a phospho-aspartate intermediate. Subsequently, the second aspartate functions as a general acid, donating a proton to the leaving group on the substrate and facilitating the de-phosphorylation reaction. Finally, the now-deprotonated second aspartate switches functions to act as a
general base, coordinating and positioning the activated water molecule that initiates nuclophilic attack on the phospho-aspartate group remaining on the enzyme. Hydrolysis of the phosphate group results in generation of inorganic phosphate and re-generation of the apo-enzyme which returns to its open conformation. Multiple residues within the catalytic pocket formed by the conserved domains are predicted to be required for enzymatic activity based on this model, the foremost being the nucleophilic first aspartate. Mutational analysis of PSP has confirmed that this amino acid is required for catalytic activity. Crystalization of SCP1, which is predicted to be highly similar to the catalytic domain of FCP1, showed a strong correspondence to the catalytic structure of PSP, with the key catalytic amino acid side chains from the two structures superimposing closely [65]. Thus, all identified HAD-family phosphatases most likely act through formation of a phospho-aspartate intermediate resulting from nucleophilic attack by the first aspartate in the signature motif on the phospho-group on the substrate.

The Eya Domain sequence contained conserved motifs in all species and homologues that closely matched the motif sequence of the known phosphatases in the HAD family, but showed no homology to other known protein classes. To test the possibility that the Eya Domain could posses intrinsic phosphatase enzymatic activity, GST-fusion proteins of various Eya family members from various species were purified and tested against the synthetic phosphatase substrate p-nitrophenylphosphate (pNPP) [3, 5, 6]. All Eya proteins tested showed significant phosphatase activity in this assay. Of the mammalian Eya homologues, Eya3 showed the strongest activity, and the Eya3 Eya Domain alone was significantly more robust that full-length protein.
The strongest phosphatase tested was full-length *arabidopsis thaliana* Eya protein [5], with an activity approximately 3.5–fold higher that Eya3 ED. Plant Eya protein consists almost solely of the Eya Domain, and thus the missing N-terminal region may include a repressor domain which inhibits phosphatase enzymatic activity. Specificity of Eya phosphatase activity was tested using various phosphorylated peptide substrates in in-vitro phosphatase assays. Eya consistently showed the strongest activity in these assays toward tyrosine phosphorylated peptides, with phospho-serine and threonine peptides showing reduced or insignificant utility as Eya substrates [3, 5, 6]. Similarly, Eya phosphatase activity was blocked in these assays by the addition of the tyrosine phosphatase inhibitor sodium vanadate, but was insensitive to inhibitors specific to serine/threonine phosphatases [6]. The known HAD-family protein phosphatases have all been shown to specifically dephosphorylate serine residues, and all known tyrosine phosphatases have significantly different catalytic motifs, with a cystine acting as a nucleophile and lacking the metal cation required by Eya proteins. Thus, Eya appears to be the founding member of a novel class of tyrosine phosphatases.

The significance of Eya phosphatase activity was confirmed using *Drosophila* transgenic experiments. While wild-type Eya protein can effectively rescue the eye formation phenotype of Eya-deficient mutant fly line eya<sup>2</sup> as well as induce the formation of ectopic eyes, Eya mutants that had been shown to abrogate phosphatase activity failed to do so [6]. Combined with experiments demonstrating that Eya mutants associated with the human developmental disorder BOR syndrome have
reduced phosphatase activity, this suggests an important role for phosphatase activity in Eya function in development.

While Eya phosphatase activity has been confirmed using artificial substrates in in-vitro assays, bona-fide in-vivo targets for Eya’s enzymatic activity have been difficult to identify. Thus far, in-vitro assays have only identified a few proteins which can be directly de-phosphorylated by Eya. One of the first to be verified was the C-terminal domain (CTD) of RNAP2. Eya shares an almost identical catalytic motif with FCP1 and SCP1, which have been demonstrated to de-phosphorylate serine 2 or serine 5 in the YSPTSPS heptapeptad repeat that makes up the CTD. FCP1 is a part of the core promoter general transcription machinery and is thought to be crucial for recycling of RANP2, which must be fully de-phosphorylated at the initiation of transcription [66]. Experiments using GST-purified CTD labeled with $^{32}$P demonstrated that purified Eya3 protein could de-phosphorylate the CTD in-vitro, while an Eya3 mutant construct lacking the nucleophilic aspartate could not [3]. Subsequent studies showed that the Arabidopsis MAPK4 homologue AtMPK4 was effectively tyrosine de-phosphorylated by either purified Arabidopsis Eya or the ED of murine Eya3 [67]. Another identified in-vitro substrate for Arabidopsis Eya is myelin basic protein (MBP), a constituent of the myelin sheath on oligodendrocytes and Schwann cells. MBP is phosphorylated on both tyrosine and serine/threonine residues, but AtEya was only active toward phospho-tyrosine in in-vitro assays. MBP is commonly used as a non-specific in-vitro substrate for kinase/phosphatase activity, and none of the mammalian Eya homologues have been shown to be expressed in glia, therefore it is unlikely that MBP is a biologically relevant in-vivo substrate for Eya. Finally, it
has been shown that *Drosophila eya* can be tyrosine phosphorylated in Drosophila S2 cells, and purified, tyrosine phosphorylated *eya* protein was shown to be effectively de-phosphorylated by GST-purified wild type murine Eya3 [6, 67]. Thus Eya may act to de-phosphorylate itself as part of a regulatory feedback loop. It is important to note that none of the substrates listed above have been confirmed in-vivo, and it has been reported that phosphatase enzymes lose much of their substrate specificity when taken out of their cellular context. Specificity is thought to be commonly mediated by binding partners and co-factors that direct the relatively promiscuous phosphatase activity toward specific phospho-proteins. Thus, while in-vitro assays are useful for assessing specificity in terms of phospho-tyrosine versus serine/threonine, they may be less amenable for identifying specific protein substrates.

The best characterized binding partners for Eya protein are *So* and *Dac*, and their mammalian homologues in the Six and Dach families. Similarly, the best characterized molecular function for Eya is in the transcriptional activation of Six target genes, thus it is tempting to suppose that either Six, Dach, or another associated transcriptional co-factor is the target for Eya phosphatase activity. Thus far, it has not been demonstrated that either Six or Dach is dephosphorylated by Eya. If Eya phosphatase activity is functioning in the context of Eya-dependent transcriptional activation, one would predict that phosphatase-inactive mutant Eya would have significantly reduced efficacy in activating Six1 target genes. Data on this so far has been somewhat contradictory. While fly transgene experiments have conclusively demonstrated that phosphatase activity is important for Eya’s biological function in development of the Drosophila compound eye [5, 6], no similar experiments have
been undertaken in mammalian systems. Transcriptional reporter assays have been undertaken using GAL4-UAS system or using Six-responsive reporters. Single-cell nuclear microinjection experiments using a GAL4-Six1 fusion and a UAS-dependent reporter demonstrated that wild type Eya3 reversed Six1-mediated repression while phosphatase-mutant Eya3 did not [3]. Similar microinjection experiments using a reporter with a multimerized Six1 binding site showed a combinatorial activation event only with Six1 and wild type Eya3, not phosphatase mutant Eya3 [3]. This data strongly argued in favor of a role for Eya phosphatase activity in Eya-mediated transcriptional activation. However, similar experiments from the Rebay lab produced contradictory results. Using Drosophila S2 cultured cells transfected with reporter containing a multimerized Six1 binding site, it was shown that Drosophila Eya constructs with mutations shown to block phosphatase activity were able to activate reporter gene expression to an equal level as wild-type Eya [6]. The discrepancies in the results could reflect differences in the experimental procedures or possibly differences between the Drosophila and mammalian Eya proteins. At this point, function of Eya phosphatase activity is largely unknown, although its importance in Eya’s biological function, at least in the context of Drosophila eye development seems clear.
References


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Chapter 2

Identification of Eya Phosphatase Substrates: Biochemical and Transcriptional Approaches
Understanding the mechanistic function of Eya phosphatase activity in the context of transcriptional activation of Six target genes and mammalian organogenesis required the identification of bona-fide in-vivo phosphatase enzymatic substrates. We initially undertook two different experimental approaches to identify substrates: biochemical purification using a substrate-trapping mutant to identify novel substrates and transcriptional assays using an in-vivo target gene for the Six/Eya transcriptional complex as a readout to assess phosphatase-dependent transcriptional activation. Ultimately, neither approach was completely successful. While the substrate-trapping mutants for Eya1 and Eya3 showed preferential binding to the C-terminal domain of RN AP2, a demonstrated in-vitro substrate, we were not able to use these mutants to purify novel substrate proteins. Microarray analysis of RNA from kidney material derived from Six1 -/- versus Six1 +/+ mouse embryos identified a number of potential target genes for the Six/Eya transcriptional complex. Of the array targets, we chose to focus on Sall1 as a confirmed direct target for Six1. While Sall1 is a direct target for Eya and is transcribed in an Eya-dependent manner in human embryonic kidney cells, this transcription was demonstrated to be wholly independent of phosphatase activity. We conclude that the phosphatase activity of Eya is not required for the transcriptional activation of all Eya target genes. Eya may only activate a subset of transcriptional targets in a phosphatase-dependent manner, or the phosphatase activity may have a novel function divorced from transcription that is nevertheless crucial for mammalian organogenesis.
2.1 Biochemical Identification of Eya Substrates: Substrate Trapping Mutations

Identification of substrates for phosphatase enzymes suggests two potential experimental approaches. Enzymes and potential phosphorylated substrates can be mixed in an in-vitro reaction and loss of the phosphate group can be monitored via western blot with a phospho-specific antibody or by pre-labeling the substrate with a marker such as $^{32}$P. Alternately, the phosphatase enzyme activity could be deleted within cells using siRNA or a specific inhibitor and accumulation of the phosphorylated form of specific substrates could be monitored by phospho-specific western blot or detected by mass spectroscopy analysis. The former approach has the benefit of demonstrating a clear, direct dephosphorylation reaction, however it has been demonstrated that many phosphatases loose substrate specificity when removed from their cellular context [1], and thus the potential for false-positive results in an in-vitro assay is quite high. The latter approach maintains the proper cellular environment for the phosphatase, but does not prove a direct substrate-enzyme reaction. Ideally both approaches would be combined to argue for a direct substrate-enzyme relationship which is still relevant in the proper cellular context for the phosphatase. However, this combined approach is not amenable to screening for novel substrates, but rather is useful for checking individual candidate substrates. As we had a limited number of candidates for Eya, we wanted a more open-ended approach for substrate identification. It has been demonstrated that for certain tyrosine
phosphatases it is possible to generate a substrate-trapping mutant protein which can be used to purify novel substrates [2-4]. We investigated whether this approach would work with Eya.

The goal of the substrate-trapping mutant is to alter the enzyme such that it retains the ability to specifically interact with phosphorylated substrate molecules, but with the catalytic reaction greatly slowed down such that the enzyme-substrate complex remains together long enough to allow for co-purification [2].

Crystallographic analysis of several tyrosine phosphatase enzymes identified a set of invariant residues that defined the catalytic domain. In all classic tyrosine phosphatases a key cysteine residue acts as the nucleophile in the de-phosphorylation reaction. One of the initial studies aimed to create a substrate trapping mutant for PTP1B, and noted that the mutation of the nucleophilic cysteine 215 to alanine had sufficient substrate trapping properties to enable capture of the enzyme-substrate complex for crystallographic analysis [4]. However, this mutant did not prove suitable for purification and identification of novel PTP1B substrates. Analysis of the crystal structure of PTP1B suggested that, when the enzyme was in the closed, substrate-bound conformation, aspartate 181 was positioned in such a way that it could act as a general acid, donating a proton to the leaving group of the tyrosine-phosphorylated substrate and aiding in cleavage of the phosphate group from the substrate protein.

Mutational analysis demonstrated that substitution of aspartate 181 with alanine generated a mutant form of PTP1B with significantly stronger substrate-trapping capability than the C215A mutant. PTP1B D181A was successfully used to purify the substrate EGFR from COS cell extract. A subsequent study focusing on the SH2-
domain containing protein tyrosine phosphatase SHP2 demonstrated that mutating the nucleophilic cysteine to serine in combination with mutation of the relevant aspartate to alanine produced an effective trapping mutant, analogous to the finding with PTP1B [5]. Studies focusing on the T-cell specific phosphatase HePTP demonstrated that mutation of the nucleophilic cysteine to serine alone, or of the conserved aspartic acid to alanine alone both produced effective substrate traps [3]. Thus, while the specific combination of point mutations that generate the most effective trapping mutant may vary from enzyme to enzyme, the nucleophilic cysteine and associated aspartate are frequently effective target residues.

