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The Role of SoxE Transcription Factors in Neural Crest and Ear Development

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ABSTRACT

The Role of SoxE Transcription Factors in Neural Crest and Ear Development

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The neural crest is a population of multipotent precursors that are found only in vertebrate embryos. These cells migrate extensively throughout the body and give rise to diverse derivatives, including craniofacial bone and cartilage, melanocytes, and the enteric nervous system. There is a large network of factors involved in neural crest formation, including SoxE factors, Sox9 and Sox10, which are homologous and contain four highly conserved domains. While both are involved in neural crest precursor formation, during neural crest differentiation stages, Sox9 gives rise to facial cartilage while Sox10 gives rise to cranial ganglia and melanocytes. Both of these factors are also involved in inner ear formation, a non-neural crest-derived tissue.

The overall aim of this thesis is to investigate the mechanism by which SoxE factors regulate events in neural crest and inner ear development. The first goal is to investigate if Sox9 and Sox10 are functionally divergent or redundant in the neural crest. In this thesis, experiments performed in *Xenopus* embryos show that these factors can perform equivalently in both inner ear formation as well as early and late neural crest development.

The second goal of this thesis is to examine how Sox9 and Sox10 can function equivalently but are able to participate in the formation of different cell types within such a close proximity to one another. Experiments here show the presence or absence of the post-translational modification, SUMOylation, is used to dynamically regulate cell fate decisions.

The third goal of this thesis is to uncover the mechanism by which SoxE SUMOylation dynamically regulates cell fate decisions, by examining the role of this post-translational modification on the regulation of the melanocyte-specific promoter, Dct. This thesis shows that the absence of SoxE SUMOylation recruits the co-activator CBP/p300 to the promoter, while the presence of SUMOylation recruits the co-repressor Groucho4 and Pax3. This is potentially a commonly used mechanism throughout development, and not specific to the Dct promoter.

The fourth goal is to provide preliminary evidence on potential SoxE-related projects. Data here shows a novel SoxE phosphorylation site, novel Sox10 interaction partners, and the effects of SoxE domain deletion constructs.

ACKNOWLEDGEMENTS

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And finally to my husband Neil - your constant support and encouragement has gotten me here. I love you very much and cannot imagine my life without you. Thank you for all of the wonderful memories you have given me and I look forward to our life together. I couldn't have finished without you.

DEDICATION

This thesis is dedicated to my parents, Doug and Jan Taylor.

LIST OF ABBREVIATIONS

ß-gal, X-gal, or Red gal	beta-galactosidase, X-galactosidase, or Red galactosidase
bHLH	basic helix loop helix
BMP	bone morphogenetic protein
C-	carboxy terminus
CBP	cyclic adenosine monophosphate response element-binding protein
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CNS	central nervous system
Dct/Trp2	dopachrome tautomerase
Dlx	distal-less homeobox gene
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial to mesenchymal transition
FGF	fibroblast growth factor
Fox	forkhead box
GSK3ß	glycogen synthase kinase 3beta
GST	glutathione-S-transferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanuesulfonic acid
HMG	high mobility group

IB	immunoblot
IP	immunoprecipitate
IPTG	isopropyl-beta-D-thiogalactopyranoside
Lef	lymphoid enhancer factor
LZ	leucine zipper
MEMFA	MOPS, EGTA, $MgSo_4$, Formaldehyde
MitF	microphthalmia-associated transcription factor
MMR	Marc's modified Ringer's solution
mRNA	messenger ribonucleic acid
MO	morpholino
N-	amino terminus
ng	nanogram
Pax	paired box
PCR	polymerase chain reaction
pg	picogram
PML	promyelocytic leukemia
Ψ	hydrophobic amino acid
RACE	rapid amplification of cDNA ends
RLU	relative luciferase unit
RNA	ribonucleic acid
RPE	retinal pigment epithelium
SAE1/2	SUMO-1 activating enzyme subunit 1/2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIM	SUMO interacting motif
SNB	SUMO-1 nuclear body
SOX	SRY box-containing
ssDNA	salmon sperm deoxyribonucleic acid
SUMO	small ubiquitin-like modifier
TGFß	transforming growth factor beta
Trp-1	tyrosinase-related protein 1
TSA	Trichostatin A
Tyr	tyrosinase
UBC9	Ubiquitin-like protein SUMO-1 conjugating enzyme
ul	microliter
UTR	untranslated region
VPA	Valproic acid

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CHAPTER 1

GENERAL INTRODUCTION

Neural crest cells

The neural crest is an embryonic population of stem cell-like precursors that arises at the border between the neural plate (central nervous system) and the non-neural ectoderm (prospective epidermis) (Figure 1.1). This pool of progenitors was vital to the evolution of vertebrates because this cell population gives rise to the structures and tissues that segregate vertebrates from invertebrates. For example, without the neural crest, vertebrate embryos would not be able to create a cavity large or strong enough to contain a brain capable of higher-level functioning. These cells are able to migrate throughout the body and contribute to the formation of many cell types including, but not limited to, the peripheral nervous system, most pigment cells, and craniofacial bone and cartilage (Figure 1.2). Because the neural crest contributes to so many different cell types, the formation of these cells is vital to proper fetal development. When defects occur in the neural crest, a wide variety of birth defects and cancers can develop.

The exact molecular mechanisms underlying neural crest formation and propagation are unknown. However, researchers have made excellent progress in elucidating some of the tissues, signaling pathways and transcription factors involved in these processes. This thesis will begin by giving an overview of the neural crest developmental process. Each individual neural crest cell has a long developmental path to follow. First, the neural crest precursors must be induced. Then, these cells must undergo an epithelial to mesenchymal cell transition (EMT). Once through this transition, these cells migrate to a final destination point in the body where they will contribute to the formation of the above-mentioned derivatives. The overview below will encompass not only the steps involved during neural crest development, but also a short summary of the current understanding about how the neural crest progresses through these steps.

Figure 1.1: The location of the neural crest precursors in the developing embryo

A) Neural crest cells arise between the neural plate, which will form the central nervous system, and the non-neural ectoderm, which will become the epidermis. The otic placode forms in a region adjacent to the neural crest precursors. **B)** Whole mount *in situ* hybridization performed on a *Xenopus* embryo shows staining that mirrors the above schematic. Staining with the Sox9 probe highlights the neural crest precursors and the otic placode.

Figure 1.1

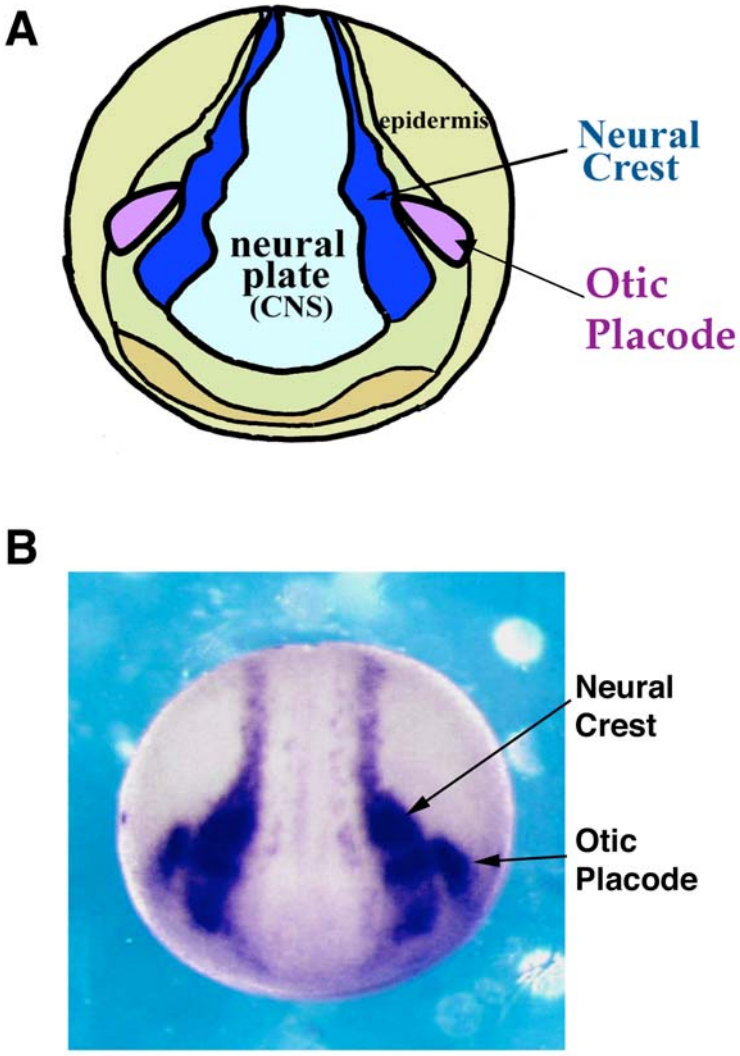
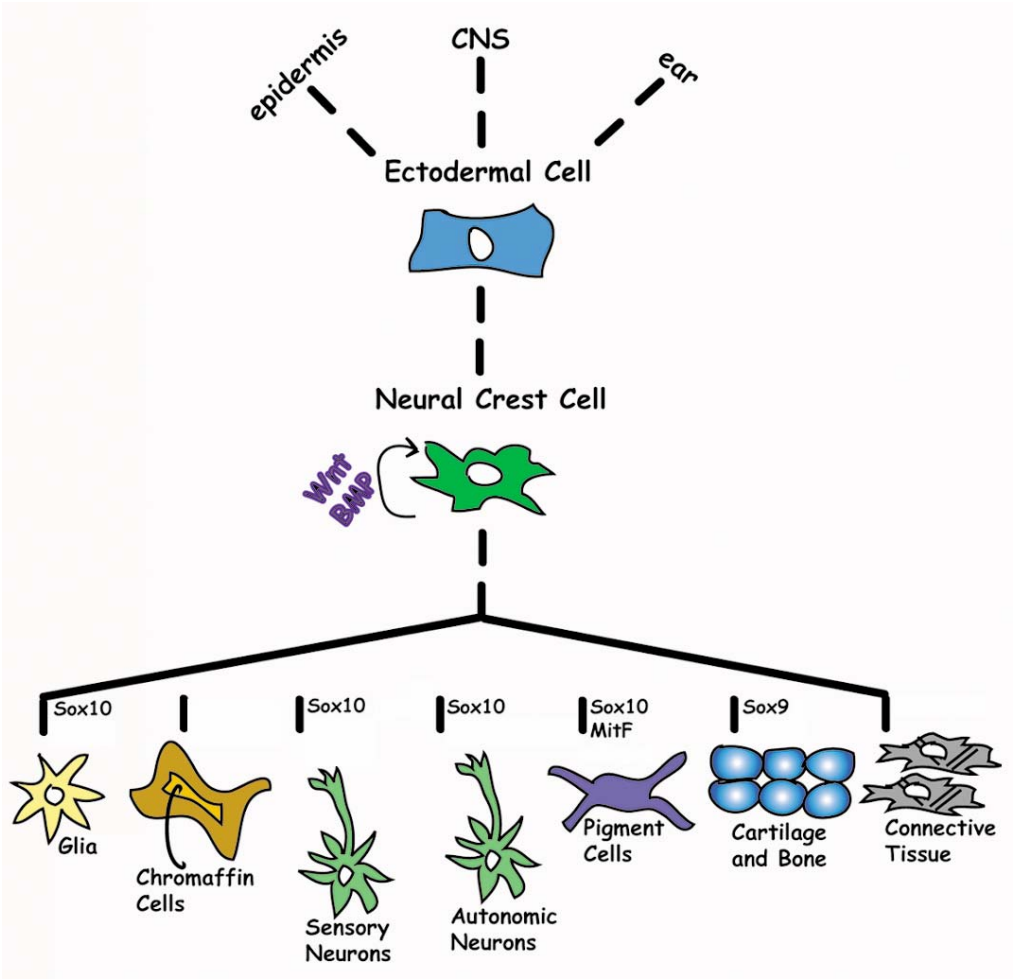

A
epidermis
Neural Crest
neural plate (CNS)
Otic Placode
B
Neural Crest
Otic Placode

Figure 1.2: Schematic of neural crest derivatives

After the neural crest forms and migrates throughout the embryo, multiple derivatives are formed throughout the body. These derivatives include glia, chromaffin cells, sensory and autonomic neurons, pigment cells, cartilage and bone, and connective tissue.

Figure 1.2


epidermis
CNS
ear
Ectodermal Cell
Neural Crest Cell
Wnt
BMP
Sox10
Sox10
Sox10
Sox10
MitF
Sox9
Glia
Chromaffin
Cells
Sensory
Neurons
Autonomic
Neurons
Pigment
Cells
Cartilage
and Bone
Connective
Tissue

Neural Crest Induction

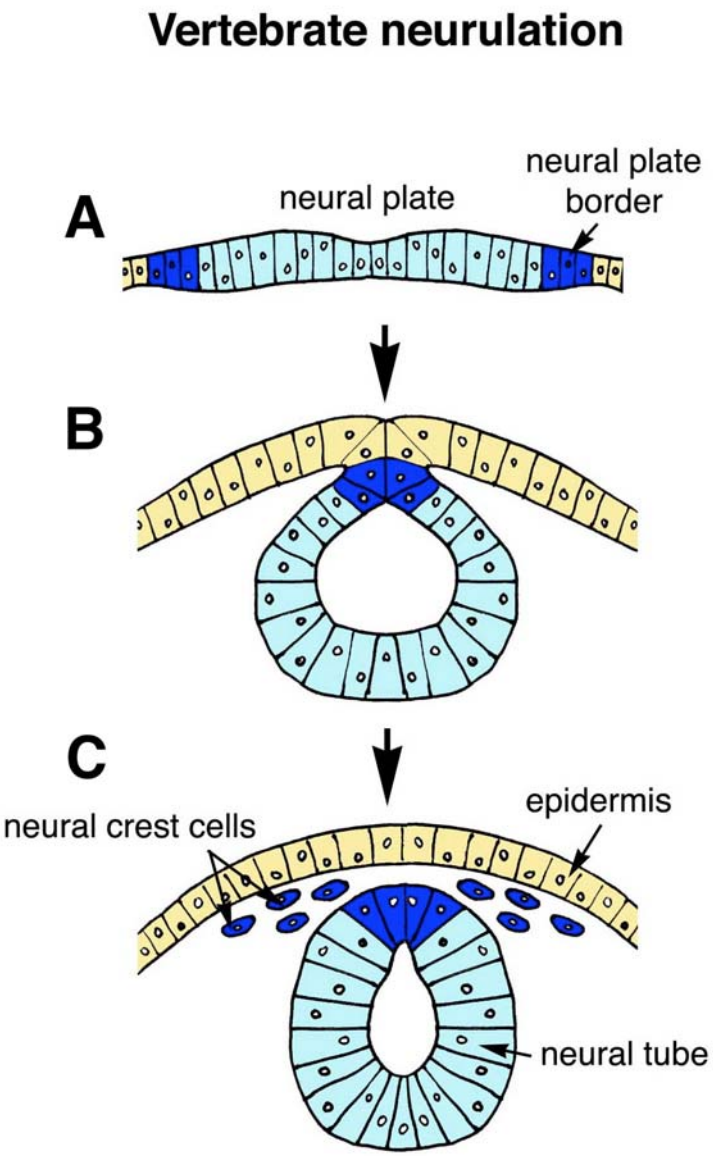
Neural crest cells arise at the neural plate border, a zone located between the neural plate and the non-neural ectoderm (Figure 1.3). During vertebrate neurulation, the neural plate folds into a tube, and at the end of this process, the prospective neural crest is located at the dorsal-most aspect of the embryo, within the neural tube. Over top of this area lies the non-neural ectoderm that will become the skin of the back. In *Xenopus laevis*, or African-clawed frog, once the neural tube has completely closed, the neural crest cells undergo an EMT, upon which these cells delaminate from the neuroepithelium and begin migration to their ultimate destination.

As mentioned, the domain in which the neural crest forms is surrounded by neural plate and non-neural ectodermal cells. One of the biggest questions surrounding these cells is how they know to contribute to the neural crest and not another surrounding cell type. In fact, through lineage tracing, it has been found that cells located within the neural plate border have the ability to populate not only the neural crest, but also the central nervous system (CNS) and the epidermis (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988). What signals are important then to make a cell turn its fate specifically to a neural crest cell? Research has suggested that a combination of signals coming from surrounding tissues, including the epidermal and neural plate layers, contribute to the formation of these cells. Several experiments have shown that culturing epidermal tissue next to neural plate tissue can induce the formation of neural crest (Moury and Jacobson, 1989; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). In addition to the epidermis and neural plate, these cells lie above another key tissue, the paraxial mesoderm. This mesoderm also appears to have an influence in determining the fate of these cells because culturing the paraxial mesoderm next to the prospective epidermis can form both neural crest cells and neural plate cells

Figure 1.3: Schematic of Neural Crest precursor formation

A) During vertebrate neurulation, the neural crest precursors form just lateral to the neural plate at the neural plate border. **B)** As neurulation proceeds, the neural plate folds up into a tube. The neural crest precursors lie at the dorsal most aspect of this tube. Over top of these cells lies the prospective epidermis, which will become the skin of the back. **C)** Once the neural tube completely closes, the neural crest cells undergo an epithelial to mesenchymal cell transition and are then able to exit the neural tube and commence migration.

Figure 1.3


Vertebrate neurulation
A
neural plate
neural plate border
B
C
neural crest cells
epidermis
neural tube

(Marchant et al., 1998; Selleck and Bronner-Fraser, 1996)).

Over the past few decades, researchers have been determining the signals that originate from these different tissues to help direct the formation of neural crest cells. One such family of molecules is the Bone Morphogenetic Proteins (BMPs). BMPs are secreted ligands belonging to the TGFß family that bind to receptor kinases to active downstream components of the pathway. BMP antagonists, such as chordin and noggin, bind BMP ligands prior to receptor binding and prevent them from activating the signaling pathway.

BMP signaling plays a large role in the developing the neural crest and has led to the formation of the neural default model. In *Xenopus*, high levels of BMPs are expressed throughout the ectoderm during early gastrulation, and these levels give rise to epidermal cell fates (Wilson and Hemmati-Brivanlou, 1995). To counteract BMP signaling, the organizer secretes BMP antagonists to prevent signaling in more proximal locations. This low level of BMP signaling allows the formation of neural tissue (Wilson and Hemmati-Brivanlou, 1997). Interestingly, a BMP gradient is produced from different concentrations of BMP antagonists secreted from the organizer. This results in an intermediate level of BMP expression and partially results in the formation of the third ectodermal tissue, the neural crest (Figure 1.4) (LaBonne and Bronner-Fraser, 1998b; Marchant et al., 1998; Nguyen et al., 1998; Wilson et al., 1997).

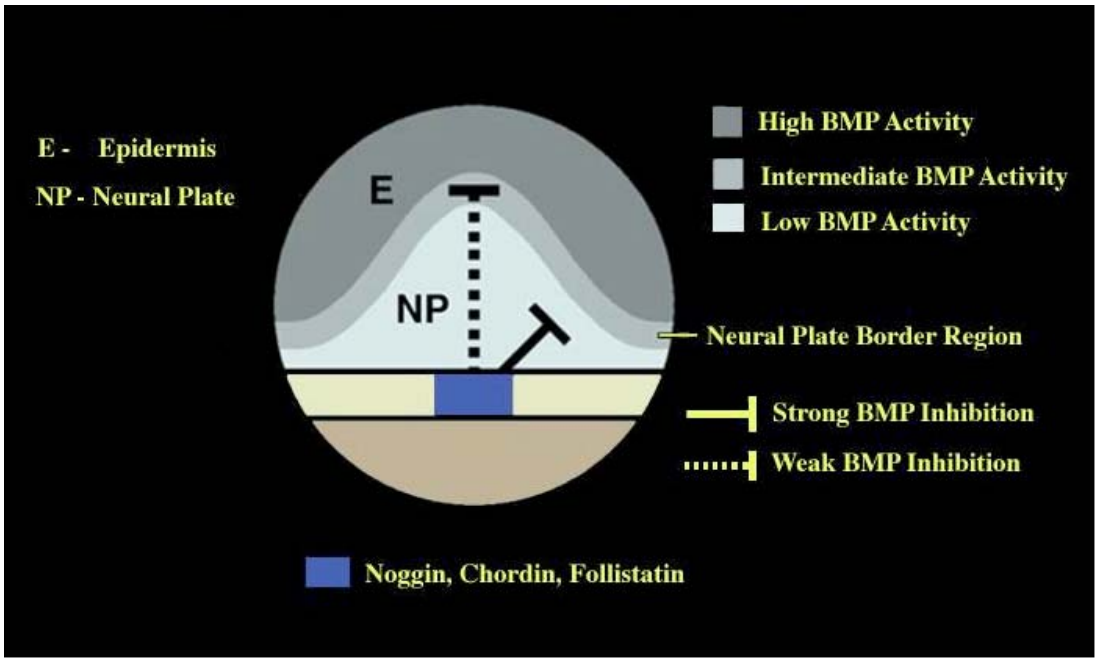
However, an intermediate BMP signal alone is not sufficient to induce the neural crest precursor marker Slug in animal cap explant assays (LaBonne and Bronner-Fraser, 1998b). If another signal is added in addition to a BMP antagonist, such as a Wnt or FGF, then Slug expression is induced in the explants (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998b; Saint-Jeannet et al., 1997).

Figure 1.4: Neural default model of neural crest induction

The neural default model of neural crest induction hypothesizes that the ectoderm secretes a high level of BMP signaling to give rise to the epidermis. To counteract this signal, the organizer secretes BMP antagonists, such as noggin or chordin. The presence of these antagonists allow for a low level of BMP signaling in the neural plate region, which promotes the formation of neural tissue. The third layer of the ectoderm, however, receives an intermediate level of BMP signaling which helps to determine the fate of the neural crest cells.

*This figure has been adapted from: (LaBonne and Bronner-Fraser, 1998a)

Figure 1.4


E - Epidermis
NP - Neural Plate
E
NP
High BMP Activity
Intermediate BMP Activity
Low BMP Activity
Neural Plate Border Region
Strong BMP Inhibition
Weak BMP Inhibition
Noggin, Chordin, Follistatin

The addition of this second signal has given rise to what is known as the two-signal model of neural crest formation and is described below (LaBonne and Bronner-Fraser, 1998b).

One of the most well-characterized and important pathways in neural crest development is the Wnt pathway. This pathway is mediated by the binding of a secreted Wnt molecule to one of the cell-surface receptors of the Frizzled pathway. This binding then creates a signaling cascade in which ß-catenin is allowed to translocate into the nucleus and initiate transcription. If a Wnt signal is not present, ß-catenin cannot go into the nucleus and is instead targeted for degradation by the addition of a series of ubiquitin moieties through GSK3ß. In the nucleus, the transcription factors Lef and Tcf actively repress transcription by binding to DNA if a Wnt signal is not present. Only if ß-catenin is present in the nucleus can this repression be relieved. Interestingly, the promoter of one of the early neural crest markers, Slug, has binding sites for Lef/Tcf, which suggests that a direct Wnt signal is involved in neural crest formation (Vallin et al., 2001).

Several Wnt molecules are excellent candidates for playing a role in neural crest induction. Wnt1, Wnt3a, Wnt7b, and Wnt8, in combination with noggin or chordin, can all induce neural crest in *Xenopus* animal cap assays (Chang and Hemmati-Brivanlou, 1998; Deardorff et al., 2001; LaBonne and Bronner-Fraser, 1998b; Saint-Jeannet et al., 1997). Likewise, overexpression studies with the Frizzled3 receptor or ß-catenin also lead to the formation of ectopic neural crest (Deardorff et al., 2001; Tamai et al., 2000). Several experiments have also indicated the necessity of a Wnt signal in neural crest formation. For example, expressing a dominant-negative form of Wnt8 blocks proper neural crest induction in the *Xenopus* embryo, as does over-expressing GSK3ß (Saint-Jeannet et al., 1997). Blocking components of the Frizzled receptor family also inhibits proper neural crest induction (Deardorff

et al., 2001; Tamai et al., 2000; Tan et al., 2001). More recently, morpholino-targeted knockdown of ß-catenin has also been shown to prevent neural crest formation (Wu et al., 2005). These data all demonstrate the necessity of Wnt signaling to proper neural crest formation.

As mentioned, combining non-neural ectoderm with paraxial mesoderm can induce neural crest cells. During *Xenopus* gastrulation, areas of the archenteron roof can induce neural tissue and neural crest in grafting experiments. If only lateral archenteron tissue is grafted, neural crest, and not neural tissue, is induced (Raven and Kloos, 1945). This result suggested that the mesoderm was perhaps the primary signaling tissue to give rise to the neural crest. This experiment was further supported by both additional *Xenopus* and chick experiments (Marchant et al., 1998; Selleck and Bronner-Fraser, 1996). Also important to note, *Xenopus* embryos lacking paraxial mesoderm are not able to form normal neural crest derivatives (Bonstein et al., 1998). This further supports the notion that a signal from the mesoderm is a necessary component for proper neural crest development, and it is thought that perhaps a member of the fibroblast growth factor (FGF) family, specifically eFGF, is this signal.

In *Xenopus*, Slug can also be induced in animal cap assays through the combination of a BMP antagonist and eFGF (LaBonne and Bronner-Fraser, 1998b). As mentioned, eFGF is a good candidate to be a neural crest inducer because it is localized to the paraxial mesoderm, along with Wnt8 (Christian et al., 1991; Isaacs et al., 1992). eFGF was found to induce Wnt8 expression, and when the Wnt pathway is inhibited, eFGF is no longer able to induce the neural crest precursor population (LaBonne and Bronner-Fraser, 1998b). This suggested that eFGF is only able to efficiently induce neural crest when it has also activated the Wnt pathway. In addition, overexpression of a dominant negative FGF receptor has demonstrated the requirement

of this receptor in the formation of the neural crest. When overexpressing this dominant-negative receptor, neural crest precursor markers are inhibited (Mayor et al., 1997).

These three types of signaling molecules, Wnts, BMPs, and FGFs, play important roles in neural crest formation. However, signaling molecules are only the first step of signal transduction. These molecules eventually lead to the activation of certain transcription factors, many of which play a large role in neural crest development. This thesis will closely examine the contribution of two of these transcription factors, Sox9 and Sox10, to not only neural crest induction, but also in later neural crest differentiation stages.

Neural Crest Migration

After induction and before differentiation, the neural crest cells must migrate along a path that is partially determined by the body axis level upon which the cells are found. For neural crest cells to delaminate and migrate away from the neural tube region, they must first undergo an EMT. This is the same transition that occurs in cancer cells that are undergoing metastasis. Within the different organisms in which neural crest is studied, migration times differ slightly. In *Xenopus*, neural crest cells begin migrating several hours after neural tube closure (reviewed in (Nieto, 2001)). Several of the same molecules that are involved in neural crest induction are also thought to be involved in the commencement of migration, such as BMPs. Other factors involved in the delamination of neural crest are the down regulation of N-CAM, N-cadherin, and cadherin 6B (Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995), as well as the up regulation of cadherin 7 and 11 (Kimura et al., 1995; Nakagawa and Takeichi, 1995). It is believed that these cadherins are tightly regulated to allow proper delamination.