Eya represents a novel class of protein tyrosine phosphatases that has homology to HAD-class phosphatases [6]. Rather than cysteine as a nucleophile, these enzymes use the first aspartic acid in a highly conserved WDLD catalytic motif. Crystallographic studies suggested that the second aspartic acid in the motif could potentially act as a general acid in a manner analogous to the conserved aspartic acid in conventional protein tyrosine phosphatases [7]. In order to identify novel, in-vivo substrates for Eya phosphatase activity, we undertook to generate effective substrate-trapping mutants for mammalian Eya factors. We focused on the second aspartic acid in the catalytic motif, Asp248 in mouse Eya3 and Asp325 in mouse Eya1, based on structural studies in the homologous PSP phosphatase that suggested this aspartic acid to function in hydrolysis of the de-phosphorylated substrate from the phospho-enzyme complex [7]. We chose to use a more conservative substitution of aspartic acid to asparagine in an effort to preserve the overall structure of the Eya substrate-binding surface. The Eya1 D325N point mutation was introduced to an HA-tagged Eya1
mammalian expression construct via targeted mutagenesis and the resulting mutant was seen to express well in transiently transfected 293T cells by western blotting against the N-terminal HA tag (Fig. 3).

To confirm that Eya1 D325N was functioning as a substrate trap, we decided to check for increased binding affinity toward one of the confirmed in-vitro substrates. We performed a co-immunoprecipitation using a FLAG-tagged construct of the RNAP2 C-terminal domain (CTD) and the HA-tagged Eya1 D325N construct or WT Eya1 construct. The CTD of RNAP2 had been previously demonstrated to be de-

| IB: anti-HA |
|---|---|
| 1 | 2 |

**Figure 3**

**HA-Eya1 D325N expression in 293T cells.** 293T cells were transfected with an expression construct for HA-tagged Eya1 D325N (Lane 1) or with an empty vector control (Lane 2). 24h post transfection, nuclear extracts were harvested and HA-tagged protein was detected by SDS-PAGE and western blotting. Specific signal was detected only in the Eya1-transfected cell extract.

phosphorylated by purified Eya protein in-vitro [8]. Eya has been primarily characterized as a functional tyrosine-specific phosphatase, and published studies indicate that RNAP2 is phosphorylated on serines two and five in the seven amino acid heptapeptad repeat that comprises the CTD [9]. However, it has been postulated that Eya could be acting as a dual-specificity phosphatase in-vitro, and, alternatively, the CTD could possess tyrosine phosphorylation which has not been fully
characterized. Immunoprecipitation using an anti-FLAG antibody and subsequent detection of HA-Eya1 in the IP product by western blot using an anti-HA antibody showed that the Eya1 D325N mutant had notably stronger affinity for the FLAG-CTD construct than wild type Eya1, suggesting a potential substrate-trapping effect (Fig. 4).

We hypothesized that other Eya homologues could also be converted into substrate trapping mutants by introducing catalytic site point mutations homologous to EYA1 D325N. Targeted mutagenesis was performed to introduce the substitution D248N into a mouse Eya3-HA expression construct. I decided to use a GST pull-down interaction assay to assess the utility of this Eya3 mutant for purification of novel substrates, due to the ease of this assay and with the ultimate goal of using GST-Eya3 protein immobilized on glutathione agarose beads for purification/mass-spectroscopy analysis. The C-terminal EYA-Domain (AA236-510) of wild-type and

![Figure 4](image-url)

**Figure 4**

*Eya1 D325N displays increased affinity for RNAP2 CTD by co-immunoprecipitation.* Co-immunoprecipitation was performed on nuclear extract from 293T cells transfected with FLAG-RNAP2 CTD in conjunction with HA-Eya1 wild type (Lane 1) or HA-Eya1 D325N (Lane 2). Anti-FLAG immunoprecipitates were probed with anti-HA by western blot, revealing enrichment for HA-Eya1 D325N in comparison to wild type.
D248N Eya3 were subcloned into the GST expression vector pGEX-6P. Both constructs were seen to express robustly in BL21 bacterial cells in response to stimulation with IPTG and were readily purified using glutathione-agarose bead resin (Fig. 5). In agreement with the co-immunoprecipitation assay using the Eya1 substrate trapping mutant, Eya3 D248N was seen to strongly interact with a FLAG-tagged construct of the RNAP2 C-terminal domain when this construct was expressed in 293T cells, while wild type Eya3 interacted weakly or not at all (Fig. 6 top panel). This data argued that these mutants of Eya1 and Eya3 were successfully acting as substrate traps and additionally supported the idea that the C-terminal heptapeptad repeats of RNAP2 could be in-vitro substrates for Eya phosphatase activity.

**Figure 5**
**GST-Eya3 wild-type and D248N mutant construct expression.**
Expression of GST-fusion proteins purified using glutathione-agarose was visualized by SDS-PAGE followed by coomassie blue staining. Lanes: (1) GST-Eya3 C-terminus (2) GST (3) GST-Eya3 C-terminus (4) GST-Eya3 D246N C-terminus.
I next used the GST-Eya3 wild-type and substrate-trapping mutant bead resin which we had produced to test several other potential substrates for Eya. Specifically, I was interested in the Eya binding partners Six1 and Dach1, both of which function with Eya in the transcriptional activation of Six1 target genes [8, 10]. Phosphorylation of Six1 and Dach1 has not been conclusively demonstrated, but we acted under the assumption that these modifications exist in vivo. A FLAG-tagged Six1 construct was expressed in 293T cells and the resulting nuclear extract was incubated with either Eya3 wild-type or trapping mutant GST-bead resin. This pull-down assay showed an approximately equal interaction between FLAG-tagged Six1 and wild-type or mutant Eya3 (Fig. 6, middle panel). The presence of the EYA domain alone seemed to be sufficient to bind to Six1, consistent with published data, however the substrate trapping mutation did not increase affinity, indicating that Six1 may not be an enzymatic substrate for Eya, at least under the conditions utilized in our assay.

Overexpression of a FLAG-Dach1 construct in 293T cells and subsequent incubation with each bead species revealed little binding in either context (data not shown). When both FLAG-Six1 and FLAG-Dach1 were overexpressed, both wild-type and substrate-trapping mutant Eya3 beads effectively pulled out both proteins with equal affinity (Fig. 6, bottom panel), suggesting that Eya3 interaction with Dach1 requires Six1, but providing no evidence that Dach1 is a phosphatase substrate for Eya.

We decided to attempt purification of novel phosphorylated substrates from 293T nuclear extracts using the GST-Eya3 wild-type and trapping mutant constructs. Our goal was to use mass-spectrophotometry to identify novel proteins purified specifically using the trapping mutant bead resin. First we wanted to confirm that
significantly more tyrosine phosphorylated proteins were purified using the trapping mutant. To enrich for tyrosine phosphorylation, we pre-treated 293T cells with a solution of sodium orthovanadate in phosphate buffered saline with .05% (W/W) hydrogen peroxide. The final concentration of sodium orthovanadate was 100uM and cells were treated for 15 minutes immediately prior to lysis. Sodium orthovanadate is a chemical inhibitor of tyrosine phosphatase activity that has been shown to increase overall tyrosine phosphorylation levels in cells by blocking dephosphorylation [11]. The resulting nuclear extract showed a dramatically increased frequency of tyrosine phosphorylated proteins on an anti-phospho-tyrosine western blot (Fig. 7). Sodium

![Figure 6](image)

**Figure 6**  
Eya3 D246N interacts preferentially with RNAP2 CTD, but not Six1 or Dach1 by GST pull-down. Beads bearing immobilized GST-Eya3 C-terminus (Lane 1), GST-Eya3 D246N C-terminus (Lane 2) or GST alone (Lane 3) were incubated with nuclear extract from 293T cells transfected with FLAG-RNAP2 CTD (Top Panel), FLAG-Six1 (Middle Panel), or both FLAG-Dach1 and FLAG-Six1 (Bottom Panel). Preferential interaction was only seen for RNAP2 CTD.
orthovanadate treated or untreated nuclear extract was incubated with GST Eya3-EYA Domain wild-type, trapping mutant, or GST tag alone immobilized on glutathione agarose and the resulting pull down was analyzed by anti-phospho-tyrosine western blot. Surprisingly, we did not see any additional phospho-tyrosine bands in the pull-down using the trapping mutant compared to the wild-type (Fig. 8). Analysis of the pull-down product by silver staining to assess total protein showed very similar profiles of associated proteins for the wild-type and mutant (Fig. 9). This suggested to us that, while the trapping mutant did show increased affinity toward the RNAP2 C-terminal domain when overexpressed in 293T cells, it did not appear to posses

![IB: anti-phosphotyrosine](image)

**Figure 7**
**Sodium Vanadate treatment greatly increases tyrosine phosphorylation in 293T cell nuclear extract.** Cells were pretreated with 100uM sodium vanadate prior to lysis and overall tyrosine phosphorylation levels were assessed via SDS-PAGE followed by western blotting with an antibody to phosphotyrosine, revealing increased overall tyrosine phosphorylation in the treated (Lane 1) sample versus untreated (Lane 2).
significantly increased affinity for endogenous nuclear proteins. However, it may be that the assays used to examine differential recruitment of endogenous phosphoproteins to the different Eya3 GST constructs were not sufficiently sensitive to clearly demonstrate the presence of low-levels of substrate proteins. It may still be possible that differential mass-spectroscopy analysis of proteins affinity purified using the Eya3 wild type and trapping mutant bead resins would reveal novel substrates. Alternately, the trapping mutant for full-length tagged Eya1 and/or Eya3 could be expressed in 293T cells, ideally via generation of a stably expressing cell line, and then immunoprecipitated using an antibody against the tag in comparison to tagged,

![IB: anti-phosphotyrosine](image)

**Figure 8**
**GST-pulldown using Eya3 constructs shows little enrichment for tyrosine phosphorylated proteins with the trapping mutant.** Nuclear extract from 293T cells treated with sodium vanadate (10% input = Lane 1) or untreated was incubated with GST (Lanes 2, 5), GST-Eya3 C-terminus (Lanes 3, 6), or GST-Eya3 D246N C-terminus (Lanes 4, 7) and pulldown material was analyzed by SDS-PAGE followed by western blot using anti-phosphotyrosine specific antibodies. The profile of associated tyrosine phosphorylated proteins for Eya3 wild-type and trapping mutant constructs were notably similar for both untreated and vanadate-treated extracts.
wild-type Eya protein. Associated proteins in the immunoprecipitate would then be analyzed by differential mass spectroscopy to identify proteins associated preferentially with the trapping mutant. These promising approaches await further investigation. However, as our initial approaches using substrate-trapping mutants for Eya1 and Eya3 met with disappointment, I decided to change strategies for the problem of substrate identification.

**Figure 9**

Analysis of total protein associated with GST-Eya3 constructs shows similar profiles for wild-type and trapping mutant. Nuclear extract from sodium vanadate-treated 293T cells (10% input = Lane 1) was incubated with GST (Lane 2), GST-Eya3 C-terminus wild type (Lane 3), or GST-Eya3 D246N C-terminus (Lane 4) and associated proteins were visualized by SDS-PAGE followed by silver staining.
Methods:

Targeted Mutagenesis:

Mutagenesis was undertaken for Eya1 using the Stratagene QuickChange Site-Directed Mutagenesis Kit (Stratagene 200515). The following primers were designed:
Forward – gattctgaccttgagagagtgttactctgggatttaaatgagaccatcattgattttccactctttgctc
Reverse – ctaagactggaactctctcaaatgagaccccttaatttactctggtagtaacaaaaggtgaggaacgag.
These primers introduced a novel DraI restriction site in addition to the D325N substitution which was subsequently used to screen for successfully mutagenized constructs. Successful mutagenesis was confirmed by sequencing.

Cell culture

Transfections were preformed using Lipofectamine 2000 (Invitrogen 11668027). Subconfluent 293T cells cultured in 10cm dishes were transfected with 10ug of each expression construct. Nuclear extracts were harvested 48 hours later using standard protocol for extraction of DNA-binding proteins [12]. 500mM Sodium Orthovanadate (SIGMA S6508) was diluted in PBS supplemented with .05% H2O2. Cells were treated at a concentration of 100uM for 15 minutes at 37 degrees Celsius and washed once in PBS prior to harvest.

GST interaction assays

GST affinity purification and protein interaction studies were performed as described [13]. Briefly, GST-fusion constructs for either Eya3 wild-type or D248N C-terminus (AA 236-510) were expressed in BL21 cells. 300ml cultures of transformant
cells were grown to OD$_{600}$ of approximately 0.6 and induced with 0.1M IPTG. Cultures were incubated for 3h, post-induction and were subsequently harvested, lysed, sonicated and incubated with glutathione-agarose beads (SIGMA G4510) for 1hr, followed by extensive washing. Purified protein immobilized on the beads was incubated with nuclear extract from 293T cells transfected with FLAG-tagged SIX1, DACH1, or RNAP2 CTD for 2h and interactions were resolved by SDS-PAGE followed by anti-FLAG western blot.

**Antibodies**

Antibodies used: anti-FLAG (SIGMA F1804, F7425), anti-HA (Roche 1867423), anti-phosphotyrosine (Upstate 05-321)

**References:**


2.2 Identification of Six/Eya Target Genes and Transcriptional Analysis of Eya Phosphatase activity

Identification of Eya phosphatase substrates using a biochemical approach proved to be challenging. Eya has been primarily characterized as a transcriptional co-factor and one of our initial goals was to understand the molecular mechanism by which Eya activates target gene expression during mammalian development. Specifically, how does Eya phosphatase enzymatic activity relate to this mechanism? I decided to shift focus to understanding Eya phosphatase activity in the context of its developmental and transcriptional functions.

One of our initial assumptions was that that phosphatase activity of Eya would significantly affect the transcriptional output of Six/Eya target genes. This assumption was based primarily on single-cell nuclear microinjection experiments using a Six1-responsive reporter construct and Eya3 wild type or phosphatase mutant expression constructs [1]. I postulated that a responsive, phosphatase-dependent target gene could be used as a read-out for potential phosphatase substrates in a relevant cell line, such as 293T human embryonic kidney cells. Mutations in the phosphorylation sites of Eya substrates should theoretically rescue changes in target gene expression in the absence of Eya. For example, if the histone acetyltransferase CBP is a key substrate, alanine mutations if the phosphorylated residues on CBP should mimic constitutive EYA activity and rescue the loss of target gene activation after EYA knockdown by
siRNA. In this manner we hoped to test a set of potential phosphatase substrates including CBP, Six1 and Dach1.

I initially investigated the utility of a Myogenin-luciferase reporter construct for these transcriptional experiments. Myogenin is a well-characterized target gene

![Myogenin-Luciferase Reporter Activity](image)

**Figure 10**

**Eya3 wild type and phosphatase mutant activate a Six1-dependent reporter to an equal extent.** A reporter construct consisting of the Myogenin proximal promoter fused to Luciferase was transfected into 293T cells in conjunction with Six1 or Six1 with Eya3 wild type or D246A mutant. Both Eya3 expression constructs were able to reverse repression seen with transfection of Six1 alone.
for Six1 in the context of skeletal muscle development, and a reporter construct consisting of the proximal promoter for Myogenin fused to luciferase was shown to be responsive to Six1 in transient transfection assays [2]. I transfected this reporter into 293T cells in combination with Six1 alone or with Eya1. Six1 alone was seen to strongly repress reporter gene expression, while transfection of Six1 with Eya3 reversed this repression, as previously published [1] (Fig. 10). To check for phosphatase-dependence of this Eya-mediated transcriptional activity, I transfected an enzymatically dead Eya3 mutant (Eya3 D246A) expression construct, instead of the wild-type. Surprisingly, this mutant showed an approximately equal ability to reverse Six1-mediated repression (Fig. 10), suggesting that phosphatase activity was not involved in Eya-dependent activation of the myogenin-luciferase reporter. I postulated that these results, which were somewhat inconsistent with published data, could be related to the artificial nature of the Myogenin-luciferase reporter construct, and not be representative of true in-vivo target genes for the Six/Eya transcriptional complex. Thus, I decided to utilize an endogenous target gene for Six/Eya in the context of embryonic kidney development, the expression of which could be assayed in 293T cells using quantitative RT-PCR.

At the time that we undertook this study, very few endogenous target genes for the Six/Eya transcriptional complex had been identified. Thus, my first step required identification of novel target genes, and in order to identify true, biologically relevant Eya targets we turned to the mouse development system. Eya1 and Six1 knockout mice show profound developmental defects in multiple organs including the inner ear, skeleton and kidney. We focused on kidney tissue due to the high penetrance and
severity of the knockout phenotype as well as the availability of an immortalized embryonic kidney cell line amenable to the transfection experiments we had planned (293T cells). As the homeodomain DNA-binding transcription factor Six1 is thought to be the primary transcriptional binding partner for Eya1, directing Eya to target gene promoters, we decided to utilize microarray analysis on RNA from Six1 wild-type and homozygous mutant mouse embryos to detect changes in target gene expression for the Six1/Eya1 transcriptional complex. In the Six1 knockout, the kidney is frequently completely absent by the latter developmental stages (e17.5-e18.5) [1]. In order to maximize available material, we harvested embryonic kidneys at e12.5 from Six1 knockouts and wild-type sex-matched littermates for RNA extraction. Dissection of Six1 knockout embryos at e12.5 revealed a significant amount of embryonic kidney material still to be present. Triplicate samples were harvested for each genotype and RNA was purified using the Qiagen RNEasy Micro Kit. Quantitative RT-PCR was performed on the purified RNA from Six1-/- and +/+ embryos using primers to the kidney-specific gene KSP-Cadherin-16 in addition to Six1 and the housekeeping gene GAPDH. KSP-Cadherin-16 is a cell-surface protein of the Cadherin superfamily that is specifically expressed in the epithelial cells of developing renal tubules [3, 4] and thus serves as an excellent marker for embryonic kidney material. KSP-Cadherin-16 was significantly expressed in both samples, while Six1 expression was virtually undetectable in RNA from the Six1-/- embryos when normalized against GAPDH (Fig. 11). RNA from each kidney sample was quantitated via nano-drop, revealing a range of sample concentrations from 10-50ng/ul. We analyzed the material using the Agilent 20K Mouse Whole Genome Array. According to the manufacturer’s
recommendations, this array platform can successfully analyze as little as 100ng of total RNA. Due to the limiting amount of material collects from some of our kidney samples, we were forced to proceed with RNA samples at this lower limit.

Initial analysis of the array results showed that overall fold changes between transcripts from the $Six1^{-/-}$ and wild-type RNA pools were quite low, with few

![Figure 11](image_url)

**Figure 11**

**Expression of Cadherin 16 and Six1 in dissected embryonic kidney material.** RNA from dissected e12.5 embryonic kidney material from $Six1^{+/+}$ or $^{-/-}$ littermates was assessed for the expression of the kidney-specific gene Cadherin 16 and for Six1 by quantitative RT-PCR. Cadherin 16 expression was comparable in both samples while Six1 message was greatly reduced in $Six1^{-/-}$ samples. Results are normalized to GAPDH.
Table 2
Selected target genes from array analysis. Genes that were consistently changed between the triplicate samples were selected by potential relevance and intensity of signal and grouped based on function.

<table>
<thead>
<tr>
<th>Developmental Transcription Factors/Cofactors</th>
<th>Gene Name</th>
<th>Accession</th>
<th>Fold Change</th>
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<td>Sall1</td>
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changes registering over 2-fold. Additionally, several known targets in addition to the Six1 transcript itself were read as essentially unchanged. This may have been related to low overall fluorescence intensity for several of these reads, which may have been indicative of problems with the spotting of the array. Problems with signal intensity suggested to us that the amount of RNA used for array analysis (100ng) was probably far less than ideal and may have resulted in the failure to identify many changed genes. Nevertheless, several interesting potential target genes were identified (Table 2). Six1 is a key transcription factor functioning early in the development of the metanephros, which ultimately forms the mature kidney [5]. As such, we anticipated that multiple downstream kidney-specific transcription factors would be among the target genes in the Six1 transcriptional program identified by the array. Multiple Zinc-Finger containing genes were seen to be specifically down-regulated in the Six1-/- embryos, including the developmentally regulated Kruppel-like zinc-finger transcription factor Glis2 which has been proposed to function in kidney development [6]. Glis2 homozygous mutant mice show adult-onset renal atrophy and ultimately complete renal failure, while in humans GLIS2 mutation has been associated with the autosomal recessive kidney disease Nephronophthisis (NPHP) [7]. Similarly, the homeodomain transcription factor Sall1 was observed to be slightly downregulated in Six1-/- embryos. Sall1 functions in embryonic kidney development and has been implicated in Townes-Brocks syndrome [8, 9]. Significantly, quantitative RT-PCR analysis of Glis2 and Sal1 using kidney RNA from e12.5 Six1 +/- and -/- sex-matched littermates showed a strong and reproducible decrease in message level for
both of these developmentally regulated transcription factors specifically in mutant embryos (Fig. 12), confirming our array results.

A set of cell surface genes with the potential to regulate cell shape, polarity, and cell-cell interactions was also identified as changed based on the array results. This included the gene Ezrin, a member of the ERM family of membrane-associated proteins with crucial function in mediating the association of cell surface proteins with the cellular actin cytoskeleton [10]. Ezrin has been strongly correlated with the metastatic potential of cancer cells from a variety of tumor types and species [11, 12] and may play an important role in cell motility and invasiveness in the context of cancer progression. Ezrin was recently identified as a target for the Six1

![Figure 12](image)

**Figure 12**

*Sall1 and Glis2 are downregulated in Six1−/− embryonic kidney.*

Transcription factors identified as array targets were confirmed by quantitative RT-PCR on RNA from e12.5 embryonic kidney material. Message levels for both Sall1 and Glis2 were significantly reduced in comparison to wild type littermates. Results are normalized to GAPDH.
transcriptional program, and overexpression of Ezrin in conjunction with Six1 in rhabdomyosarcomas correlates with malignancy [13]. Also changed was Talin1, another gene that functions in the organization of cell membrane proteins and actin filaments [14] at certain points on the cell membrane and which shares a FERM domain with Ezrin [10]. One possibility suggested by these array results is that Six1 regulates a set of genes that control the complicated cell migration and morphology changes that accompany the condensation of mesenchymal cells around the uteric bud during nephrogenesis. Simultaneously, these target genes may function during tumor progression to promote metastatic potential, and may explain and expand the role of Six1 as an oncogene. The expression changes of these genes in response to Six1 depletion and their functional relevancy to the Six1-/- phenotype remains to be confirmed by independent experiments.

We also identified the gene Granulin in our array set as being significantly downregulated in the absence of Six1. Granulin is a secreted growth factor of approximately 6kDa derived from a larger precursor protein, progranulin [15]. Granulin functions to promote cell proliferation during development and has been implicated as an oncogenic factor promoting proliferation and metastasis in ovarian cancer [16]. As previously mentioned in the context of the putative target Ezrin, Six1 has increasingly been linked to tumor progression [17-19]. In addition to activating cell proliferation genes such as cMyc [1] which could have an active role in Six1-dependent cancer progression as well as survival and proliferation of progenitor cell populations during devleopment, Six1 may activate a set of cytoskeletal genes and
secreted growth factor genes that are involved in the cell-cell interaction and cell migration processes that are crucial for invasive cancer cells.

Several cytochrome P450 genes were identified on the array as being downregulated in the absence of Six1. These well-studied enzymes function in the metabolism of specific endogenous compounds as well as the processing of exogenous factors such as therapeutic drugs or xenobiotics [20, 21]. Of note, the cytochrome most dramatically downregulated in the absence of Six1 was Cyp26b1, which has been implicated in the metabolism of retinoic acid and is thought to maintain low levels of RA signaling in specific tissues during development [22, 23]. Cyp26b1 has been reported to be expressed in the developing kidney [24]. Thus Six1 may have a

**Figure 13**

A set of cytochrome P450 genes are downregulated in *Six1*/*- embryonic kidney. Several genes encoding cytochrome P450 enzymes were identified as possible Six1 targets based on array analysis. Quantitative RT-PCR was performed for cytochrome P450 family members 26, 2, and 1 using RNA from e12.5 embryonic kidney material. Message levels for all three were modestly reduced in the knockout in comparison to wild-type littermates. Results are normalized to GAPDH.
function in cellular metabolic events which help to control the responses of developing kidney cells to external stimuli. The message levels of the three cytochromere P450 genes identified in the screen were separately checked by qRT-PCR on material from e12.5 Six1 wild-type and homozygous mutant kidneys. A modest decrease in gene expression was observed (Fig. 13).