Undergoing an EMT allows the neural crest cells to travel long or short distances to achieve their final destination. Several transcription factors, such as Slug, Snail, Twist and Sox9, are thought to be involved in the neural crest cell EMT. Snail can trigger an EMT in cultured mammalian epithelial cells (Cano et al., 2000). Interestingly, it has been found that Snail acts as a strong repressor of E-cadherin, thereby potentially setting up a role for Snail in the down-regulation of cadherins in neural crest EMT (Batlle et al., 2000; Cano et al., 2000). In addition, work done in *Xenopus* has shown that early inhibition of the Snail-related family member, Slug, prevents neural crest formation, while later inhibition prevents cell migration (LaBonne and Bronner-Fraser, 2000). Also, a recent publication working with chick explants suggested that Sox9 plays a role in promoting EMT in neural crest cells. Sakai and colleagues found that Sox9 was required for BMP-mediated Slug induction and subsequent EMT. They found that this was perhaps through a physical interaction between Sox9 and Slug and henceforth synergistic activation of the Slug promoter (Sakai et al., 2006). However, these investigators used Phalloidin staining to investigate cells undergoing an EMT. The cells would have been better labeled with an EMT-specific marker, such as a cadherin. Therefore, the role of Sox9 in this process is somewhat unclear and this will not be specifically discussed in this thesis.

Once the neural crest cells have begun their journey to reach their final destination, they are thought to receive a number of cues, such as BMP signaling, during their migratory path that help to guide them to their ultimate placement (reviewed in (LaBonne and Bronner-Fraser, 1999)). It is here where they will differentiate and contribute to the formation of a specific neural crest derivative.

Neural Crest Differentiation

The neural crest cells located within the most anterior portion of the embryo, including the midbrain and hindbrain, migrate through the head and give rise to craniofacial bones and the neurons and glia of the cranial ganglia. Neural crest cells located just posterior to this region, the vagal crest, migrate and contribute to the formation of the heart and enteric nervous system. The trunk neural crest, located posterior to the vagal crest, migrates to destinations that will become the sensory and sympathetic ganglia, as well as the adrenal medulla. Finally, the tail portion of the embryo, which contains the sacral neural crest, also helps contribute to the formation of the enteric nervous system. Interesting to note is that melanocytes, which are a neural crest derivative, are created by neural crest cells from every section of the embryo (reviewed in (Barembaum and Bronner-Fraser, 2005)). Data from this thesis will not only contribute to the knowledge of how the melanocyte lineage forms and is regulated, but also to the formation of another non-neural crest derived tissue, the inner ear.

Inner Ear Development

Interestingly, the embryonic ear is an important structure that begins forming at the same time that the neural crest precursors are forming (Figure 1.2). These two cell populations are located very close to one another during all aspects of development. In fact, many of the same signaling molecules and transcription factors that are involved in neural crest development are also involved in ear development.

Hearing impairments rank among the most common birth defects, and half of all cases of deafness in children are caused by inherited defects in genes that control the development and/or function of the inner ear (Torres and Giraldez, 1998). Despite its obvious clinical significance,

however, surprisingly little is understood about the early development of this complex sensory organ, particularly at the molecular level. The inner ear is unique to vertebrates and plays an essential role in hearing, the detection of acceleration, and the maintenance of balance (Riley and Phillips, 2003). This organ forms from an ectodermal thickening known as the otic placode that arises adjacent to the neural plate at the level of the hindbrain. As the otic placode develops, it invaginates and separates from the surface ectoderm to initially form a simple otic vesicle or otocyst, and ultimately an elaborate fluid filled labyrinth that houses both auditory and vestibular structures (Figure 1.4) (Barald and Kelley, 2004). As mentioned, the otic placode is located just lateral to the neural crest precursors, while the otocyst is found close to many neural crest-derived tissues (Figure 1.5).

The control over both early and late aspects of otic placode development is thought to stem from inductive interactions with surrounding tissue, such as the hindbrain and mesoderm. The most widely accepted model suggests that mesoderm underneath the region that will become the otic placode signals the overlaying ectoderm to become competent for ear formation. Then, a second signal from the hindbrain actually induces otic placode formation in the proper location (reviewed in (Torres and Giraldez, 1998)). Important to note, however, is that this model is not fully based on experimental evidence. A number of signaling pathways have been implicated in this process, the best studied of these being the fibroblast growth factor (FGF) and Wnt pathways (reviewed in (Streit, 2002)). Downstream of these secreted signals, a number of transcription factors have been implicated as essential regulators of inner ear development, including *Dlx3b* and *Dlx4b* (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997), *Pax8* (Heller and Brandli, 1999; Pfeffer et al., 1998), and *Foxi1* (Nissen et al., 2003; Solomon et al., 2003). Mutations in any of these factors cause either a complete failure of otic placode formation and/or

Figure 1.4: Schematic of developing otic placode and otocyst

A) The otic placode forms from the ectoderm, adjacent to the hindbrain (HB) and notochord (NC). The otic placode competency zone is depicted by blue staining, while the neural crest precursors are highlighted in pink. **B)** As development progresses, the otic placode invaginates and eventually pinches off to form the otocyst, which is depicted by the purple staining. **C)** The otocyst (purple) then becomes completely closed.

*This figure was adapted from: (Barald and Kelley, 2004).

Figure 1.4

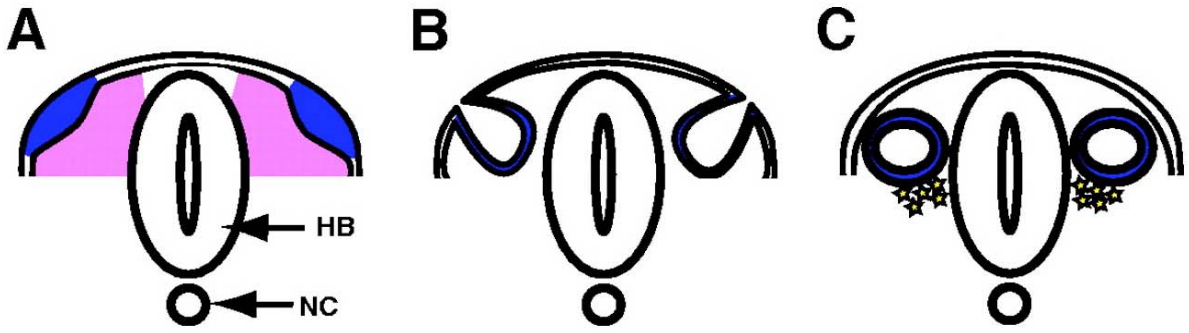
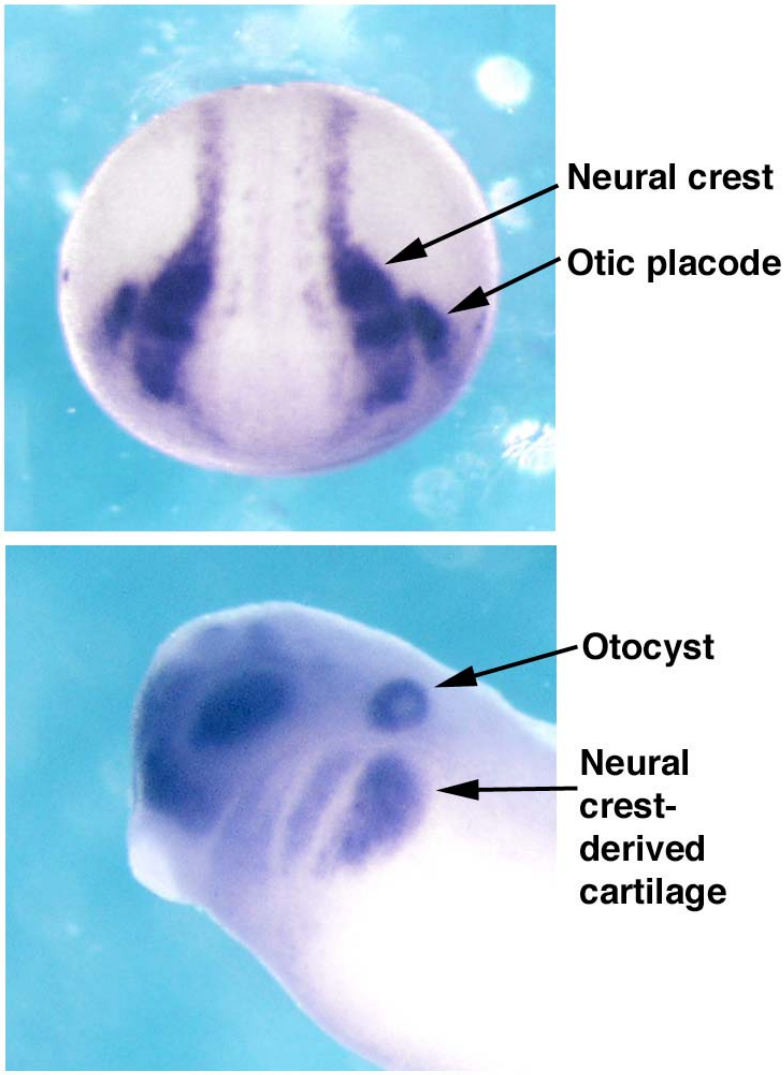


Figure 1.5: The otic placode and otocyst develop very close to the neural crest precursors and their derivatives.

Whole mount *in situ* hybridization performed in *Xenopus* embryos stained with the probe Sox9. In the upper embryo, the otic placode can be seen in close proximity to the neural crest precursors at neurula stages. In the lower embryo, the otocyst forms on the side of the head, adjacent to many neural crest derivatives, such as the facial cartilage, which is depicted here.

Figure 1.5


Neural crest
Otic placode
Otocyst
Neural crest-derived cartilage

severe auditory and vestibular deficits (Liu et al., 2003). Other factors, including *Six1* (Oliver et al., 1995; Ozaki et al., 2004; Zheng et al., 2003), *Pax2* (Heller and Brandli, 1999; Herbrand et al., 1998; Hutson et al., 1999; Lawoko-Kerali et al., 2002; Nornes et al., 1990; Pfeffer et al., 1998; Riley et al., 1999; Torres et al., 1996) and BMP4 (Chang et al., 1999; Chang et al., 2002; Gerlach et al., 2000), have been implicated in the patterning of the otocyst, which becomes divided into three discrete domains: a ventral cochlear region responsible for hearing, a medial region important for detecting motion and position, and a dorsal region responsible for vestibular function (Saint-Germain et al., 2004). Clearly many transcription factors play a role in ear development. An additional set of factors involved in ear formation, as well as neural crest formation, is the SoxE factors and the role of these factors in ear formation will be discussed in this thesis.

SoxE Transcription Factors

The SOX family of transcription factors belongs to the high mobility group (HMG) box superfamily of DNA-binding proteins (Bowles et al., 2000; Koopman et al., 2004). Sox proteins regulate many developmental processes, such as cell specification, organ development, and germ layer formation (Wegner, 1999). All Sox proteins possess transcriptional activation domains; however, they bind DNA with low affinity, and are thought to require DNA binding co-factors to stabilize their interactions with DNA (Kamachi et al., 2000). There are several published examples of Sox family members interacting with specific partners in order to synergistically activate transcription (Bondurand et al., 2000; Lang and Epstein, 2003). The SOX family comprises subgroups A-J, which are each characterized by an HMG DNA binding domain, which shows greater than eighty percent amino acid homology across the subgroups. Members

of Group E, comprised of *Sox8*, *Sox9*, and *Sox10*, are further characterized by the presence of two homology regions termed E1 and E2 that are of unknown function but which have been proposed to be protein-protein interaction domains (Figure 1.6) (Bowles et al., 2000).

Their high degree of sequence identity suggests that Sox9 and Sox10 could be at least partially functionally redundant, and these factors display both overlapping and distinct patterns of expression during early embryonic development. At neural plate stages, neural crest precursor cells express both *Sox9* and *Sox10*, although expression of *Sox9* precedes that of *Sox10* in the neural crest. Similarly, while both factors are expressed in the otic placode, here again expression of *Sox9* is initiated considerably earlier than that of *Sox10* (Figure 1.7). Importantly, although expression of *Sox9* and *Sox10* continues to overlap in the developing otocyst, by these neural crest differentiation stages, the expression of these factors in the neural crest is completely non-overlapping. Expression of *Sox9* is maintained in the cells that give rise to the facial cartilage, while *Sox10* expression becomes restricted to developing melanoblasts and cranial glia (Figure 1.7 and (Aoki et al., 2003; Spokony et al., 2002)).

Inner ear development and SoxE factors

As eluded to, the group of transcription factors emerging as key regulators of inner ear development is the SoxE proteins, Sox9 and Sox10. *Sox9* is one of the earliest transcription factors to be expressed in the ectoderm that will give rise to the otic placode, and recent work in both zebrafish and *Xenopus* has shown that this factor is essential for early otic placode development (Liu et al., 2003; Saint-Germain et al., 2004). By the end of gastrulation, expression of *Sox9* marks a patch of cells just lateral to the neural crest precursors, prior to the

Figure 1.6: Schematic of Sox9 and Sox10 protein domains

SoxE factors, Sox9 and Sox10, are highly homologous. They contain a centralized, highly conserved HMG, or DNA binding domain, and a C-terminal activation domain. Sox9 and Sox10 also contain two other highly conserved domains of unknown function, termed E1 and E2.