The array results identified multiple sets of potential target genes from the Six/Eya transcriptional complex. Our goal was to find direct targets with a strong transcriptional response to changes in the transcriptional activity of the Six/Eya complex. Co-committant with our array studies, work from Li Chai and colleagues confirmed that the transcription factor Sal1 was a bona-fide Six1 target gene in embryonic kidney cells [25]. These authors identified a single Six1 binding site within the proximal promoter of Sall1, approximately one kilobase upstream of the transcriptional start site. A reporter construct of the Sall1 promoter fused to luciferase was shown to be activated by various Six-family homologues and Six1 was shown to directly bind to the promoter region of Sall1 by gel-shift assay [25]. As Sall1 was also identified as a Six1 target by our array analysis, I chose to focus on Sall1 as a transcriptional read-out of Eya phosphatase activity. In order to confirm that Sall1 was a direct target for Eya as well as Six, I performed chromatin-immunoprecipitation using a guinea-pig antibody against Eya3 which I had previously generated and characterized. Using PCR primers specific to the Six1 binding site in the Sall1 promoter, I observed that that Eya3 was specifically recruited to this site in proliferating 293T cells and not to an intronic control region approximately 8Kb downstream (Fig. 14). Thus, Eya3 seems to be directly recruited to the promoter of
actively transcribed Sall1 along with Six1 in 293T cells. To assess the functional significance of Six/Eya recruitment to the Sall1 promoter, I checked changes in the level of Sall1 expression in response to overexpression or depletion of Eya and Six gene levels in 293T cells using quantitative RT-PCR. Similar to the results previously obtained using the Myogenin-luciferase reporter, transfection with Six1 alone resulted in a decrease in the expression level of endogenous Sall1 in 293T cells, while transfection of Six1 with Eya1 reversed this repression and actually drove expression above the level seen in a GFP control tranfection (Fig. 15). I next depleted endogenous Eya3 by transfecting 293T cells with siRNA specific to human Eya3. A
modest but reproducible decrease in the expression of Sall1 was observed 48 hours post-transfection. Eya3 activity was rescued by co-transfecting Eya3 siRNA with murine expression constructs to wild-type Eya3 or an enzymatically dead Eya3 mutant (Eya3 D246A) [1]. As expected, the wild-type Eya3 restored Sall1 expression to near-endogenous levels. However, this same rescue effect was seen with the enzyme-mutant, suggesting that a phosphatase-incompetent Eya3 construct is still capable of activating gene transcription as well as the wild-type, at least in the context of Sall1 (Fig. 16).

![Figure 15](image)

**Figure 15**

*Overexpression of Six1 and Eya3 activates expression of Sall1 in 293T cells.* Six1 alone or in conjunction with Eya3 was overexpressed in 293T cells via transient transfection. Changes in endogenous Sall1 expression were assessed via quantitative RT-PCR in comparison to GFP control transfected cells. All results are normalized to GAPDH.
Transcriptional assays using different domains of Drosophila Eya fused to the Gal4 DNA-binding domain have previously demonstrated the potential for the N-terminus of Eya to act as a transcriptional activator [26]. In these assays, addition of the C-terminal EYA domain, which contains the phosphatase catalytic motif, actually decreased the transcriptional activity of fusion protein. This suggested that the N-terminal putative transactivation domain functions independently of the phosphatase.

**Figure 16**

Eya3 wild type and phosphatase mutant both activate Sall1 expression in 293T cells.

Knockdown of endogenous Eya3 expression of 293T cells with specific siRNA resulted in a decrease in endogenous Sall1 expression as assessed by quantitative RT-PCR. Rescuing Eya activity by transfecting back either Eya3 wild-type or Eya3 D246A resulted in a comparable restoration of Sall1 message level. Results are normalized to GAPDH.
domain, which may actually inhibit transcriptional activation mediated by Eya. To explore the function of the N-terminus of mammalian Eya3 in transcriptional activation of Sall1, I used expression constructs of Eya3 that the lab had previously generated representing only the C-terminal EYA domain. This construct, representing amino acids 236-510 contains the binding motif for Six-family transcription factors and had been demonstrated to be a functional phosphatase in-vitro [1], but completely lacks the N-terminal transactivation domain. Endogenous Eya3 was depleted from 293T cells using siRNA and Eya function was rescued by transfecting back either wild-type Eya3 or Eya3 C-terminus. When the expression level of Sall1 was assessed by qRT-PCR 48 hours post siRNA transfection, we observed a near complete rescue effect with the wild-type Eya3 transfection, but no rescue with the N-terminal deletion (Eya3 deltaN) (Fig. 17). Indeed, the C-terminal EYA-Domain, which is a functional phosphatase and should still be recruited to Six1 was wholly incompetent in activating transcription, not only of endogenous Sall1, but also of the Myogenin-luciferase reporter construct (data not shown). My conclusion from this data is that the transcriptional activity of Eya in regards to Sall1, and potentially many other target genes, is wholly attributable to the N-terminal transactivation domain, and is not related to phosphatase function.

The results of my transcriptional studies on Sall1 were surprising in the context of our original assumptions as to Eya phosphatase function. However, a recent publication from the laboratory of Ilaria Rebay supported the idea that Eya primarily activates its transcriptional program in a phosphatase-independent manner [27]. Array
analysis was performed on *Drosophila* transgenic lines expressing either wild-type or two different phosphatase-dead mutant *eya* constructs. The vast majority of changes in target gene expression seen with the wild-type transgene were recapitulated with the phosphatase mutants, indicating that loss of phosphatase activity had little effect on the overall transcriptional output of these transgenes, although a small number of genes were identified that were upregulated specifically by the wild-type transgene. The current data suggests that the original hypothesis that phosphatase activity would
have a crucial role in Eya transcriptional function was incorrect. Instead, phosphatase activity may have role in the transcription of a small set of target genes with key function in organogenesis, or it may function in some cellular process unrelated to transcription, but still crucial for successful organogenesis. Our exploration of the phosphatase activity of Eya had to again change course in order to arrive at the desired goal of substrate identification and mechanistic understanding.

**Methods:**

*Cell culture*

Transfections were performed using Lipofectamine 2000 (Invitrogen 11668027). Subconfluent 293T cells cultured in 10cm dishes were transfected with 10ug of each expression construct or 10nM of each siRNA.

*Quantitative RT-PCR*

RNA was harvested from transfected cells using the RNEasy mini kit, (QIAGEN). cDNA was generated via reverse transcriptase reaction using the Superscript III RT kit (Invitrogen). Quantitative message level analysis was performed using the Mx3000p Real-Time PCR System (Stratagene).

*Animal Care*

Six1 homozygous mutant mice were described previously [1]. Embryos were harvested at embryonic day 12.5 and kidney material was dissected and stored in
RNA-lysis buffer at -80 degrees Celsius. RNA was subsequently purified using the RNEasy micro kit (QIAGEN) for array analysis.

*Luciferase Assays*

Luciferase assays were performed as previously described [1]. Sample values were normalized against Renilla Luciferase control.
References:


Chapter 3

Eya and the DNA Damage Response: Prevention of Aberrant Developmental Apoptosis
Loss of Eya activity results in the failure of organogenesis at least in part due to increased apoptotic cell death in the progenitor cells of target tissues. It has been postulated that increased apoptosis is related to failure of these cells to activate the developmental transcriptional program that specifies them toward proper differentiation, with apoptotic death serving to clear out these non-specified cells as development proceeds. However, data from our lab and others has indicated that the activation of this transcriptional program is largely independent of the phosphatase enzymatic activity of Eya, which is nevertheless crucial for organ development. We hypothesized that Eya could be playing a more direct role in prevention of apoptosis in a phosphatase-dependent manner via a mechanism unrelated to transcriptional regulation. During development, cells are exposed to multiple stress signals that have the potential to trigger genotoxic stress response pathways, including hypoxic and osmotic stress. These stress-response pathways frequently result in apoptotic death and are mediated by signaling cascades that include phosphorylation of nuclear proteins. What if Eya prevents aberrant developmental apoptosis in response to endogenous stress signals by blocking these apoptotic pathways by de-phosphorylating key nuclear signaling factors? We found that, \textit{in-vivo}, phosphorylation of the DNA-damage response factor H2AX was elevated in the metanephric mesenchyme of \textit{Eya1 -/-} mouse embryos in comparison to wild-type embryos. We postulated that H2AX could be a substrate for Eya phosphatase activity and found that Eya1 and Eya3 interacted with H2AX in response to damage signals in human embryonic kidney cells and that Eya3 was specifically phosphorylated itself by
the DNA-damage response kinase ATM, directing it to bind H2AX. H2AX is classically phosphorylated on serine 139 by ATM kinase after a damage event in the vicinity of DNA double strand breaks. We found that Eya dephosphorylated H2AX at a tyrosine residue that is phosphorylated under basal conditions. Dephosphorylation of this tyrosine promotes efficient recruitment of repair factors to the induced phosphoserine mark, and persistent tyrosine phosphorylation after damage promotes the binding of pro-apoptotic factors including JNK1, directly promoting apoptotic cell death. Thus, Eya seems to play a key role in the life or death decisions in response to DNA damage and genotoxic stress. In the absence of Eya, H2AX remains tyrosine phosphorylated with the result being an increased apoptotic response. We propose that in-vivo Eya exists as a dual-function nuclear factor, driving a transcriptional program key for organogenesis in a manner independent of phosphatase activity, and blocking apoptotic cell death by de-phosphorylating H2AX on tyrosine in response to DNA damage events.

3.1 H2AX and the DNA damage response

DNA double strand breaks (DSB) are potentially the most dangerous form of DNA damage a cell can suffer. If not repaired, the portion of the chromosome distal from the centromere will be lost during mitosis, resulting in cell death. To prevent this, a complicated, highly efficient system has evolved to halt cell cycle progression in the event of DSB, and to repair the damaged DNA. This system involves chromatin with specific histone variants and histone tail modification, as well as a host of repair factors, chromatin modifying enzymes and cell cycle checkpoint genes. Many of the
systems activated upon DSB to repair DNA and halt cell cycle progression also function in apoptosis and in the event that the breaks cannot be effectively repaired, cells are removed via apoptotic cell death. Much of the mechanism involved in DNA DSB recognition and repair has been elucidated in recent years, specifically the initial response to the break and the factors recruited around the breakpoint early in the response.

One of the central features of the cellular response to double strand breaks centers around the histone H2A variant H2AX [1]. H2AX is thought to comprise 10 to 20 percent of the H2A in a given cell and differs from H2A in its C-terminal tail. Significantly, the tail region of H2AX contains an SQE motif at serine 139 which is a consensus target site for PI3-K family kinases. It has been established that upon DNA damage H2AX is rapidly phosphorylated on serine 139 for long stretches at both ends of a DNA double strand break. These stretches of serine 139 phosphorylated H2AX (γH2AX) extend for over a megabase and are easily visible using a phospho-specific antibody to stain nuclei of cells treated with a DSB-inducing agent such as gamma irradiation [2, 3]. A characteristic pattern of punctate γH2AX foci rapidly forms, presumably with a break or cluster of breaks at the center of each focus. Interestingly, while flanked by very large stretches of γH2AX, DSBs are free of γH2AX in the regions immediately proximal to the breakpoint (1-2kb) according to chromatin-IP experiments performed in yeast [4]. The kinases that produce γH2AX were found to be PI3-K family members ATM, ATR, and DNA-PK. How these kinases are activated in response to DNA damage is not known, but evidence suggests that ATR primarily phosphorylates H2AX in response to replication stress, possibly due to DSB
arising from stalled replication forks, and to UV radiation [5]. ATM is the primary kinase in response to gamma-irradiation, although some experiments have suggested redundancy between ATM and DNA-PK in this context. Thus, these kinases function in semi-distinct pathways. Additionally, dependent of cellular context, still other kinases outside of the PI3K family are capable of phosphorylating serine 139 on H2AX.

Serine phosphorylated H2AX serves as a binding site for multiple repair factors which are also recruited into the foci that form around breaks. Primary among these factors is MDC1, which has been demonstrated to bind directly to phosphorylated serine 139 via its BRCT domain [6]. MDC1 also directly binds to a set of repair factors known as the MRN complex which includes MRE11, RAD50, and NBS1, and can be thought of as an adaptor protein as it recruits these factors to break sites. Furthermore, NBS1 has been shown to directly bind activated ATM [7], and it is thought that constant recruitment of ATM to MRN complex bound at the break site results in the phosphorylation of the next adjacent H2AX-containing nucleosome, causing the spreading of phosphorylated H2AX typically seen in foci. Although MCD1 plays a central role in recruiting repair factors to γH2AX-decorated chromatin, it is not absolutely required for initial recruitment of all repair factors. Experiments in H2AX-deleted mouse embryonic fibroblasts revealed that the repair factors 53BP1 and BRCA1 were able to effectively form foci similar to those seen for gamma-H2AX after exposure to IR, even in the absence of H2AX protein, although disassociation of these factors from the foci was much more rapid [8, 9]. Thus, other histone marks are hypothesized to function in the repair response in conjunction with γH2AX. For
example, the Tudor domain of 53BP1 has been shown to effectively bind histone H4 tri-methylated on lysine 20, although there is no evidence that this mark is induced upon DNA damage. Recently, the ubiquitin ligase RNF8 was found to be recruited to damage foci and its activity was seen to be required for effective 53BP1 recruitment [10, 11]. RNF8 specifically binds to MDC1 that has been phosphorylated by ATM, and subsequently ubiquitylates H2A, H2AX or both. A combination of H2AX phosphorylation, H4 methylation, and histone ubiquitylation seems to function in effective 53BP1 recruitment and retention in repair foci.