Figure 1.6

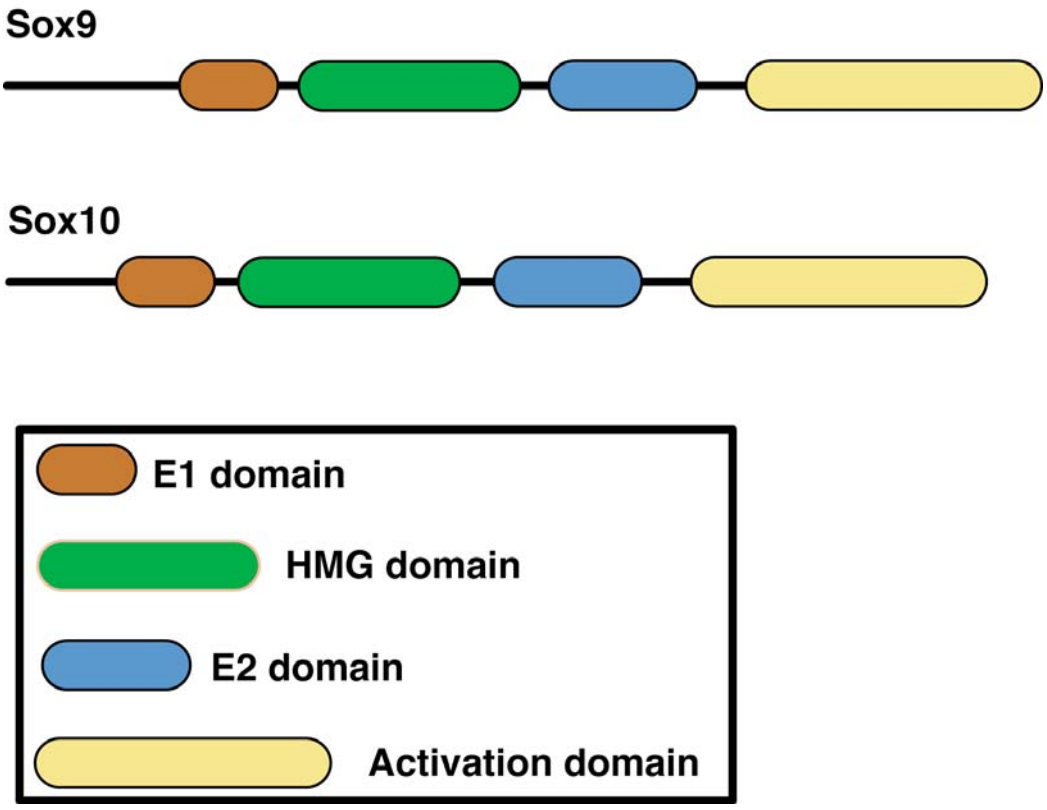
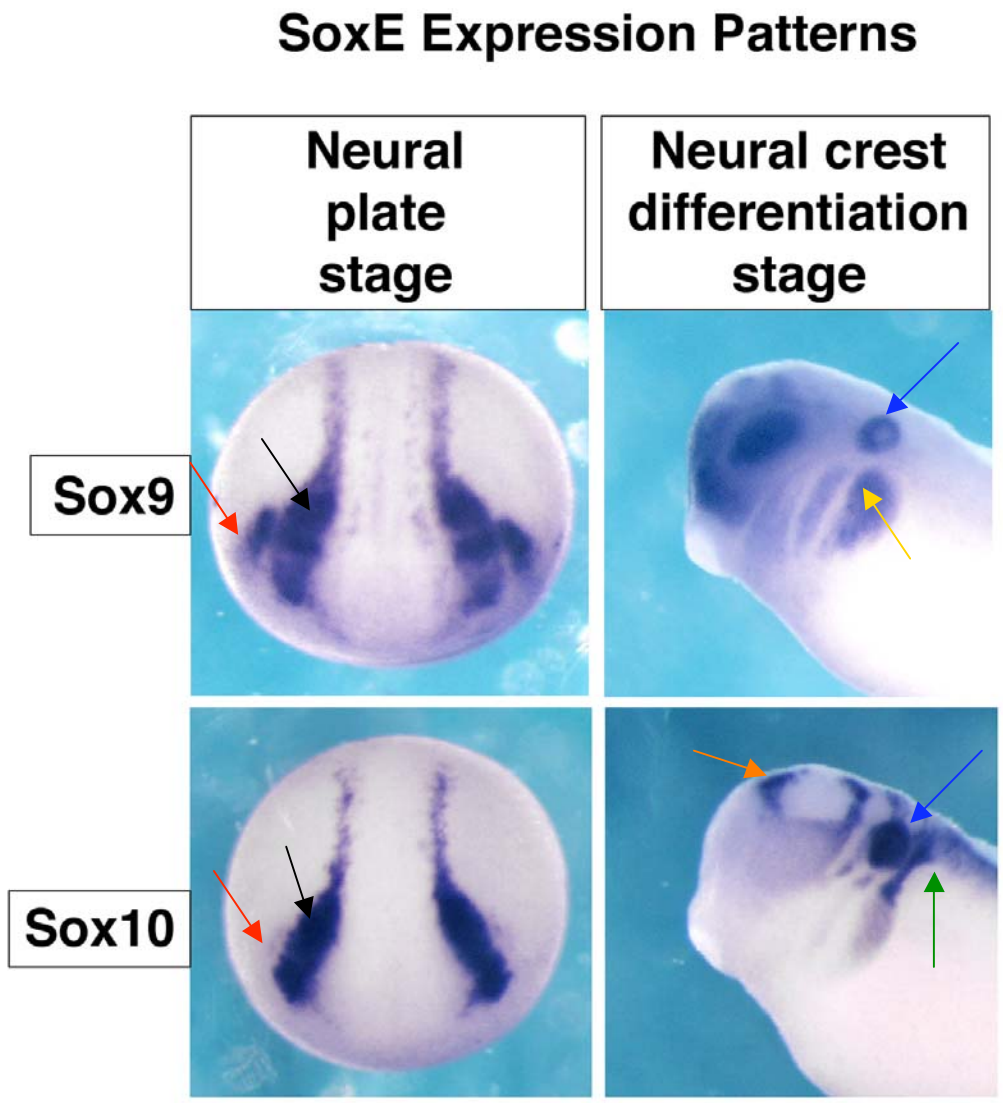

Sox9
Sox10
E1 domain
HMG domain
E2 domain
Activation domain

Figure 1.7: Sox9 and Sox10 Expression Patterns in *Xenopus* Embryos

At neural plate stages, Sox9 and Sox10 have overlapping expression patterns. Both factors are expressed by the neural crest precursors (black arrows), as well as the otic placode (red arrows). Sox9, however, is more highly expressed in the otic placode at this stage. However, at neural crest differentiation stages, the expression patterns of Sox9 and Sox10 are completely non-overlapping with respect to neural crest derivatives. Sox9 is found in the facial cartilage (yellow arrow), while Sox10 is found in glia (orange arrow) and melanoblasts (green arrow). Both Sox9 and Sox10 are expressed in the otocyst at this stage (blue arrows).

Figure 1.7


SoxE Expression Patterns
Neural plate stage
Neural crest differentiation stage
Sox9
Sox10

onset of *Pax8* expression in this region (Saint-Germain et al., 2004). Importantly, morpholino oligo-mediated depletion of Sox9 leads to loss of expression of early ear markers, such as *Pax8*, as well as failure of a recognizable otic vesicle to form (Saint-Germain et al., 2004). Using a hormone-inducible inhibitory *Sox9* construct, these authors found that *Sox9* is required for early otic placode specification, but not for latter patterning events of the otic vesicle (Saint-Germain et al., 2004). Also, in zebrafish, simultaneous morpholino-mediated depletion of the *dlx3b*, *dlx4b*, *sox9a* and *sox9b* genes leads to the complete inhibition of the otic placode (Liu et al., 2003). This same study found that the ability of *Fgf3* and *Fgf8* to facilitate otic placode induction is partially mediated by *Sox9a* and *Sox9b*, but not by *Dlx3b* and *Dlx4b* (Liu et al., 2003). As the otocyst becomes regionalized, *Sox9* is most highly expressed in the dorsal region that contains the semicircular canals and endolymphatic duct (Saint-Germain et al., 2004).

Sox10 in the mouse inner ear is expressed in the otic vesicle epithelia by embryonic day 11.5 (Watanabe et al., 2000). This expression is localized to the developing cochlea and vestibule at embryonic day 13.5. In adult mice, *Sox10* expression is found in the organ of Corti support cells and in Schwann cells of the spiral ganglion (Watanabe et al., 2000). Interestingly, *Sox10* has a well-documented role in glia cells (Britsch et al., 2001). The expression of *Sox10* in the developing otic vesicle suggests that like *Sox9*, it too will prove to be an important regulator in the development and maintenance of the inner ear (Watanabe et al., 2000). It thus seems likely that understanding the function of SoxE factors will prove essential to understanding both early and late aspects of inner ear development. Both the close proximity of the embryonic ear to the neural crest cells and the fact that SoxE factors have been implicated in the development of the ear make this organ another excellent cell type to study in this thesis. This thesis will provide novel findings with respect to the contribution of Sox9 and Sox10 to ear formation.

SoxE Factors and Neural crest

As can be inferred from their expression patterns in the early neural crest precursors, SoxE factors are important to the neural crest from early stages of development. Depletion of Sox9 or Sox10 in *Xenopus* embryos leads to a loss of neural crest precursor formation, while overexpression of these factors leads to an increase in the neural crest progenitor population (Aoki et al., 2003; Honore et al., 2003; Spokony et al., 2002; Taylor and Labonne, 2005). Thus, by the criteria of their effects on neural crest precursor formation, these two closely related Sox E factors appear to have similar activities. However, as mentioned, these factors are completely non-overlapping with respect to their expression patterns at later neural crest differentiation stages (Figure 1.7). In fact, research has shown that Sox9 and Sox10 have different roles during this developmental window. For example, it has been found that overexpression of Sox10 but not Sox9 induces melanocyte formation (Aoki et al., 2003), indicating that each of these factors may possess some distinct activities. In addition to its role in melanocyte formation, *Sox10* has also been identified as a key player in the development of glia (Britsch et al., 2001; Kelsh et al., 2000; Southard-Smith et al., 1998; Stolt et al., 2002). From expression pattern studies and mouse knockout models, it has been hypothesized that *Sox9* is instructive of cartilage fates (Bi et al., 2001), while *Sox10* does not contribute to the formation of this tissue, again implying that these highly related factors have at least some distinct activities.

However, there have been some instances where Sox9 and Sox10 have been shown to perform similar developmental duties with respect to neural crest derivatives. Investigators have implicated Sox10 in the maintenance of multipotency in neural crest stem cells and in the inhibition of neuronal differentiation (Kim et al., 2003; Paratore et al., 2002). While these studies did not address whether other SoxE factors had similar activities, more recent work has

demonstrated that forced expression of Sox9 blocks neuronal differentiation of neural crest cells in avian embryos (Cheung and Briscoe, 2003). These data suggest that perhaps Sox9 and Sox10 both have a role in maintaining a stem cell-like state. Also, although Sox9 has been specifically demonstrated to not play a role in melanocyte formation (Aoki et al., 2003), of late, a few groups, including data presented here in this thesis, have shown Sox9 to be involved in melanocyte formation. Passeron and colleagues found that Sox9 was present in human melanocytes and was able to positively regulate certain melanocyte-specific promoters (Passeron et al., 2007). Additionally, Cook and colleagues found that upon a down-regulation of Sox10, Sox9 expression increased in differentiated melanoblasts (Cook et al., 2005). Taken together, these data suggest that Sox9 and Sox10 may be able to functionally substitute for one another at later neural crest differentiation stages and certainly in the melanocyte lineage. This thesis closely examines the functional redundancy of the SoxE factors in both the ear and neural crest formation, including the specific neural crest derivative, the melanocyte.

Melanocytes

Melanocytes are specialized dendritic cells derived from the neural crest that produce melanin, the polymer that gives rise to color in the skin. The melanocyte lineage is the last neural crest lineage to form, coming mainly from the trunk neural crest. Interestingly, although this is the last neural crest derived cell type to become specified, these cells are already fated to become melanocytes as they migrate. As the neural crest cells become situated along the dorsal midline of the embryo, prior to migration, they become melanocyte precursors cells, or melanoblasts. Upon exiting the midline, these fated melanoblasts then migrate to their ultimate

destination where they will become melanocytes (Figure 1.8) (reviewed in (Sulaimon and Kitchell, 2003)). At the cellular level, melanocytes make melanin in distinct organelles called melanosomes (Sulaimon and Kitchell, 2003). These organelles are found at the level of the epidermal cells, keratinocytes, and hair shafts. It is at this level where melanin will be secreted and the pigmentation pattern can thus be formed (Hearing and Tsukamoto, 1991). Many different pathways and transcription factors, several of which are discussed below, regulate all of the steps involving melanocyte formation.

MitF Transcription Factor

One key regulator of melanocyte formation is the basic helix-loop-helix leucine zipper factor, microphthalmia-associated transcription factor (MitF) (Figure 1.9). MitF, which belongs to the MiT family, binds to DNA through its basic domain and uses its HLH and Zip domains to form homo- or heterodimers (Hemesath et al., 1994). MitF, a transcriptional activator, is involved in a variety of developmental processes that are regulated by different MitF isoforms, and only MitF-M is specific to neural crest-derived melanocytes (Fuse et al., 1996). In this thesis, the term MitF will be used in substitution for MitF-M. MitF, known as the master regulator of melanocytes, is crucial for all aspects of melanocyte development, including their commitment, migration, differentiation, and survival (reviewed in (Steingrimsson et al., 2004)).

MitF expression is first seen in *Xenopus* pigment precursor cells during neural crest migratory stages. This expression runs along the dorsal midline and is also found in the retinal pigment epithelium (RPE), which is a non-neural crest-derived lineage. MitF continues to be

Figure 1.8: Mature melanocytes on the flank of a *Xenopus* embryo

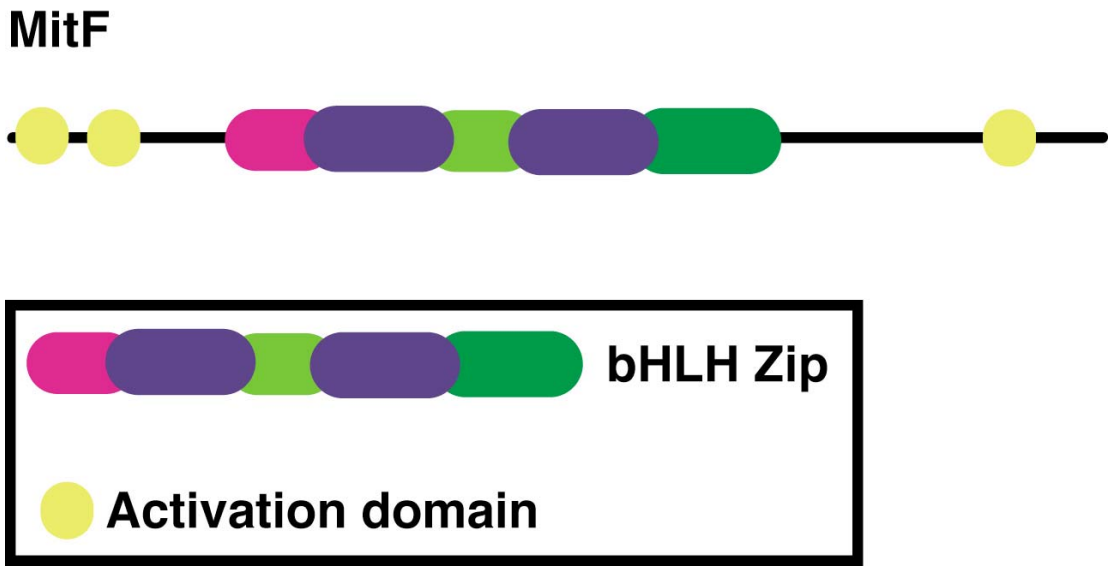
Xenopus laevis swimming tadpole stage has mature, melanin-producing melanocytes scattered along the flank of the embryo (black arrows).

Figure 1.8

Figure 1.9: MitF protein schematic

The MitF protein contains three activation domains. It also contains a centralized, basic helix-loop-helix leucine zipper domain. The basic region binds DNA and the HLH-LZ forms homo- or heterodimers.

Figure 1.9



expressed in these locations until after neural crest differentiation stages (Figure 1.10).

MitF and melanocyte formation

As a master regulator, MitF has been found to be required for the development of neural crest-derived pigment cells. In both the mouse and in zebrafish, a loss of MitF correlates with a loss of melanocytes (Lister et al., 1999; Moore, 1995). Furthermore, Kumasaka and colleagues found that in *Xenopus*, misexpression of MitF leads to an increase in melanophores, while a dominant-negative form of MitF leads to a decrease in melanophore number (Kumasaka et al., 2005).

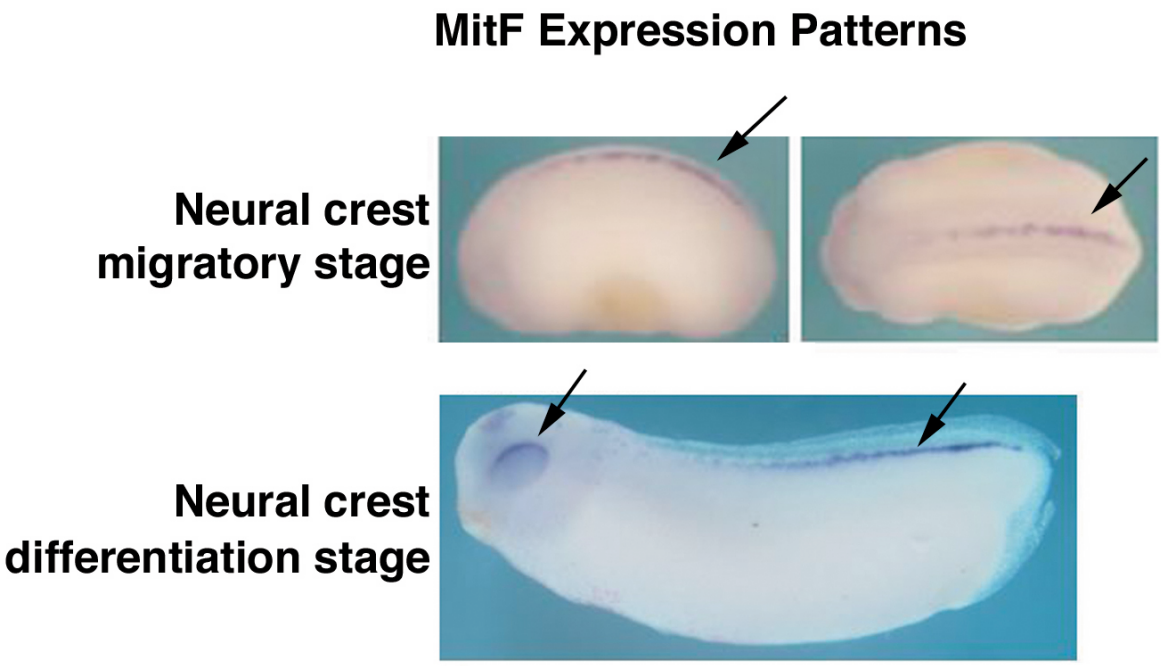
MitF is tightly regulated by several different signaling pathways, including the Wnt pathway and α-melanin-stimulating hormone (α-MSH) pathway. The MitF promoter is also regulated by several transcription factors, including Pax3 and Sox10 (reviewed in (Steingrimsson et al., 2004)). Interestingly, in zebrafish, Elworthy and colleagues found that Sox10 null embryos lacked Mitf-a *in vivo*. However, in this system, they were able to rescue for the loss of MitF-a expression by misexpressing Mitf-a, suggesting that the role of Sox10 was limited to the induction of MitF-a and not the maintenance (Elworthy et al., 2003). Pax3 and Sox10 have also been shown to work synergistically on the MitF promoter, with the caveat that both of these factors must bind to the promoter independently in order to elicit this response (Lang and Epstein, 2003).

Clearly, as it is a master regulator of melanocyte formation, MitF must regulate the expression of multiple downstream factors. These factors include enzymes required for melanin biosynthesis, such as Tyrosinase (Tyr), Tyrosinase-related protein 1 (Trp-1) and Dopachrome

Figure 1.10: MitF Expression Pattern in *Xenopus* embryos

Whole mount *in situ* hybridization performed in *Xenopus* embryos stained for MitF expression patterns. MitF expression turns on during neural crest migratory stages along the dorsal midline (black arrow). During neural crest differentiation stages, MitF is expressed by the RPE and along the dorsal midline (black arrows) by melanoblasts.

Figure 1.10


MitF Expression Patterns
Neural crest migratory stage
Neural crest differentiation stage

tautomerase (Dct/Trp2) (reviewed in (Ferguson and Kidson, 1997; Shibahara et al., 2000)). The promoters of these target enzymes all contain a highly conserved, 11 bp element flanking the site where MitF binds. This conserved sequence has been termed an M-box and is thought to be the MitF recognition site specific to melanogenesis (Yasumoto et al., 1997). Interestingly, both MitF and Sox10 regulate one of the MitF targets, Dct, and this thesis will uncover a potential mechanism used by these factors to regulate Dct activation.

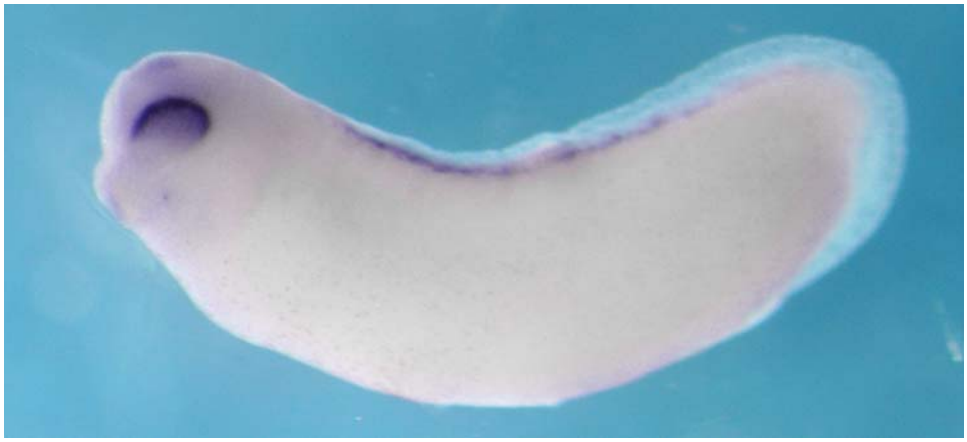
Regulation of the melanocyte-specific enzyme Dct

Dct is responsible for converting the melanin biosynthetic intermediate dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which is more stable and less toxic to cells (Tsukamoto et al., 1992). Dct is expressed in both the RPE and melanoblasts (Figure 1.11) (Steel et al., 1992). The Dct promoter is regulated by a number of transcription factors, including MitF, Sox10 and Pax3 (Budd and Jackson, 1995; Hemesath et al., 1994; Jiao et al., 2004; Lang et al., 2005; Ludwig et al., 2004; Potterf et al., 2001; Yasumoto et al., 1994). It has been found that mice lacking either one or both copies of Sox10 have no Dct expression and that the presence of Sox10 is able to activate the Dct promoter (Potterf et al., 2001; Southard-Smith et al., 1998). Interestingly, the ability of MitF alone to activate the Dct promoter comes with conflicting reports. Potterf and colleagues found that MitF alone could not activate the Dct promoter (Potterf et al., 2001). Yasumoto and colleagues showed that only a form of MitF containing the N-terminus of the protein could activate Dct expression (Yasumoto et al., 1997). However, Murakami and Arnheiter found that MitF could activate the Dct promoter above basal levels (Murakami and Arnheiter, 2005). These conflicting reports suggest a more complex system is involved in regulating the production of this enzyme.

Figure 1.11: Dct Expression Pattern in *Xenopus* embryos

Whole mount *in situ* hybridization performed in a *Xenopus* embryo stained for Dct expression. Dct is first expressed during neural crest differentiation stages along the dorsal midline and in the RPE.

Figure 1.11



However, taken together, MitF and Sox10 can synergistically activate the Dct promoter (Jiao et al., 2004; Ludwig et al., 2004; Yasumoto et al., 2002). Interestingly, the role of Pax3 in Dct regulation is that of an inhibitor. Lang and colleagues found that Pax3 competes with MitF to bind to the Dct promoter in the absence of a Wnt signal. The displacement of MitF by Pax3 leads to the inhibition of synergistic Dct activation. However, when ß-catenin is present, Pax3 can no longer bind to the Dct promoter due to its sequestration by Groucho4, allowing MitF to take its proper place and activate transcription (Lang et al., 2005).

Clearly these regulatory transcription factors are all working on multiple melanocyte-specific promoters throughout development to elicit very different responses. For example, with respect to the MitF promoter Pax3 works as an activator and in the case of the Dct promoter it works to inhibit transcription. One way in which transcription factors are dynamically regulated is through the use of different post-translational modifications.

Post-translational modifications of SoxE factors and MitF

Post-translational modification is the chemical modification of a protein after it has been translated. These modifications, which add different functional groups to the target protein, allow the role of the protein to be altered. Two examples of post-translational modifications include, but are not limited to, phosphorylation and SUMOylation. Briefly, phosphorylation is the addition of a phosphate group to a target amino acid, usually a tyrosine, serine, or threonine. The addition of a phosphate group to a protein can have many different effects on its target, including activating the phosphorylated protein, silencing the target protein, altering protein-protein interactions, and in some cases, targeting the protein for degradation. Phosphorylation will be discussed in more detail in Chapter 4 of this thesis.

SUMOylation of a protein occurs over a number of steps, where the end result is the covalent linkage of a SUMO moiety to a target lysine. The first step of SUMOylation begins with the SUMO precursor being cleaved to open up an activation site for the E1 activating enzyme. The E1 enzyme is a heterodimer consisting of SAE1 and SAE2 subunits and it is this enzyme that begins the SUMOylation process. This E1 enzyme creates a form of SUMO in which the C-terminus of SUMO is linked to AMP. This link is then broken, upon which the C-terminus of SUMO forms a thioester bond with one of the E1 enzyme subunits. Next, SUMO is transesterified from the E1 subunit to the E2 SUMO conjugating enzyme, UBC9. Interestingly, UBC9 can recognize the SUMO-targeted protein and the UBC9 and SUMO thioester can catalyze the bond between SUMO and the target lysine in the protein of interest. While this demonstrates that only the E1 and E2 enzymes are necessary to SUMOylate a protein, there are also E3 ligases specific to the SUMOylation cycle. E3 ligases increase the efficiency of SUMOylation. The SUMOylation cycle is completed when the SUMO moiety is deconjugated from the target protein. This process occurs through the use of SUMO-specific proteases (SUMOylation cycle reviewed in (Hay, 2005)).