Repair of DSB generally occurs via homologous recombination driven by the recruited factors, and as the repair process proceeds γH2AX foci are removed. Removal seems to be mediated by a combination of de-phosphorylation of S139 and removal of the entire histone in a ubiquitin-dependent manner. In yeast, H2AX is first released from the chromatin and then de-phosphorylated by the heterotrimeric HTP-C complex which contains the phosphatase Pph3 [12]. In contrast, metazoan cells primarily remove H2AX via directly dephosphorylating it on chromatin via the serine phosphatase PP2A [13]. PP2A has been shown to be directly recruited to repair foci late in the repair process by an as-yet unknown mechanism. Loss of either PP2A in mammalian cells or Pph3 in yeast results in defects in DNA repair and increased sensitivity to DNA damage, indicating that removal of γH2AX foci is crucial for successful completion of the repair process.

Of note, recent work has suggested that a portion of H2AX is released from chromatin shortly after induction of DSB in a ubiquitin-dependent manner. It has been known for some time that the histone acetyltransferase TIP60 and its yeast
homologue NuA4 are recruited to repair foci and have an important function in the repair process. Work by Ikura, et. al. suggests that TIP60 is recruited in a complex with the ubiquitin ligase UBC13 [14]. TIP60 acetylates H2AX on lysine 5 immediately after damage induction and subsequently, in manner dependent on K5 acetylation, UBC13 ubiquitylates H2AX on lysine 119. After treatment with IR, both monoubiquitylation and polyubiquitylation of H2AX was seen to increase, and prevention of either acetylation of K5 or ubiquitylation of K199 by mutation of these residues reduced cell viability in response to IR. Polyubiquitylation of H2AX occurred quickly, within 5 minutes post-IR treatment, and coincided with release of H2AX from chromatin, as seen by fluorescence recovery after photobleaching (FRAP) experiments [14]. While rapid removal of H2AX from chromatin immediately after induction of DSB seems contradictory to the established role for H2AX in mediating the repair response, it may reflect the local removal of H2AX from the area immediately proximal to the breakpoint. It is theorized that incorporation of gamma-H2AX leads to chromatin condensation which co-insides with transcriptional repression, and thus it may be necessary to free up the area surrounding the break such that the recruited repair factors have access to the broken DNA ends.

It is becoming increasingly apparent that γH2AX foci are characterized by a number of post-translational modifications in addition to phosphorylation of S139. However, our understanding of why these large foci form is still far from complete. One interesting distinction is the vastly increased size of the spreading of γH2AX in mammalian cells in comparison to yeast. The increased size may reflect an amplification of the initial break signal in order to trigger cell cycle arrest, as the foci
effectively titrate away factors required for cell cycle progression, in addition to
transcriptionally silencing large portions of the genome. It has also been proposed that
γH2AX foci serve to hold the two broken DNA strands together [15]. H2AX-deficient
mice have been shown to be predisposed to deleterious chromosomal translocations,
presumably resulting from repair of mismatched chromosomes following DSB
induction, that can lead to thymic lymphoma [8]. The protein complexes that form on
γH2AX feature multiple protein-protein and protein-DNA interactions, and thus the
large foci could serve to cross-link broken ends together based on these interactions.
It has also been demonstrated in yeast that a Cohesin domain is established at sites of
DSB based on γH2AX foci formation as a late step in DSB repair [16]. Cohesin
complex serves to tie sister chromatids together and thus may aid in proper repair of
DSB via homologous recombination. Another insight to the function of γH2AX foci
may be the recent observation that foci form preferentially in euchromatic rather than
heterochromatic regions [17]. Double-labeling experiments performed on IR-treated
cells with antibodies to γH2AX and marks for heterochromatin including histone H3
tri-methyl lysine 9 and HP-1 suggested that there is little overlap of these marks. If
these results are accurate, it would suggest two possible conclusions; radiation-
induced DSB preferentially form in euchromatic regions, or that there are differing
mechanisms for the repair of breaks that occur in euchromatic or heterochromatic
regions, with γH2AX foci being specific to the repair of euchromatic breaks.

The well characterized function of H2AX in the repair response is not the only
role H2AX plays in the nucleus. The cellular response to DNA damage hinges on a
choice between repairing the damage and removing the damaged cell via apoptotic
cell death. Many factors that are crucial for DNA repair also function in apoptotic pathways. One example is the kinase ATM, activated by DNA double strand breaks. ATM directly phosphorlylates H2AX and is required for the formation of gamma-H2AX foci, as discussed previously, but ATM also phosphorylates and stabilizes p53, the classic pro-apoptotic tumor suppressor gene, as well as the checkpoint kinases Chk1/2, which can promote apoptosis via activation of p73 [18]. One potential explanation for the dual functions of ATM involves the level to which p53 is activated. Upon initial induction of DSB, p53 is activated by ATM at a low level that is sufficient to activate transcriptional targets such as p21 which promote cell cycle arrest, preventing cell division until the damage is repaired. However, if the breaks are not efficiently repaired and ATM activation is sustained, stabilized p53 accumulates in the cell beyond a certain threshold level whereupon it activates pro-apoptotic transcriptional targets such as FAS, PUMA, and BAX. Similarly, evidence supports a direct role for H2AX in apoptotic response in addition to its role in DSB repair. Mice homozygous null for H2AX display a mild phenotype featuring increased sensitivity to DNA-damaging agents [19]. Combinatorial deletion of H2AX and p53 results in a much more severe phenotype characterized by increased propensity for lymphomas [20]. As lymphoma is known to frequently arise from oncogenic translocations, the H2AX/p53 phenotype may be attributed to defects in proper repair of DSB, particularly during V(D)J recombination in B-cells. However, H2AX has also been proposed to have tumor suppressor function not directly related to its DSB repair activity. Studies in cells from gastrointestinal stromal tumors treated with the apoptosis inducing agent imatinib (Gleevec) suggested that H2AX protein is
specifically downregulated in these cells and part of the drug-induced apoptotic pathway requires increased cellular levels of serine-phosphorylated H2AX protein [21].

A detailed study from Lu, et. al. demonstrated the requirement for H2AX in apoptosis mediated by JNK kinase [22]. JNK kinases are MAPK-family kinases that are activated in response to cell stress. They are selectively activated in response to UV radiation, hypoxic stress, and DSB among other stimuli and function to promote apoptosis both at the transcriptional level via the activation of AP-1 through the phosphorylation of c-JUN, and by phosphorylation of pro-apoptotic targets in the cytoplasm such as Bcl2-family proteins on the mitochondrial membrane [23]. It has been demonstrated that JNK-induced apoptosis is dependent on cytochrome-C release and subsequent caspase activation, possibly mediated by the phosphorylation of mitochondrial membrane targets [24]. Lu, et. al. demonstrated that in a mouse epidermal cell line, UV radiation induced rapid phosphorylation of S139 of H2AX, but did not result in DSB or ATM/ATR activation. H2AX S139 phosphorylation in this context was shown to be due primarily to activated JNK, and this phosphorylation event was shown to be required for UV-induced apoptosis mediated by JNK activation. H2AX -/- MEF cells were resistant to UV or etoposide induced apoptosis, and pre-treating the H2AX WT MEF cells with a chemical JNK inhibitor blocked apoptotic response to UV to a similar level as seen in the H2AX -/- cells. H2AX phosphorylation occurred independently of caspase 3 activation. H2AX was hypothesized to aid in DNA cleavage by caspase-activated DNAse (CAD), possibly through a direct interaction between CAD and H2AX, helping to recruit the enzyme to
DNA. Another possibility is that phosphorylation of H2AX alters local chromatin structure, making DNA more accessible to activated CAD. These results provide an intriguing mechanism for the previously identified pro-apoptotic function of H2AX and support the idea that H2AX, like many other factors activated in response to DNA damage, plays a role in both DNA repair and apoptotic cell death.

Although our understanding of H2AX function in DNA damage response is rapidly expanding, many questions still remain. In a typical cellular response to DNA DSB-inducing agents, both JNK and ATM/ATR kinases are activated. What cellular mechanisms regulate the choice of ATM/ATR phosphorylation of H2AX as part of a repair process versus JNK phosphorylation of H2AX in apoptosis? Why does H2AX phosphorylated by ATM/ATR form into discrete foci, while H2AX phosphorylated by JNK does not? How is it that the same modification (phosphorylated serine 139) is the target of two distinct pathways with two differing outcomes? Answers may lie in a more detailed analysis of the modifications of H2AX other than S139 phosphorylation. It is becoming apparent that while histone tail modifications play a key role in regulation of transcription, replication and repair, the individual marks exist within a complex scheme where specific combinations of post-translational modifications mediate key interactions with specific outcomes.
3.2 Eya-Dependent Dephosphorylation of Tyrosine-Phosphorylated H2AX Regulates Apoptosis and Cell Survival Decisions

DNA lesions with the potential to trigger apoptotic cell death are frequently incurred during cellular processes such as DNA replication and transcription, and particularly during embryonic development, where cell stress conditions are a frequent occurrence and improper regulation of cell survival and apoptotic responses can have profound effects on organogenesis [25]. The developmentally regulated transcriptional co-factor Eya is a component of the retinal determination (RD) pathway that controls the development of various organ systems in metazoans, including the kidney [26-28]. The primary phenotypic consequence of loss of Eya activity is increased apoptotic cell death in early tissue primordium and subsequent agenesis of target tissues [29, 30]. Previous work by our lab and others identified a phosphatase enzymatic domain in mammalian Eya1-4 as well as the Drosophila homologue eyes absent (eya), and demonstrated that Eya is a functional tyrosine phosphatase [31-33]. Here, we demonstrate that increased apoptosis seen in the absence of Eya is due to persistent phosphorylation of H2AX Y142, a mark which is a component of the mechanisms that distinguish between apoptotic and repair responses to genotoxic stress.

Eya-H2AX interactions

Consistent with its importance in mammalian organogenesis, an increase in TUNEL-positive cells can be seen within the metanephric mesenchyme of the
developing kidney in transverse sections from Eya1−/− embryos at embryonic day 10.5 (Fig. 18a) [30]. TUNEL positive cells were also seen within the developing renal tubules of Eya1−/− embryos at e11.5 (Fig. 18a). In sagittal sections from embryonic day 11.5, loss of kidney tissue was apparent by H&E staining as well as immunostaining with the kidney-specific marker KSP-cadherin-16, which labels developing renal tubules (Fig. 18b). In order to understand the potential role of the phosphatase enzymatic activity of Eya in a biological context, we examined nuclear phosphoproteins known to regulate apoptosis that could be substrates for Eya phosphatase activity. Nuclear phosphorylation of the histone variant H2AX was recently shown to be a crucial component of apoptosis induced by the activation the JNK/SPAPK stress response pathway [22], in addition to having a well-studied role in DNA damage repair [8, 34-36]. We therefore checked for increased H2AX phosphorylation in the absence of Eya, in kidney material from sagittal sections of embryonic day 11.5 Eya1−/− and Eya1+/+ littermates and using an antibody to serine139-phosphorylated H2AX (γH2AX). This revealed a distinct increase in γH2AX whole-nuclei staining specifically in Eya1−/− metanephric mesenchyme, which was absent in the wild-type sections (Fig. 18b).

Because the developing kidney is exposed to localized hypoxia during early development as the rapidly proliferating organ outgrows the local vasculature, leading to activation of stress response pathways and increased generation of reactive oxygen species [37, 38], we considered the possibility that apoptosis induced in the absence of Eya might be related to altered DNA damage response pathways. To mimic the events
in the Eya1−/− kidney in a cell model, we depleted endogenous Eya1 or Eya3 in 293T human embryonic kidney cells using specific siRNAs (Figure 19) and then subjected the cells to hypoxic conditions for 24 hours. Eya1 and Eya3 have been previously qualified as effective tyrosine phosphatase enzymes and both are expressed in 293T cells. Interestingly, knockdown of either Eya1 or Eya3 using specific siRNAs caused a significant increase in TUNEL-positive apoptotic nuclei in response to hypoxia (Fig. 18c). Next, we directly induced DNA double strand breaks by subjecting the cells to ionizing radiation after Eya knockdown by siRNA. Analogous to the results observed in response to hypoxia, Eya1- or Eya3-depleted cells were more sensitive to radiation-induced DNA damage, undergoing apoptosis at an approximately 10-fold higher rate than control siRNA transfected cells (Fig. 18d). Thus, in embryonic kidney cells, both in vivo and in culture, an increase in apoptotic cell death is observed in the absence of Eya that may be related to the cellular response to DNA damage, which involves γH2AX [1, 34].

Figure 19. Quantitation of siRNA knockdown. siRNA knockdown of Eya1 (A), Eya3 (B), and Fe65 (C) in 293T cells as assessed by quantitative RT-PCR demonstrates effective loss of message in comparison to a nonspecific control siRNA.
We therefore investigated a potential interaction between Eya and H2AX by co-immunoprecipitation assays using 293T embryonic kidney cells before and after exposing the cells to ionizing radiation to induce DNA damage. We could detect interactions between H2AX and wild-type Eya1 or Eya3 only under DNA damage conditions (Fig. 20a). Eya was capable of interacting with H2AX in the context of chromatin, based on co-immunoprecipitation experiments using fixed sonicated chromatin from 293T cells as input (Fig. 20b). In response to IR-induced double stranded DNA breaks, H2AX is phosphorylated by ATM/ATR PI3K-family kinases on chromatin forming long stretches of serine phosphorylated γH2AX flanking the break visible as γH2AX immunostained foci [2]. Immunostaining of transfected HA-tagged Eya1 or Eya3 protein in 293T embryonic kidney cells revealed a clear co-localization of Eya with γH2AX foci after treatment with IR (Fig. 20c, d). These results suggest that in response to damage, Eya is recruited to H2AX foci that mark DNA double-strand breaks. To formally test this, we utilized the estrogen receptor-I Ppol system [39, 40], in which 4-hydroxytamoxifen (4-OHT) is used to induce activation of the eukaryotic homing endonuclease I-Ppol which then generates double stranded breaks at defined genomic loci, including a site on chromosome 1 within an intron of the DAB1 locus. ChIP analysis following 4-OHT induction of I-Ppol in 293T cells revealed that γH2AX and Eya3 were present at a 6 hour time point at a 4kb region flanking the I Ppol cut site, which is in consistent with a direct role for Eya in the cellular response to genotoxic stress (Fig. 20e).

Interestingly, we found that Eya3 is serine phosphorylated in 293T cells in response to genotoxic stress (Fig. 21a), consistent the recent identification of Eya3 as a
potential substrate for the DNA damage-response protein kinases ATM and ATR [18, 41, 42]. Inhibition of ATM/ATR function, by pretreating cells with the PI3K inhibitor caffeine, blocked the interaction between Eya3 or Eya1 and H2AX in response to ionizing radiation (Fig. 21b). Because serine 219 of Eya3 was identified by mass spectroscopy as a target residue for ATM/ATR phosphorylation [42], a S219A Eya3 mutant was tested for co-localization with γH2AX and interaction with H2AX in response to IR. The non-phosphorylatable mutant Eya3 construct failed to form damage-dependent nuclear foci or interact with H2AX after IR treatment (Fig. 21c, d), indicating that ATM/ATR phosphorylation of Eya3 on serine 219 is crucial for directing Eya-H2AX interactions. Because Eya1 and Eya3 are seen to interact in 293T embryonic kidney cells both before and after treatment with ionizing radiation (Fig. 21e), we suspect that regulation of Eya3 via damage-dependent phosphorylation at serine 219 is one cue that may direct both Eya1 and Eya3 to gamma-H2AX, indicating that these covalent modifications of H2AX and Eya may act as sensors for the DNA damage response pathway.

**H2AX is an Eya tyrosine phosphatase substrate**

We next tested whether the interaction between H2AX and Eya could represent a substrate-enzyme relationship, with H2AX acting as a direct substrate for Eya phosphatase activity, dependent on ATM/ATR licensed interactions. Preliminary support for this hypothesis came from experiments using a putative substrate-trapping mutant [43] Eya3 (EYA3 D248N) which showed increased affinity for H2AX in untreated 293T cells by co-immunoprecipitation (Fig. 22). Because purified Eya
exhibited no in-vitro phosphatase activity toward phosphorylated S139 of γH2AX (data not shown), and Eya appears to be a tyrosine-specific phosphatase [31-33], we assessed its activity as a tyrosine phosphatase on γH2AX. H2AX purified either from 293T cells or from bovine histone fraction possesses tyrosine phosphorylation as seen using a phosphotyrosine-specific antibody (Fig. 23a). This tyrosine phosphorylation mark on H2AX decreased in response to DNA damage induced by ionizing radiation, the topoisomerase I inhibitor CPT, or hypoxia (Fig. 23b). To determine whether this H2AX phosphorylation mark might be a target of Eya phosphatase activity, we utilized an in-vitro phosphatase assay, mixing purified HA-tagged Eya1 or Eya3 with H2AX protein. Wild-type Eya effectively removed the phosphotyrosine mark from H2AX, while the phosphatase-inactive mutant Eya proteins (Eya1 D323A or Eya3 D246A) had little or no effect (Fig. 23c). These data establish the biochemical ability of Eya to directly dephosphorylate tyrosine phosphorylated H2AX in-vitro.
To confirm this activity in a cellular context, 293T human embryonic kidney cells were transfected with siRNA against Eya1 or Eya3 or control siRNA and subsequently exposed to ionizing radiation. In contrast to untransfected cells or cells receiving control siRNA, which displayed a loss of γH2AX tyrosine phosphorylation in response to damage as seen previously, Eya siRNA-treated cells showed significantly increased γH2AX tyrosine phosphorylation levels as assessed by western blot analysis (Fig. 23d). Rescuing Eya function by expressing wild-type murine Eya1 or Eya3 constructs, not targeted by the siRNAs, into these siRNA-depleted cells reversed this increased H2AX phosphorylation, while a phosphatase-dead mutant for either homologue failed to rescue H2AX phosphorylation (Fig. 23e). Together, these data indicate that Eya specifically de-phosphorylates H2AX on tyrosine both in-vitro and in the context of embryonic kidney cells undergoing DNA-damage response. The observation that depletion of either Eya1 or Eya3 alone proved to be sufficient to fully block H2AX tyrosine de-phosphorylation in these cells suggested a lack of compensatory activity by these two homologues, suggesting instead cooperative function. Because Eya1 and Eya3 co-purify in 293T cells before and after damage (Fig. 21e), we are tempted to suggest that, specifically in the context of the embryonic kidney, Eya1 and Eya3 may form a stable complex with exhibits phosphatase activity toward γH2AX with both components required for the overall stability of this enzymatic complex.

**Eya-dependent H2AX Y142 de-phosphorylation: function in apoptosis**

To begin to evaluate a possible connection between Eya-mediated tyrosine dephosphorylation of H2AX and modulation of the apoptotic response, we first sought
to identify precisely which H2AX tyrosine residue(s) were phosphorylated. Mutagenesis of each of the four tyrosine residues in H2AX revealed that only mutation of tyrosine residue 142 blocked H2AX tyrosine phosphorylation as assessed by western blot analysis (Fig. 24a), indicating that Y142 was the only phosphorylated tyrosine. FLAG-tagged H2AX Y142F mutant was phosphorylated on S139 in response to damage, although at levels significantly lower than FLAG-tagged wild-type H2AX. (Fig. 24b), indicating that Y142 phosphorylation does not function as a pre-requisite for S139 phosphorylation in the DNA damage response, but may promote efficient serine phosphorylation.

It has been established that a key function of H2AX S139 phosphorylation is to provide a docking site for DNA repair factors near or at DNA double strand breaks [2]. These factors include Mediator of DNA Damage Checkpoint protein 1 (MDC1) which has been shown to bind directly to phosphorylated S139 of H2AX at the sites of double strand breaks [6] based on tandem BRCT1 repeats within the C-terminus of MDC1 [44]. MDC1 functions in the recruitment of a set of ancillary repair factors including MRE11, RAD50, NBS1 (the MRN complex), 53BP1 and BRCA1 [45, 46], although these factors are not wholly dependent on MDC1 and γH2AX for recruitment to breaks [9]. Because an intact H2AX COOH-terminal tyrosine has been found to be required for MDC1-H2AX interaction and productive DNA repair [6], it was of particular interest to determine whether persistent phosphorylation of Y142 in the absence of Eya could negatively impact MDC1 recruitment to the tail of H2AX. We first generated peptides corresponding to the C-terminal tail of H2AX with phosphorylation of both S139 and Y142, or of S139 alone. Affinity purification of
nuclear extract from irradiated 293T cells with each peptide revealed that, in the absence of Y142 phosphorylation, a set of DNA repair factors including MDC1, MRE11 and Rad50 were bound to the S139 phosphorylated H2AX peptide (Fig. 24c). Intriguingly, when phosphorylated tyrosine 142 was present with phosphoserine 139, none of these factors were found to bind; instead, the established pro-apoptotic factor JNK1 was now present (Fig. 24c). The stress-response kinase JNK1, activated by DNA damage and initiating a pro-apoptotic program, has been recently shown to translocate into the nucleus upon activation where it phosphorylates substrates including H2AX S139, an event critical for DNA degradation mediated by caspase-activated DNase (CAD) in apoptotic cells [22]. In agreement with our peptide purification experiments, we were able to detect a robust interaction between transfected wild-type H2AX and endogenous JNK1 in 293T cells in response to high-dose radiation; this interaction was markedly reduced in the case of the H2AX Y142F mutant (Fig. 24d).

It is well established that proteins containing SH2 or PTB domains are capable of directly binding to phosphotyrosine residues. JNK1 does not contain such domain and its recruitment to tyrosine phosphorylated H2AX may be mediated by an adaptor protein. Therefore, it was of particular interest to identify proteins containing SH2 and PTB phosphotyrosine-binding domains that could bind directly to H2AX phosphotyrosine 142 under conditions of genotoxic stress. We tested a set of known nuclear proteins containing these domains for binding to tyrosine-phosphorylated H2AX (Table 3) and found that, while most exhibited no interaction, the PTB-domain protein Fe65 [47], a co-factor for several cell-surface receptors that has been shown to
translocate to the nucleus during DNA damage response and suggested to exert a pro-apoptotic role [48, 49], bound specifically to wild type γH2AX under DNA damage conditions, but not to the γH2AX Y142A mutant (Fig. 24e). This is consistent with recruitment of Fe65 to the Y142 phosphorylated form of γH2AX, in concert with other pro-apoptotic factors, including JNK1. The second PTB domain on Fe65 is proposed to be key for this interaction based on co-immunoprecipitation experiments (Fig. 25).

We postulated that Fe65 may function as an adaptor protein, binding directly to the phosphotyrosine reside on γH2AX and facilitating the recruitment of pro-apoptotic factors such as JNK1. To test this, we knocked down endogenous Fe65 in 293T cells using specific siRNAs (Fig. 19) and assessed the interaction between H2AX and JNK1 in response to genotoxic stress by co-immunoprecipitation. While control siRNA had no effect on the ability of H2AX to co-immunoprecipitate JNK1, knockdown of Fe65 strongly abrogated this interaction (Fig. 24f).

**Figure 25. Fe65 interacts with H2AX via PTB2.** Myc-tagged PTB2 domain of Fe65 was seen to be sufficient to interact with H2AX in 293T cells treated with 30uM Etoposide to induce apoptosis by co-immunoprecipitation.
Our studies suggest that the phosphorylation status of Y142 serves as a key determinant for recruitment of either repair or apoptotic factors to the serine-phosphorylated tail of H2AX in response to DNA damage. Specifically, Y142 must first be de-phosphorylated in order for MDC1 and its associated repair factors to be effectively recruited to γH2AX, while persistent Y142 phosphorylation prevents MDC1 binding and permits active recruitment of pro-apoptotic factors, including Fe65 and JNK1, to γH2AX.

To confirm the function of tyrosine 142 phosphorylation in regulation of the apoptotic response, we transfected H2AX−/− mouse embryonic fibroblasts (MEFs) [19] with either wild type or Y142F H2AX expression constructs. When these cells were subjected to high-dose ionizing radiation, cells expressing H2AX Y142F displayed a greatly reduced apoptotic response in comparison to cells expressing wild-type H2AX (Fig. 24g). In contrast to wild type-transfected cells, in which numerous apoptotic cells were detected by whole-nuclei γH2AX and TUNEL staining, H2AX-Y142F-transfected cells produced approximately 6-fold fewer apoptotic cells. Instead, virtually all MEF cells expressing the mutant H2AX protein displayed a staining pattern of γH2AX foci indicative of a productive DNA repair response (Fig. 24g). These data suggested to us that lack of H2AX Y142 phosphorylation promotes a damage repair response instead of an apoptotic response to DNA damage, in part by promoting successful recruitment of MDC1 and associated repair factors. The presence of Y142 phosphorylation in wild type-H2AX transfected MEF cells is proposed to lead to the recruitment of pro-apoptotic factors such as JNK1 to H2AX,
while blocking the recruitment of the damage repair complex, directly promoting apoptotic response to genotoxic stress.