SUMOylation of a protein can modify a number of cellular processes, such as altering protein-protein interactions, transcriptional activity, sub-cellular localization, protein stability and DNA binding. Generally, with respect to transcriptional activity, SUMOylation acts to repress transcription (reviewed in (Gill, 2003)). SUMOylation is thought to repress transcriptional activity through a number of mechanisms, which will be discussed in detail during Chapter 3 of this thesis.

Sox9 is modified by phosphorylation and SUMOylation, while Sox10 has been shown to be modified by SUMOylation only (Girard and Goossens, 2006; Huang et al., 2000; Komatsu,

2004; Malki et al., 2005; Oh et al., 2007; Taylor and Labonne, 2005). The SUMOylation of SoxE factors will be discussed in great detail in Chapter 2 and Chapter 3 of this thesis and evidence presented here clearly demonstrates that this modification plays an extremely important role in the formation of both the neural crest and inner ear.

While Sox10 has yet to be demonstrated as a phosphoprotein in published journal format, Sox9 does have documented phosphorylation sites. Huang and colleagues found that Sox9 was phosphorylated by protein kinase A (PKA) at two amino acids. They found that phosphorylation of these sites led to more efficient DNA binding and enhanced transcriptional activity (Huang et al., 2000). An additional, novel site for SoxE factor phosphorylation will be discussed in Chapter 4 of this thesis.

MitF is a SUMOylated protein and this modification will be discussed in detail during Chapter 3 of this thesis (Murakami and Arnheiter, 2005). MitF is also a phosphoprotein. It is phosphorylated by mitogen-activated protein kinase (MAPK), ribosomal S6 kinase (RSK), glycogen synthase kinase-3ß (GSK3ß) and p38 and while the exact roles of all of these modifications remains to be elucidated, they will surely play an important role in the regulation of MitF (reviewed in (Levy et al., 2006)).

The regulation of proteins by post-translational modification is a common molecular mechanism used to control the activity of individual proteins. This thesis will examine the use of SoxE and MitF protein modifications to regulate to formation of the neural crest and inner ear. Studying the regulation of these proteins is extremely important not only to enhancing the scientific community's knowledge, but also to gain insight into the misregulation of these proteins, which can lead to very devastating human diseases.

Human Disease and SoxE and MitF Transcription Factors

Consistent with the data emerging from developmental studies in model organisms, mutations in Sox9 and Sox10 have been linked to human congenital defects that are correlated with a deficiency in proper neural crest and inner ear development. The deletion of one *Sox9* allele in humans causes Campomelic Dysplasia (CD), characterized by severe skeletal defects, autosomal XY sex reversal, and deafness (Bi et al., 2001; Savarirayan et al., 2003; Spokony et al., 2002). This human syndrome is mirrored by a Sox9 heterozygous mouse, in which defects in the craniofacial cartilage and palate occur (Bi et al., 2001).

Mutations in Sox10 are associated with Waardenburg-Hirschsprung's disease (WS4), a syndrome associated with aganglionic megacolon, pigmentation defects, and deafness (Bondurand et al., 2001; Chan et al., 2003; Herbarth et al., 1998; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). Another Sox10 missense mutation is found in patients with Yeminite deaf-blind hypopigmentation syndrome (YDBS), which is characterized by pigmentation defects and deafness, but not aganglionic megacolon (Bondurand et al., 1999; Lang et al., 2000; Lang and Epstein, 2003). A mouse knockout model, Dom, mirrors human Sox10 mutations. These mice have cranial ganglia, enteric nervous system and melanocyte defects (Herbarth et al., 1998).

Mutations in MitF are associated with Waardenburg syndrome 2 (WS2A) (Read and Newton, 1997). This disease is commonly associated with hypopigmentation and sensorineural deafness. Tietz syndrome is another disease caused by MitF mutations. Tietz syndrome patients have severe congenital deafness and hypopigmentation (Amiel et al., 1998; Smith et al., 2000). While these two dominant syndromes appear to be similar, Tietz syndrome is more severe than WS2A. MitF and some of the MitF responsive genes are also up regulated in many, but not all

melanoma cell lines (Selzer et al., 2002; Vachtenheim and Novotna, 1999; Vachtenheim et al., 2001). While the role that MitF plays in melanoma has not been thoroughly investigated, it does indicate the potential to use MitF as a melanoma marker.

By elucidating the mechanisms by which Sox9, Sox10, and MitF regulate normal development of the neural crest and inner ear, light will be shed on the defects underlying these and other developmental disorders.

Xenopus ***as a model system***

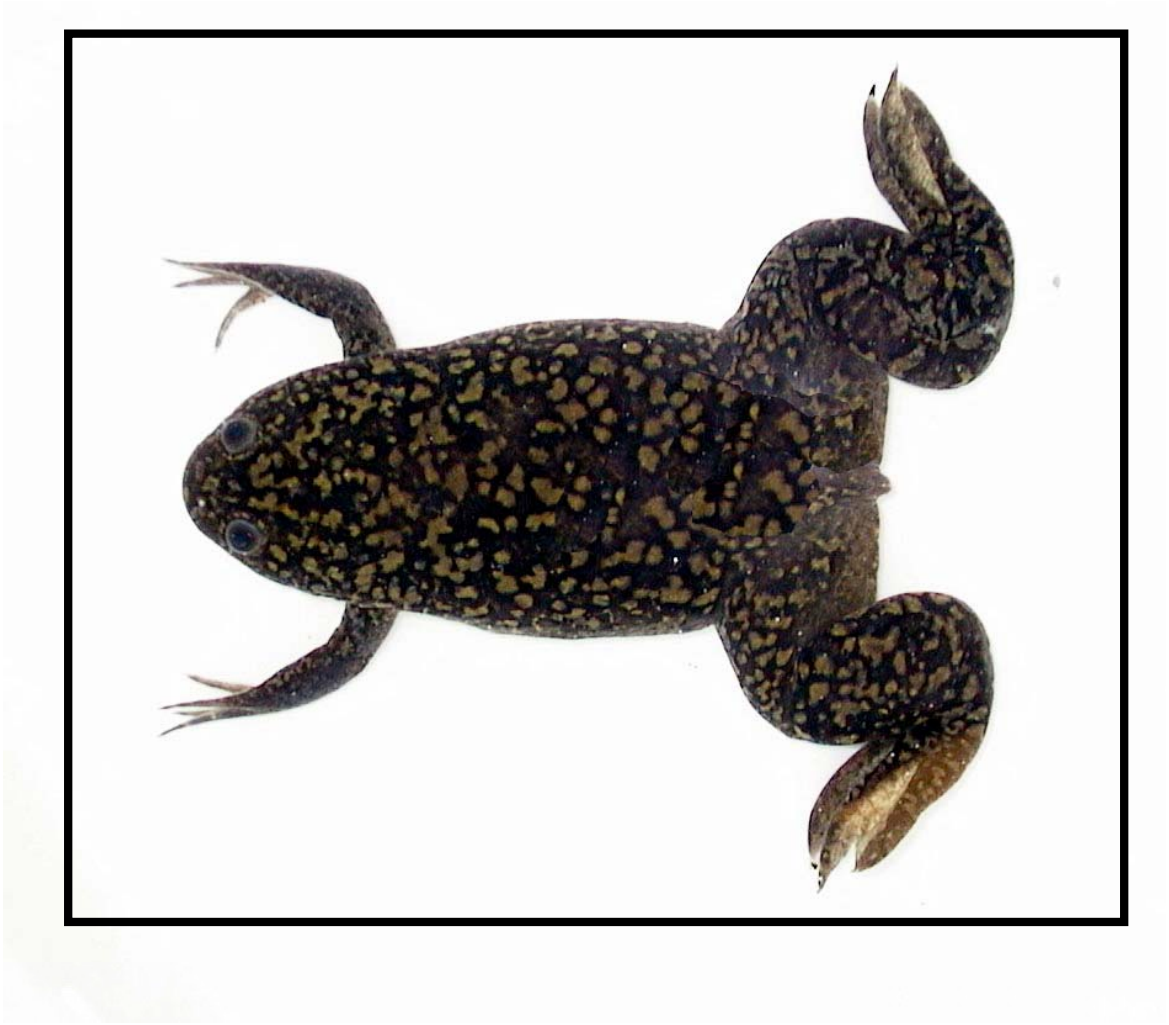
The experiments in this thesis, unless stated otherwise, were performed in the African clawed frog, *Xenopus laevis* (Figure 1.12*)*. *Xenopus* is a powerful system in which to investigate the molecular mechanisms underlying gene function, and the major advances in the understanding of early development have come from studies of these embryos. *Xenopus* is a particularly advantageous system for studying the neural crest and inner ear development, as both early and late stages of neural crest and inner ear development are accessible to experimental manipulation. A major advantage of the *Xenopus* system is the ease with which gene expression can be perturbed. Several methods are available for carrying out loss-of-function experiments, allowing rapid inhibition of either individual proteins or groups of related proteins. Constitutive and inducible over-expression experiments provide additional tools for investigating gene function. Furthermore, these embryos are large and easy to obtain in large numbers, facilitating the collection of material for biochemical studies. *Xenopus* provides advantages not readily available in any other model organism, making this an excellent system for the experiments described in this thesis.

Figure 1.12: Xenopus laevis (African clawed frog)

A mature, female *Xenopus laevis* frog. *Xenopus* serves as a good model organism because of the easy embryonic manipulations that can be performed, the large embryo size, and the numerous progeny that can be obtained for experiments on any given day.

*image taken from www.xenbase.org

Figure 1.12



Specific Aims of Thesis Project

Interestingly, embryonic development repeatedly uses the same signaling pathways and transcription factors to yield very different results. How the embryo has the ability to exploit single factors in a variety of ways is unclear. Two cell types where this phenomenon is present is the neural crest and inner ear. Pathways such as the Wnt pathway and factors such as the SoxE factors are used in early precursor formation, neural crest migration, and during neural crest differentiation steps to acquire very different outcomes. In addition, during the same developmental window when SoxE factors contribute to the development of the neural crest cells, they also contribute to the development of the inner ear. The overall goal of this thesis is to enhance our understanding of how SoxE factors help to regulate the formation of two cell types, the neural crest and the inner ear.

The first aim, presented in Chapter 2, was to investigate if Sox9 and Sox10 had redundant or divergent activity during neural crest and inner ear formation. Experiments performed in *Xenopus* embryos demonstrated that these factors could perform equivalently in both inner ear formation as well as early and late neural crest development. This data was of particular interest because at neural crest differentiation stages, Sox9 and Sox10 are not expressed in the same neural crest derivatives. Also, the embryonic ear is formed at the same time as these derivatives and is located very close spatially to these different tissues. These observations led to the fundamental biological question of not only how could these factors be used over and over during development to perform very different functions, but also how these functionally equivalent factors are then able to contribute to the formation of very different tissues during the same developmental window.

The second line of investigation focused on how these factors were able to perform divergent activities. Experiments performed in the second half of Chapter 2 demonstrated that Sox9 and Sox10 were post-translationally modified by SUMOylation and that this modification is a mechanism used by SoxE proteins to give rise to one cell fate over another. Specifically, only SoxE proteins that cannot be SUMOylated preferentially gave rise to ectopic neural crest precursors and ectopic melanocytes, while only SoxE proteins that were constitutively SUMOylated gave rise to enlarged embryonic ears.

The third line of investigation is presented in Chapter 3 and narrows in on the specific mechanism used by SUMO to prevent ectopic melanocyte formation. The melanocyte-specific promoter, Dct, was used in these experiments. Experiments presented in this chapter revealed that the presence of SUMOylation is inhibitory to the synergistic activation of this promoter. Not only does this chapter rule out several common mechanisms used by SUMOylation to prevent transcriptional synergy, it also presents evidence suggesting that SUMOylation blocks access of a co-activator to this promoter and hence decreases the transcriptional response, while at the same time recruiting a co-repressor to the promoter. Evidence provided at the end of Chapter 3 suggests that this may be a common regulatory mechanism used by SoxE factors throughout development.

Then, in Chapter 4, preliminary evidence is provided on potential SoxE-related projects that I will not publish in journal format. Data here demonstrates a novel SoxE phosphorylation site, several novel Sox10 interaction partners, and the effects of several SoxE domain deletion constructs on neural crest and inner ear formation.

Taken together, the studies presented in this thesis describe a specific mechanism used by SoxE proteins to regulate the formation of different cell types. These studies also present several

new ways in which SoxE proteins could be regulated during development, including phosphorylation and the use of different partner proteins. Chapter 5 describes the impact that these results have in the field and the future directions that have evolved from this thesis.

CHAPTER 2

SOXE FACTORS FUNCTION EQUIVALENTLY DURING NEURAL CREST AND INNER EAR DEVELOPMENT AND THEIR ACTIVITY IS REGULATED BY SUMOYLATION

Introduction

The SoxE factors Sox9 and Sox10 are both essential for the formation of the neural crest precursor cells. Sox9 and Sox10 also play divergent roles in the process by which neural crest cells are directed to form specific derivatives. Sox9 is involved in facial cartilage formation, while Sox10 is involved in cranial ganglia and melanocyte formation (Aoki et al., 2003; Spokony et al., 2002). These two factors have also been implicated in the development of the vertebrate inner ear, with Sox9 being one of the earliest known otic placode markers (Liu et al., 2003; Saint-Germain et al., 2004; Watanabe et al., 2000). Interestingly, the loss of Sox9 directly correlates with the loss of the otic placode at early neurula stages (Saint-Germain et al., 2004). Despite their importance, however, the mechanisms that allow SoxE proteins to regulate such a diverse range of cell types have remained poorly understood. Given their overlapping expression patterns and proposed roles in neural crest precursor formation, neural crest lineage diversification, and otic placode formation, it seemed possible that individual SoxE factors might possess some divergent activities, and that the complement of SoxE factors expressed in a cell might play an instructive role in dictating that cell's fate.

To test this hypothesis, I expressed Sox9 or Sox10 in early *Xenopus* embryos and compared and contrasted their activities. As demonstrated in this chapter, I found that each factor could direct the formation of neural crest precursors and the development of a range of neural crest derivatives, and I detected no differences in the activities of Sox9 and Sox10 in these assays. Moreover, I found that misexpression of Sox9 or Sox10 frequently resulted in the formation of enlarged or ectopic otocysts, demonstrating that both factors are potent effectors of inner ear formation. A central question in developmental biology is how factors with conserved

activity can mediate very different functional outcomes when expressed in different tissues. To gain insight into the mechanisms by which SoxE proteins might be regulated such that they can direct development of diverse cell types, I carried out a yeast two-hybrid screen that identified SUMO-1 and UBC9 as SoxE-interacting proteins.

In the following chapter, I will demonstrate that SoxE factors are regulated by SUMOylation. SUMOylation is a post-translational modification that is involved in numerous cell regulatory events, including but not limited to, altering protein-protein interactions, altering sub-cellular localization, and altering transcriptional activity (reviewed in (Hay, 2005)). I will show that SoxE mutants that cannot be SUMOylated, or that mimic constitutive SUMOylation, are each able to mediate a subset of the diverse activities characteristic of wild-type SoxE proteins. These findings provide important mechanistic insight into how the activity of widely deployed developmental regulatory proteins can be directed to specific development events.

Methods

DNA Constructs and Embryo Methods

A partial *XSox10* cDNA was isolated from an arrayed cDNA library, and a full-length clone was generated by 5' RACE (BD Clontech, Mountain View, CA). *XSox9* was isolated from stage 17 cDNA using low copy number PCR and a high-fidelity polymerase (Tgo, Roche, Indianapolis, IN). cDNAs were cloned into a pCS2 variant that adds five C-terminal myc tags (gift of R. Davis) and confirmed by sequencing. The Sox9 K61R and K365R mutations and Sox10 K44R and K333R mutations were generated using the Quick Change method (Stratagene, La Jolla, CA). hSUMO-1 was inserted in the vector pCS2-FlagN and used for *in vivo* assays.

$Sox9^{K61,365R}$/SUMO-1 and $Sox10^{K44,333R}$/SUMO-1 were created by ligating SUMO-1 in-frame C-terminal to the full-length $Sox9^{K61,365R}$ or $Sox10^{K44,333R}$ mutants using PCR methods, inserting a proline and a glycine between the two sequences. The fusion proteins were N-terminally epitope tagged by insertion into vector pCS2-Myc (provided by D. Turner). All constructs were confirmed by sequencing. All results shown are representative of at least two independent experiments. RNA for injection was produced *in vitro* from linearized plasmid templates using the Message Machine kit (Ambion, Austin, TX). mRNA concentrations injected were in the range of 5-50 pg. Embryo methods are as described in (LaBonne and Bronner-Fraser, 1998b). Briefly, pigmented and albino embryos were obtained and fertilized *in vitro* by established methods, dejellied and cultured in 0.1 X MMR. Staging of embryos was done according to the Nieuwkoop and Faber's normal table. Embryos were injected in 1 X MMR + 3% Ficoll then transferred to 0.1 X MMR until harvesting. At all times, nuclear ß-galactosidase mRNA was co-injected as a lineage tracer to allow identification of the injected side of the embryo. Embryos were fixed for 1 hour in MEMFA, and stored dehydrated in 100% ethanol. For ß-galactosidase staining, embryos were washed 2 X in PBS and 2 X in staining solution. Color reaction was carried out in the presence of X-gal (50 mg/ml) or Red Gal (100 mg/ml) at 37˚C until desired color was achieved. *In situ* hybridization was carried out with digoxigenin-labeled antisense RNA probes. Embryos were photographed in 100% ethanol (LaBonne and Bronner-Fraser, 1998b). Sox10 probe is directed against the 3'UTR of the message and does not recognize the coding region construct used for misexpression experiments. Constructs for making Pax8 and Dlx3 probes were provided by A. Brandli and T. Moreno, respectively.

Yeast Two-Hybrid Assays

Chapter 4 discusses this methodology in greater detail. Briefly, Sox10, amino acids 1-333 inserted in the vector pEG202, was used as bait to screen a gastrula stage yeast two-hybrid library in the pJG4-5 vector (gift of S. Sokol) essentially as described (Itoh et al., 2000). Positive interactors were recovered from yeast, shuttled through bacteria, and reteseted in yeast for stringency of interaction by growth on selective medium and by assaying ß-gal activity. Clones that retested were sequenced, and a number of these were identified as either Xenopus UBC-9 or Xenopus SUMO-1 (Genbank accession numbers BC046273 and Z97073, respectively). Baits consisting of Sox9 amino acids 2-305, $Sox9^{K61R}$ amino acids 2-305, and $Sox10^{K44R}$ amino acids 1-333 were also constructed in pEG202, and interactions with both SUMO-1 and UBC9 were compared by plating serial dilutions of the transformants on selective medium.

Western Blots and SUMOylation Assays

Wild-type or mutant Sox9 or Sox10 proteins were expressed in the presence or absence of SUMO-1, and embryos were collected at gastrula stages unless otherwise noted. For Western blots, embryos were lysed in RIPA buffer (50mM Tris HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with phenylmethylsulfonyl fluoride, aprotinin, leupeptin, N-ethylmaleimide, cytochalasin B, and 1,10-phenanthroline. Samples were resolved on SDS-PAGE and proteins were detected using antibodies against the epitope tags (Myc: 9E10, Santa Cruz Biotechnology, Santa Cruz, CA; Flag: M2; Sigma-Aldrich, St. Louis, MO). For loading controls, blots were stripped and

reprobed for actin (α-actin; Sigma-Aldrich). Secondary antibodies were horseradish-peroxidase coupled and detected by chemiluminescence (Pierce, Rockford, IL).

Morpholino Oligonucleotide Rescue Experiments

A Sox10 morpholino antisense oligonucleotide designed against the 5' UTR and coding region of Xenopus Sox10 (5'-AGCTTTGGTCATCACTCATGGTGCC-3', Sox10 MO) was obtained from Gene Tools, LLC (Philomath, OR). To deplete Sox10, 10 ng of Sox10MO was injected into a single blastomere at the eight-cell stage. For rescues, mRNA encoding epitope-tagged forms of either Sox9, Sox10, or $Sox9^{K61,365R}$ was subsequently injected, together with mRNA encoding the lineage tracer ß-gal, and harvested for *in situ* hybridization.

Results

Sox9 and Sox10 have equivalent effects on neural crest formation

Although Sox9 and Sox10 have recently emerged as key determinants of both neural crest and inner ear development, the extent to which SoxE factors play functionally equivalent roles in these processes has been less clear. I therefore expressed these factors in early *Xenopus* embryos and compared their ability to influence neural crest and inner ear fates. To control for dosage-dependant effects, epitope-tagged forms of both Sox9 and Sox10 were generated. The activities of the tagged and untagged forms of each protein were compared, and the epitope tags were found to have no effect on function. Experiments directly comparing Sox9 and Sox10

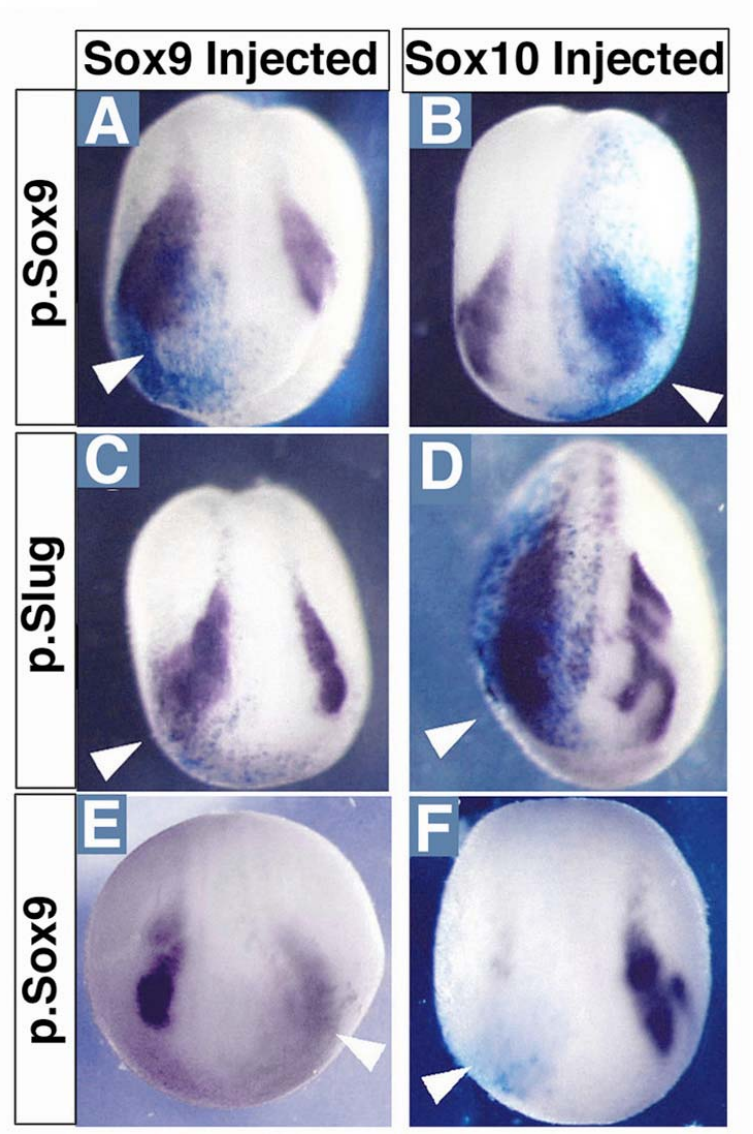
activity were carried out using the epitope-tagged factors, and protein expression levels were monitored via western blots. mRNA encoding epitope-tagged Sox9 or Sox10 was injected into one cell of 2-cell stage *Xenopus* embryos, and the injected embryos were allowed to develop to neurula stages, when the effects on neural crest precursor cells could be assayed by *in situ* hybridization. β-galactosidase mRNA was co-injected as a lineage tracer, and the uninjected side of the embryo served as a control for normal development.

I found that both Sox9 and Sox10 were able to increase the formation of neural crest precursor cells as evidenced by expanded expression of markers such as *Slug* and *Sox9* (Figure 2.1 A-D), consistent with previous reports (Aoki et al., 2003; Honore et al., 2003). Significantly, however, I also noted that neural crest markers were sometimes inhibited in Sox9- and Sox10-injected embryos. For example, in an experiment in which most embryos showed expanded expression of neural crest markers, I also noted embryos in which expression of the same markers had been inhibited (Figure 2.1 E,F) (Sox9 injected: 75% increased, 10% decreased, N=47; Sox10 injected: 68% increased, 8% decreased, N=38). This suggested that in the embryos showing a decrease, Sox9 or Sox10 activity might have led to the formation of some other cell type at the expense of neural crest precursors. When in the same experiment five-fold higher levels of Sox9 or Sox10 were expressed, this inhibition of neural crest precursor formation was noted more frequently than at lower doses (Sox9 injected: 45% increased, 34% decreased, N=29; Sox10 injected: 40% increased, 45% decreased, N=40). Importantly, I observed no differences in the abilities of Sox9 and Sox10 to expand or inhibit the formation of neural crest precursor cells. Moreover, the observation that the differences in these phenotypes were not fully dose-dependant suggested the possibility that the activity of SoxE proteins might be dynamically regulated, perhaps by post-translational modification.

Figure 2.1: Effects of Sox9 and Sox10 on neural crest development

In situ hybridization examining the expression of neural crest markers *Sox9* **(A, B, E, F)** and *Slug* **(C, D)** in Sox9- **(A,C,E)** or Sox10- **(B,D,F)** injected embryos. Injection of either Sox9 or Sox10 frequently leads to an increase in neural crest precursor formation (**A-D**, white arrowhead) but could also result in loss of neural crest precursor formation (**E, F**, white arrowhead).