**Conclusions**

Cells are confronted with DNA-damage resulting from a variety of stimuli under normal, physiological conditions and at each instance the cell must make a fundamental decision between DNA repair or apoptosis. Our data suggest that γH2AX serves as a pivotal component of the adjudication between these two outcomes, with a single post-translational modification, phosphorylation of tyrosine 142, being capable of discriminating between recruitment to γH2AX of functional apoptotic or repair complexes. In the presence of Y142 phosphorylation, binding of repair factors to the phosphorylation mark on serine 139, which is mediated by MDC1, is inhibited (Fig. 24h), while recruitment of pro-apoptotic factors, including JNK1, is promoted. Because *Eya* genes have a specific temporal and spatial expression pattern, we expect that other phosphatases are also capable of removing the γH2AX Y142 mark during genotoxic stress in cellular contexts where Eya gene products are not expressed.

Mutations in the human *Eya1* gene, several of which have been shown to abrogate its phosphatase activity, have been identified as causative for the developmental disorder BOR syndrome, a disease characterized by kidney defects as well as well as inner ear malformation frequently resulting in congenital deafness [50] [51, 52]. In this context the phosphatase activity of Eya may have a conserved function in mammalian organogenesis, acting to block an improper apoptotic response to physiological levels of genotoxic stress in both mice and humans.
The present study indicates that the phosphorylation of tyrosine 142 of H2AX prevents recruitment of repair complexes to phospho-serine 139 of γH2AX, although it is likely that there are additional aspects that underlie the full molecular logic for the dual phosphorylation-mediated events. We hypothesize that the presence of both phosphorylated resides results in direct binding of the PTB-domain factor Fe65, which then mediates the effective recruitment of other pro-apoptotic factors, including JNK1.

3.3 Discussion and Implications

Over the course of this thesis, the study of Eya has lead in directions never originally predicted, but the final destination has been extremely rewarding. Eya is a complicated factor. Indeed, the studies detailed in this thesis have argued that it possesses at least two distinct functions: transactivation of target genes of the Six/Eya transcriptional complex, and prevention of aberrant developmental apoptosis in response to environmental and/or endogenous cell stress. These two functions can be neatly assigned to two different functional domains within Eya itself. While transactivation activity is wholly dependent on the N-terminal transactivation domain and independent of the C-terminal phosphatase domain, inhibition of apoptotic signaling pathways in response to cell stress and DNA damage is completely dependent on phosphatase enzymatic activity. These separate functions will almost certainly both contribute to the overall phenotype seen in the absence of Eya: failure of organ formation due to loss of progenitor cells to apoptosis.

The transcriptional function of Eya family proteins had been conclusively demonstrated prior to the start of this thesis [31, 53]. The N-terminus of all known
Eya homologues, with the possible exception of *Arabidopsis Thaliana* Eya, can serve as an independent transactivation domain, capable of driving expression of reporter genes when fused to an artificial DNA binding domain [54]. The addition of Eya to transcriptional reporter systems responsive to Six-family homeodomain transcription factors switches these reporters from repression to activation in a manner dependent on Six/Eya protein-protein interactions. Thus Eya, which has no intrinsic DNA-binding activity, is thought to synergize with Six proteins to form a heteromeric transcription factor complex capable of driving target gene expression. The N-terminal transactivation domain has not been particularly well characterized however, and its mechanism of action remains obscure. Possibly, the N-terminus recruits co-activators such as CBP and P/CAF to target gene promoters [31], although experiments to demonstrate these interactions remain to be performed. In contrast, interactions have been confirmed between Six and Eya proteins, and proteins of the Dach family. These Ski/Sno-related factors seem to function in the same overall genetic pathways as Six and Eya and are suspected to serve as co-repressors based on sequence homology, although the mechanism of transcriptional repression by Dach has not be demonstrated. One model suggests that Six proteins on DNA recruit Dach for repression prior to engaging Eya activity [31]. Precisely how Eya switches this complex from repression to activation and how Eya recruitment to Six-occupied promoters is regulated is unknown.

While little is known mechanistically in regards to how these transcriptional factors function, the list of known target genes which they regulate is being slowly elucidated. Recent work from the laboratory of Ilaria Rebay provided a set of target
genes from Drosophila based on microarray analysis of transgenic files overexpressing Eya in the head, including genes known to be involved in cell proliferation such as *string (stg)* [55]. Array analysis of embryonic kidney RNA from *Six1* +/+ and +/- littermates undertaken by our lab revealed a set of putative target genes involved in cell proliferation, migration, kidney development, and cellular metabolism. Many of these genes remain to be formally confirmed as direct targets for the Six/Eya transcriptional complex; however they offer interesting hints as to how these factors work to drive kidney development in mammals, and may help our understanding of the function of Six proteins in cancer. *Six1* has been shown to be amplified in certain human breast and cervical cancer cell lines (see Chater 2). Array analysis of *Six1* +/- mouse embryonic kidneys identified a set of developmentally regulated transcription factors including Glis2 and Sall1 which are known to function in mammalian kidney development. Additionally we identified pro-growth signals such as Granulin, cell-cell interaction factors such as Ezrin and Talin1, and Cytochrome P450 metabolic genes such as Cyp26b1. These factors may play significant roles in the mammalian kidney development program which Six and Eya regulate, as well as possessing potential implications for cancer progression. While most of these genes remain to be confirmed as direct transcriptional targets for *Six1*, they represent an intriguing list of potential target genes and imply that Six may regulate a complex genetic program involving multiple cellular processes. While this data does not provide mechanistic insight to the operation of the Six/Eya transcriptional complex, it should help to increase our understanding of how Six and Eya help promote successful organogenesis
during embryonic development, and how they may function in human diseases such as cancer.

Our primary goal was to understand how the phosphatase enzymatic activity of Eya functioned in the context of Eya’s role in embryonic development. Surprisingly, accumulating data from our lab and others has suggested that Eya phosphatase activity is largely unrelated to its transcriptional function. Array analysis by the Rebay lab indicated that wild-type and phosphatase-mutant Eya transgenes both efficiently upregulated a remarkably similar set of target genes [55]. Our own studies have indicated that the mammalian Six/Eya target gene Sall1, a kidney-specific transcription factor, is transcriptionally activated by either wild-type Eya3 or phosphatase-dead Eya3 to an equal extent, while a truncated Eya3 with a functional phosphatase domain but no transactivation domain was incapable of activating Sall1 expression (see Chapter 2-2). This data contrasted to transgenic studies in Drosophila demonstrating that Eya phosphatase activity is ultimately important for proper eye development [32, 33].

While the bulk of current data now indicates that Eya phosphatase activity plays a very limited role in Eya-dependent transcriptional activation, our work has demonstrated that the phosphatase activity of Eya plays a crucial role in regulating cell survival decisions during development in a manner unrelated to transcriptional regulation. During development, primordial cells are subjected to cell stress from a variety of sources, including hypoxia and oxidative stress, which can result in the activation of cell-stress/DNA damage response pathways and ultimately apoptosis. When the DNA-damage response is activated in mammalian embryonic kidney cells,
Eya is directed to the histone variant H2AX at the sites of DNA double strand breaks. H2AX is a component of the damage response network that is basally tyrosine phosphorylated at residue 142 on the C-terminal tail. In response to DNA damage this tyrosine phosphorylation mark is rapidly removed by Eya, as the proximal serine residue 139 is phosphorylated by the DNA-damage response kinase ATM. In the absence of Eya, the phosphotyrosine mark persists, resulting in inefficient binding of repair factors, including MDC1, to the phosphoserine, and instead promoting the recruitment of pro-apoptotic factors including JNK1. This ultimately leads to an increased apoptotic response to cell-stress/DNA damage conditions, which may deplete the progenitor cell pool to such a degree that kidney organogenesis is blocked, as seen in Eya1-/- mouse embryos. Thus, normally functioning Eya has a role as a DNA damage response factor, creating a binding surface on the tail of H2AX via de-phosphorylation of tyrosine 142 that is permissive for the recruitment of MDC1 and associated repair factors. This is a novel function for Eya, completely separable from its more classic role as a transcriptional co-activator. According to our ChIP and immunostaining data, Eya is actively recruited to DNA repair foci during the DNA damage response. These repair foci are thought to be centered around sites of DNA double strand breaks, and as such we suspect that nuclear Eya is relocalized from target gene promoters where it is bound with Six1 to break sites where it associates with H2AX in a complex which probably includes other DNA repair factors. While we have characterized only Eya-dependent de-phosphorylation of H2AX in the context of repair, it is likely that Eya has other functions in the context of DNA repair that have yet to be elucidated, including novel phosphatase targets.
We propose a new model for Eya function based on these findings (Figure 26). In cells of the developing embryonic kidney under basal conditions, Eya functions in conjunction with Six1 based on direct protein-protein interactions as a conventional transcriptional co-activator. The combinatorial transcription factor comprised of Six and Eya drives transcriptional activation of a set of target genes crucial for both proliferation/cell survival and proper differentiation specification of the metanephric mesenchyme. However, in the event of cell stress and activation of the DNA damage response, we hypothesize that Eya is pulled away from Six target genes in a manner dependent on phosphorylation by ATM/ATR. Eya is subsequently directed to H2AX at sites of DNA damage, most likely after H2AX itself has become serine phosphorylated by ATM/ATR. Eya then dephosphorylates tyrosine 142 on H2AX, generating a phosphorylation profile on the tail of H2AX that is conducive for binding of repair factors and which favors productive repair and cell survival over apoptotic death. Finally, after repair has been successfully completed, Eya must be removed from the repair sites and return to its task of driving the expression of Six target genes. This model may help to explain why the phosphatase activity of Eya is crucial for proper embryonic development, yet plays an exceedingly minor role in activation of the Six/Eya transcriptional program. We propose that both activities are required for proper organogenesis, and the kidney agenesis phenotype associated with Eya1-/- mice results from defects in both transcriptional activation and life or death fate decisions in response to cell stress/DNA damage conditions. To what extent each activity contributes to the overall phenotype is unknown, and will most likely require complex transgenic experiments to address.
Many key questions from our work remain unanswered and several components of our model have not been conclusively demonstrated. We demonstrate that Eya1 and Eya3 are both capable of dephosphorylating H2AX in embryonic kidney cells in response to a DNA damage signal. However loss of either factor individually results in a complete loss of H2AX dephosphorylation in response to damage (Fig. 23). Why do Eya1 and Eya3 show a lack of complementarity in regards to phosphatase activity? Preliminary data suggests that Eya1 and Eya3 may exist functionally in a complex *in-vivo*, as they are seen to co-immunoprecipitate with each other both before and after damage. The two factors may influence each other’s stability, such that loss of one leads to loss of the entire complex and loss of total phosphatase activity. This model remains to be formally proven. Additionally, how and to what extent is Eya removed from the Six1 transcriptional complex in the event of DNA damage? How does phosphorylation by ATM/ATR result in Eya relocating en-masse to sites of DNA damage and how is the Six/Eya transcriptional complex ultimately restored? Chromatin immunoprecipitation experiments may help to elucidate the kinetics of Eya re-localization on DNA in response to damage and are currently under consideration.

Eya possesses a distinct spatio-temporal expression pattern, yet the model of H2AX tyrosine dephosphorylation would seem to be ubiquitous component of the DNA damage response in all cells. How is apoptosis resulting from persistent tyrosine phosphorylation of H2AX avoided in cells that do not express Eya, a population that includes most on the cells in an adult organism? We suspect that other protein tyrosine phosphatases function to dephosphorylate H2AX on tyrosine 142 in contexts
where Eya is not expressed and experiments to identify these phosphatases are currently underway.

Our work greatly expands our understanding of the nuclear factor Eya and how it functions in organogenesis. It also adds significant mechanistic insight to the function of H2AX in the decision between survival and apoptosis during the DNA damage response. The connection between Eya and H2AX was unanticipated, but underscores how nuclear transcription factors can possess multiple, separable functions dependent on cellular context. Under basal conditions Eya is a transcription factor in the embryonic kidney, yet under DNA damage conditions it functions as a repair factor controlling survival and cell death decisions.

Eya has been strongly implicated in the human genetic disorder BOR syndrome [50]. This disease results in congenital deafness, kidney defects and craniofacial malformations among other symptoms and mutations of human EYA1 resulting in BOR phenotype have been shown to compromise phosphatase enzymatic activity [51]. Our findings suggest that BOR syndrome may be due in part to inappropriate apoptotic response to genotoxic stress conditions encountered normally during embryonic development in tissues expressing EYA1. Additionally, both Six1 and Eya2 have been shown to be amplified in certain cancers [56-58]. The putative function of these factors was presumed to be related to transcriptional activation of target genes related to proliferation and cell survival. However, our findings suggest that, at least for Eya2, oncogenic function may also be related to a block in apoptosis in response to genotoxic stress due to excessive Eya phosphatase activity.
In identification of H2AX Y142p as a substrate for Eya phosphatase activity, we have greatly increased our understanding of both the function of the Eya phosphatases and the embryonic response to genotoxic stress at the cellular level. The dephosphorylation of H2AX Y142 by Eya in embryonic kidney cells is a key regulatory event determining the cellular response to genotoxic stress and DNA damage. This couples with the separate function of Eya in transcriptional regulation, and contributes to downstream phenotypic consequences of loss of Eya. While much still remains to be elucidated in regards to this system, we feel confident that the work in this thesis represents a significant contribution to the fields of molecular and developmental cell biology.
Methods

Antibodies, Reagents and Cells

The following commercially available antibodies were used: anti-H2AX (Cell Signaling Technology and Abcam), anti-γH2AX (Cell Signaling Technology and Upstate), anti-phosphotyrosine (Zymed and Upstate), anti-KSP-Cadherin 16 (Abcam), anti-HA (Berkeley Antibody Company), anti-FLAG (Sigma), anti-MDC1 (Abcam and Bethyl laboratories), anti-RAD50, MRE11, JNK1 (Abcam and Santa Cruz Biotechnology). Antibody to Eya3 were generated by immunizing guinea pigs with GST-purified peptides representing the amino-terminus of human EYA3 (AA 1-239).

The following commercially available reagents were used: caffeine (Calbiochem). Eya1 and Eya3 siRNAs were purchased from Qiagen. H2AX−/−MEF was kindly provided by Drs. Yang and Song (UCSD). Standard molecular cloning and tissue culture were performed as described by Sambrook and Russell (2001).

Animal Care

Mouse embryos from E10.5 to E11.5 were fixed in 2% paraformaldehyde, penetrated with 24% sucrose in PBS, and embedded in OCT compound for cryosectioning. Serial 14um sections were blocked in 10% normal goat serum/PBS/0.1% Triton-X 100 and immunostained using antibodies to γH2AX or KSP-Cadherin16. Immunostaining was visualized using secondary antibodies conjugated to AlexaFluor-595 (Invitrogen) and sections were mounted using Vectashield mounting media plus DAPI (Vector Laboratories). Parallel sections were stained with Haematoxylin and Eosin as described (Li, et. al., 2003).
TUNEL Staining

TUNEL assay was performed using ApopTag In Situ Apoptosis Detection Kit (Chemicon). Tissue sections were post-fixed in ethanol:acetic acid 2:1 at -20°C for 5 minutes and incubated with TdT enzyme at 37°C for 1 hour. DIG incorporation was visualized using anti-digoxigenin-rhodamine secondary (Roche) and stained sections were mounted using Vectashield mounting media plus DAPI (Vector Laboratories).

Cell Treatment and Transfection/ RNA interference

For hypoxia experiments, 293T cells were transferred to an 8% CO₂, 1% O₂ incubator and maintained for approximately 16 hours. Cells were immediately fixed or lysed upon removal from the hypoxia incubator. Gamma-irradiation of cultured cells was performed at the UCSD Medical Teaching Facility according to established protocols. Cells were transfected using Lipofectamine 2000 (Invitrogen). The cells were gamma-irradiated approximately 36-48 hrs after transfection.

Immunoprecipitation/Western Blot Analysis

For immunoprecipitation and Western blotting, cells were rinsed in PBS, harvested, and lysed in Lysis buffer containing 10% glycerol, 0.5 mM EDTA, 25 mM Tris-HCl (pH8.0), 150 mM NaCl, 1 mM Na₂VO₃, 10 mM β-glycerophosphate, 0.1% NP-40 and 1 mM DTT in presence of protease inhibitors (Roche) and 1 mM PMSF. After clearing by centrifugation, the concentration of the extracts was determined using the bicinchoninic acid protein assay kit (Bio-Rad) and the extracts used for immunoprecipitation. The extracts were incubated with the specific antibody
overnight at 4°C, followed by incubation with protein A/G agarose beads (Santa Cruz Biotech.), washed extensively, and separated by electrophoresis. Proteins were transferred onto nitrocellulose membranes (Bio-Rad) and Western blotting was performed following standard protocols.

**Immunocytochemistry**

Cells were fixed for 15 min with 2% paraformaldehyde in PBS and permeabilized with 0.05% Triton X-100 in PBS for 30 min. After blocking with PGBA solution (0.1% BSA, 0.1% gelatine, 0.1% FBS), cells were incubated with specific antibodies for 2 hrs at RT. Antigen was detected with secondary antibodies conjugated to AlexaFluor-595 or AlexaFluor-488 (Invitrogen). Cells were coverslipped using Vectasheild mounting media plus DAPI (Vector Laboratories).

**In vitro phosphatase assay**

The HA-tagged Eya phosphatase was immunoprecipitated from gamma-irradiated 293T cells using anti-HA affinity resin (Roche). After extensive washing, Eya phosphatase was eluted with HA peptide. The reaction mixture containing purified Eya protein in 100 μl phosphatase buffer (50 mM Tris-HCl, pH 7.0, 5 mM MgCl₂, 10% glycerol, 3mg/ml BSA) and bovine histone (Sigma) was incubated for 60-90 min at 30 °C. The H2AX was immuno precipitated with anti-H2AX antibody and Western blotting was performed.
Peptide affinity chromatography

Biotinylated synthetic peptides (hH2AX 128-142 amino acid) were purchased from Sigma Genosys and Anaspec. For peptide affinity chromatography, biotinylated phosphopeptides and unphosphorylated peptides were coupled to streptavidin-coated Dynabeads M-280 (Invitrogen) for 2 hrs at RT. Beads were incubated with nuclear extract from 200 Gy-irradiated 293T cells and washed extensively with Tris buffered saline (pH 7.5) containing 0.5% Tween 20. The bound proteins were separated by SDS-PAGE using 4%–12% Bis-Tris NuPAGE gel (Invitrogen), followed by Western blot analysis.
References:


**Acknowledgements**

*A revised form of Chapter 3.2 of this thesis has been submitted for publication in the journal Nature under the title “Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions”. The dissertation author was the primary investigator and author of this paper.*
Figure 18. Loss of Eya leads to increased γH2AX-positive apoptotic cells. (A) TUNEL staining reveals apoptotic cells within the developing kidney of Eya1-/- embryos at e10.5 and e11.5 not present in wild type littermates. (B) Abnormal morphology and loss of developing renal tubules within the urogenital ridge (dotted line) in Eya1-/- embryos coincides with increased γH2AX-positive nuclei by immunostaining. In culture, 293T human embryonic kidney cells depleted for Eya1/3 using siRNA displayed increased apoptotic response to hypoxia for 20hrs (2% O₂) (C) or 5Gy IR (D). Cell counts were performed on TUNEL stained cells co-stained with DAPI in triplicate to identify the proportion of TUNEL-positive nuclei. Bar graphs represent mean +/- SEM of fold apoptotic cells normalized to control siRNA from triplicate samples. “*” p < .05.
Figure 20. Eya interacts with H2AX in a DNA-damage dependent manner. HA-tagged Eya1 or Eya3 interacts with FLAG-tagged H2AX in 293T cells in response to IR (5Gy), but not under basal conditions, both in nuclear extracts (A) and on sonicated chromatin (B). Immunostaining of 293T cells demonstrates that transfected, HA-tagged Eya1 (C) or Eya3 (D) localizes to DNA-damage induced foci coincident with γH2AX. (E) HEK293 cells were transfected with ER-I-PpoI, and 48 h after transfection cells were treated with 4-OHT for the indicated times, then fixed and used for ChIP analyses using the indicated antibodies and primers downstream to the I-PpoI cut site on human chromosome 1. Results are the average of duplicate qRT-PCR values, differing by <3%. 
Figure 21. Eya3 phosphorylation by ATM/ATR DNA-damage dependent kinases regulates interaction between Eya and H2AX. (A) Western blot with antibody specific to the phosphorylated target site of ATM/ATR demonstrates phosphorylation of Eya3 in response to DNA damage (5Gy IR). (B) Eya1/3 interaction with H2AX is lost in the presence of a PI3K inhibitor (5 mM caffeine). (C) Mutation of the ATM/ATR phosphorylation site of Eya3 (S219) prevents formation of damage-induced Eya3 foci. Representative examples of foci formation are shown. (D) HA-Eya3 (S219A) fails to interact with FLAG-H2AX in response to DNA-damage (5Gy IR) by co-immunoprecipitation in 293T cells. (E) DNA damage-independent interaction of Eya3 and Eya1 was assessed by co-immunoprecipitation in 293T cells.
Figure 23. Tyrosine phosphorylated H2AX is a substrate for Eya phosphatase. (A) Immunoprecipitation (IP) of H2AX followed by western blot using a specific anti-phosphotyrosine antibody. (B) IP-western of tyrosine phosphorylated H2AX in response to DNA-damage signals. Bars represent quantified western blot signals normalized to untreated cells. (C) In vitro phosphatase assay using wild type Eya1/3 or enzymatically-inactive mutant proteins (Eya1 D323A, Eya3D246A) and bovine histone. Bars represent quantified western blot signals normalized to input. Mean values +/- SEM from triplicate western blot experiments are shown. **p-value <.001. (D) siRNA knockdown of endogenous Eya1/3 in 293T human embryonic kidney cells (48h) and subsequent IP-western for H2AX using anti-H2AX and anti-phosphotyrosine antibodies. (E) Rescue of endogenous Eya function by co-transfection of human siRNA and murine wild type or enzymatically inactive mutant Eya1/3 constructs for 48hrs in 293T human embryonic kidney cells reveals that loss of H2AX phosphotyrosine mark is dependent on Eya phosphatase activity.
Figure 24. H2AX Y142 phosphorylation discriminates between apoptotic and repair responses to DNA-damage. (A) Individual substitution mutations of the four tyrosine residues in H2AX followed by IP-western to detect phosphotyrosine. (B) H2AX (Y142F) mutant maintains phosphorylation of S139 in response to DNA damage (5Gy IR). (C) Differential phosphorylation of Y142 regulates binding of DNA repair factors. Affinity purification performed on nuclear extract from irradiated 293T cells using synthetic peptides representing the C-terminal tail of H2AX bearing S139 phosphorylation +/- Y142 phosphorylation followed by western blot analysis. Co-immunoprecipitaition confirms interaction between wild-type H2AX and JNK1 (D) or Fe65 (E) but not H2AX (Y142F) mutant. Asterisks on Figure 5e indicate Fe65 band. (F) siRNA knockdown of Fe65 in 293T cells blocks the damage-dependent interaction of JNK1 and γH2AX by co-immunoprecipitation in cells transfected with Fe65 siRNA or control siRNA 48 hours prior to harvest. (G) The inability to phosphorylate H2AX Y142 abrogates apoptotic response to DNA damage. H2AX/- MEF cells were transfected with wild type or mutant H2AX (Y142F) expression constructs and exposed to high-dose IR (100Gy). Apoptotic response among transfecants was assessed by γH2AX staining and TUNEL. Bar graphs represent mean +/- SEM of fold apoptotic values for triplicate or greater cell counts of transfected (green) nuclei. Values were normalized to WT-H2AX-transfected samples. “**”p < .001. (H) Proposed model for Y142 phosphorylation status of H2AX in regulation of apoptotic versus repair response.
Table 3 - Selected SH2/PTB-domain containing factors. Nuclear SH2 or PTB-domain containing factors assessed for interaction with the tyrosine-phosphorylated tail of H2AX by peptide pull-down assay. Additionally, the listed nuclear tyrosine kinases were assessed for H2AX-interaction by co-immunoprecipitation.
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<th>SH2/PTB proteins evaluated for H2AX binding</th>
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Figure 26 – Proposed model for the dual nuclear functions of Eya in embryonic kidney cells. Under basal conditions Eya functions as a conventional transcriptional co-activator in conjunction with Six-class homeodomain transcription factors. Eya exists in a complex with Six and other transcriptional co-activators on the promoters of target genes and drives a genetic program required for proper kidney cell development. Under conditions of genotoxic stress, Eya is relocalized to DNA sites proximal to DNA double strand breaks where it interacts with γH2AX and mediates dephosphorylation of tyrosine 142 on γH2AX. The damage-dependent interaction between Eya and H2AX is mediated in part by serine phosphorylation of Eya by the damage response kinases ATM/ATR. Efficient dephosphorylation of Y142 of H2AX results in a phosphorylation profile of the H2AX tail that is permissive for binding of DNA repair factors, including MDC1.