Figure 2.1



Sox9 and Sox10 promote melanocyte and glial formation and inhibit neuronal differentiation

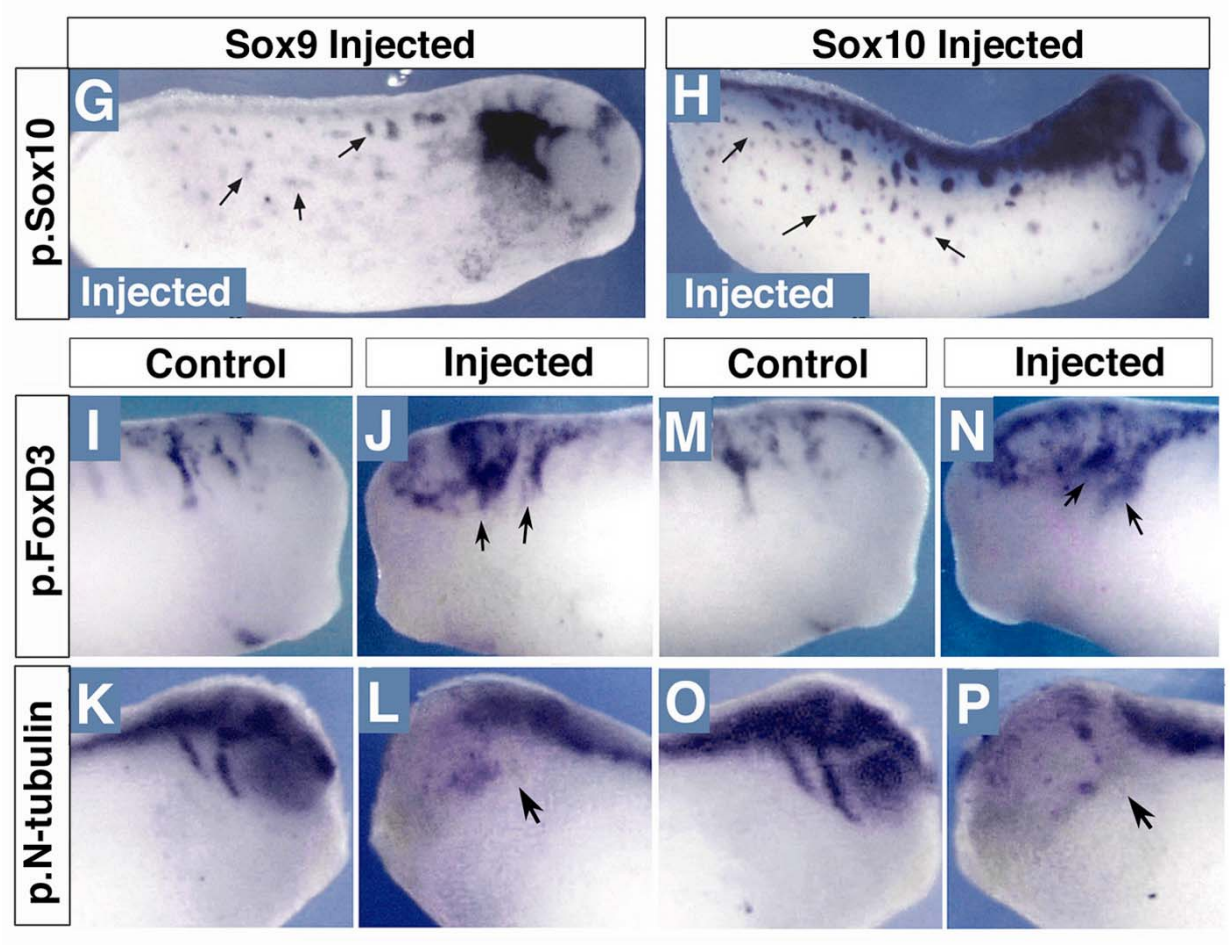
I next compared the ability of Sox9 and Sox10 to direct the formation of specific neural crest derivatives. In particular I was interested in comparing the effects of these two proteins on development of melanoblasts and glia, as previous studies have suggested that Sox10 is an important determinant of these lineages (Britsch et al., 2001; Kelsh et al., 2000; Southard-Smith et al., 1998; Stolt et al., 2002). Sox9 and Sox10 were expressed at levels that predominantly expand the neural crest progenitor pool, and injected embryos were harvested at tailbud stages for *in situ* hybridization with markers of developing melanoblasts, or allowed to develop to stages when formation of differentiated melanocytes could be directly evaluated. In these experiments I found that both Sox9 and Sox10 potently induced the formation of melanoblasts as evidenced by scattered ectopic cells on the embryonic flank that expressed melanocyte markers such as *Sox10*, *Mitf* and *Dct* (Figure 2.2 G,H and not shown; Sox9 injected: 51% of embryos, N=129; Sox10 injected: 53% of embryos, N=103), as well as by the formation of supernumerary melanocytes at swimming tadpole stages (Figure 2.11 C and not shown).

At the stages examined in these experiments, *Sox10* expression marks three cell populations: melanoblasts, glia, and the developing ear. In addition to numerous ectopic melanoblasts in caudal regions, I noted dramatically increased *Sox10* expression in cranial regions. Such staining is consistent with an increased number of cells adopting glial and/or otic fates. To distinguish between these possibilities, I examined expression of *FoxD3* at stage 28, when it serves as a glial marker (Gilmour et al., 2002; Kelsh et al., 2000). I found that Sox9- and Sox10-injected embryos showed significantly enhanced *FoxD3* staining in the cranial ganglia (Figure 2.2 I,J,M,N) (Sox9 injected: 69% increased, N=55; Sox10 injected: 74% increased,

Figure 2.2: Effects of Sox9 and Sox10 on neural crest development

G, H) Both Sox9 and Sox10 induce the formation of supernumerary and ectopic melanocytes (black arrows). **I-P)** An increase in *FoxD3*-expressing glia (**I** vs. **J**; **M** vs. **N**) and a decrease in *N-tubulin* expressing neurons in the cranial ganglia (**K** vs. **L**; **O** vs. **P**) are found in Sox9 **(I-L)** and Sox10 **(M-P)** injected embryos. Light blue or red stain is the lineage tracer β-gal.

Figure 2.2


Sox9 Injected
Sox10 Injected
p.Sox10
G
H
Injected
Injected
Control
Injected
Control
Injected
p.FoxD3
I
J
M
N
p.N-tubulin
K
L
O
P

N=46). Together these findings indicate Sox9 and Sox10 can both direct the formation of at least two cell types commonly associated with Sox10 function, melanocytes and glia.

Recent work has also implicated Sox10 in the maintenance of multipotency in neural crest stem cells and in the inhibition of neuronal differentiation (Kim et al., 2003; Paratore et al., 2002). Interestingly, another group showed that in avian embryos, misexpression of Sox9 blocks neuronal differentiation of neural crest cells (Cheung and Briscoe, 2003). I therefore asked if Sox9 or Sox10 could influence the adoption of neuronal fates in *Xenopus* neural crest cells. I found that both factors inhibited the differentiation of *N-tubulin*-expressing cells in the cranial ganglia, even at doses that significantly increased formation of *FoxD3*-expressing glia in sibling embryos (Figure 2.2 K,L,O,P) (Sox9 injected: 69% decreased, N=49; Sox10 injected: 72% decreased, N=42).

Sox9 can rescue neural crest formation in Sox10-depleted embryos

The above data demonstrate that in overexpression assays, Sox9 and Sox10 are functionally equivalent with respect to their ability to mediate neural crest formation. However, these experiments do not exclude the possibility that these factors perform equivalently in these assays secondary to an ability to positively cross-regulate each other's expression. For example, the ability of Sox9 to induce pigment cells might be due to its ability to induce expression of endogenous Sox10, which would then initiate a program of melanocyte differentiation. To explore this possibility, I compared the ability of Sox9 and Sox10 to rescue neural crest development in embryos depleted for Sox10. Embryos injected at the eight-cell stage with morpholinos targeting Sox10 show reduced or absent expression of early neural crest markers (Figure 2.3 A,B; 90% inhibited, N=59). Subsequent injection of mRNA encoding either Sox9 or

Sox10 significantly rescued formation of neural crest progenitors in these embryos (Figure 2.3 C-F; Sox9: 18% inhibited, N=40; Sox10: 21% inhibited N=52). These findings confirm that Sox10 does not have functions during neural crest precursor formation that cannot be compensated for by Sox9. Importantly, when rescued embryos were allowed to develop to tailbud stages, I found that Sox9 was still able to induce formation of ectopic melanoblasts despite Sox10 depletion (Figure 2.3 I-J). This finding demonstrates that the ability of Sox9 to direct melanocyte formation is not secondary to an ability to activate *Sox10*.

Sox9 and Sox10 direct the formation of enlarged and ectopic otic vesicles

Because SoxE factors have also been implicated in otic placode formation, I compared the effects of Sox9 and Sox10 activity on inner ear development. The consequences of SoxE misexpression on the development of this cell type have not been previously reported. I found that injection of mRNA encoding either factor frequently led to expanded expression of otocyst markers, or "enlarged ears" (Figure 2.4 A,B) (Sox9 injected: 55% enlarged, N=94; Sox10 injected: 61% enlarged, N=154). Moreover, in 3-5% of cases Sox9- and Sox10-injected embryos developed supernumerary otocysts, such that between two and four distinct otic vesicles formed on a single side of the injected embryo (Figure 2.4 C,D,F). To ask if the appearance of enlarged or ectopic otic vesicles correlated with an increase in size of the otic placode at neural plate stages, I examined the expression of *Pax8*, which is among the earliest markers of this structure (Heller and Brandli, 1999). I found that both Sox9 and Sox10 could expand the domain of *Pax8* expression corresponding to this placode (Figure 2.4 G,H) (Sox9 injected: 52% increased, N=50; Sox10 injected: 48% increased, N=62). Similarly, both factors could mediate increased *Dlx3*

Figure 2.3: Sox9 and Sox10 rescue neural crest development in Sox10-depleted embryos

Embryos injected with Sox10 morpholinos (MO) show reduced or absent expression of early neural crest markers such as *Sox10* (**A,B)** and reduced otic vesicles and cranial neural crest **(G,H)**. These defects could be rescued equally as well by Sox9 or Sox10 **(C-F, I-L).** Sox9 retains its ability to induce ectopic melanoblasts in Sox10 depleted embryos (**J**, black arrows). Light red stain is the lineage tracer β-gal.

Figure 2.3

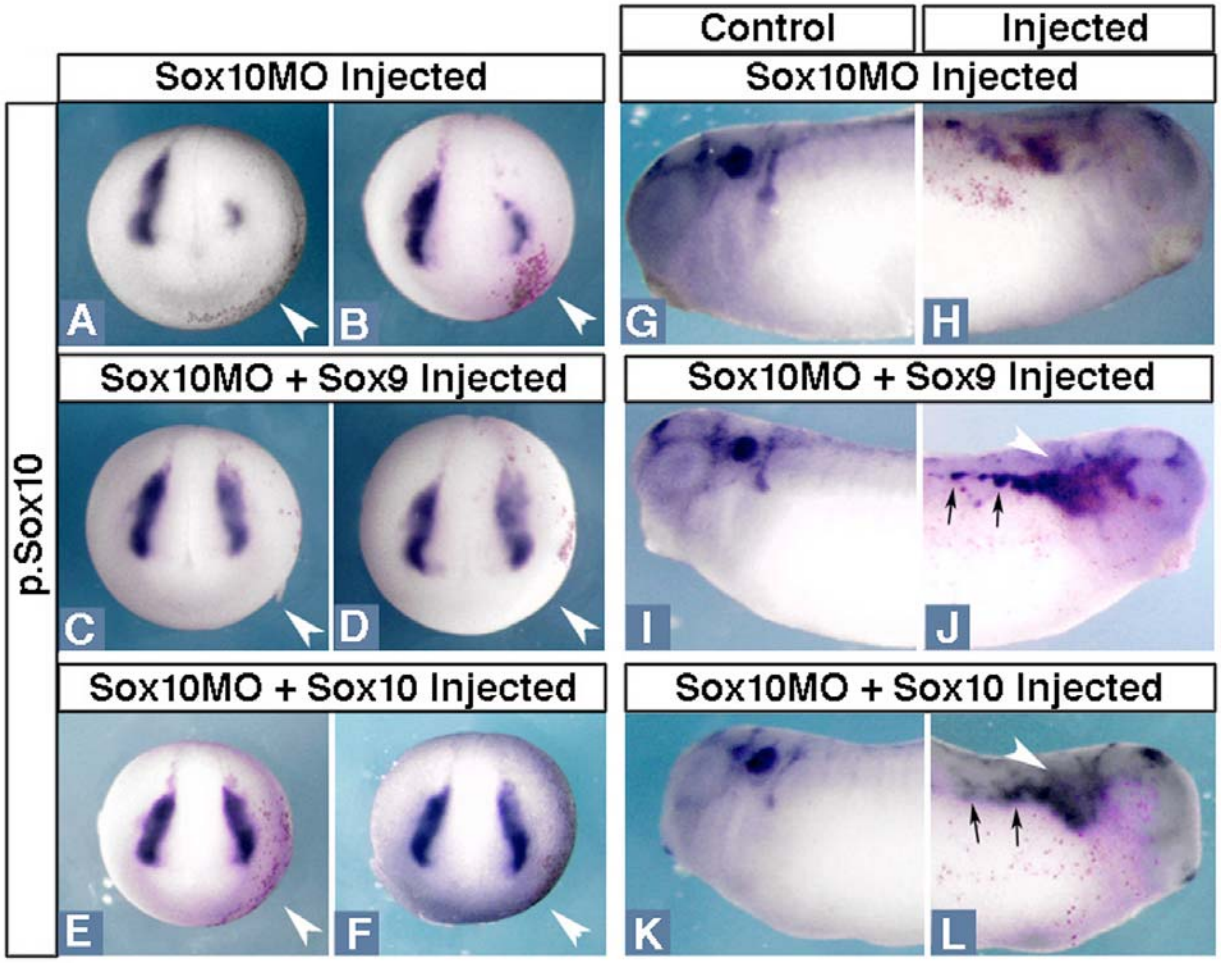
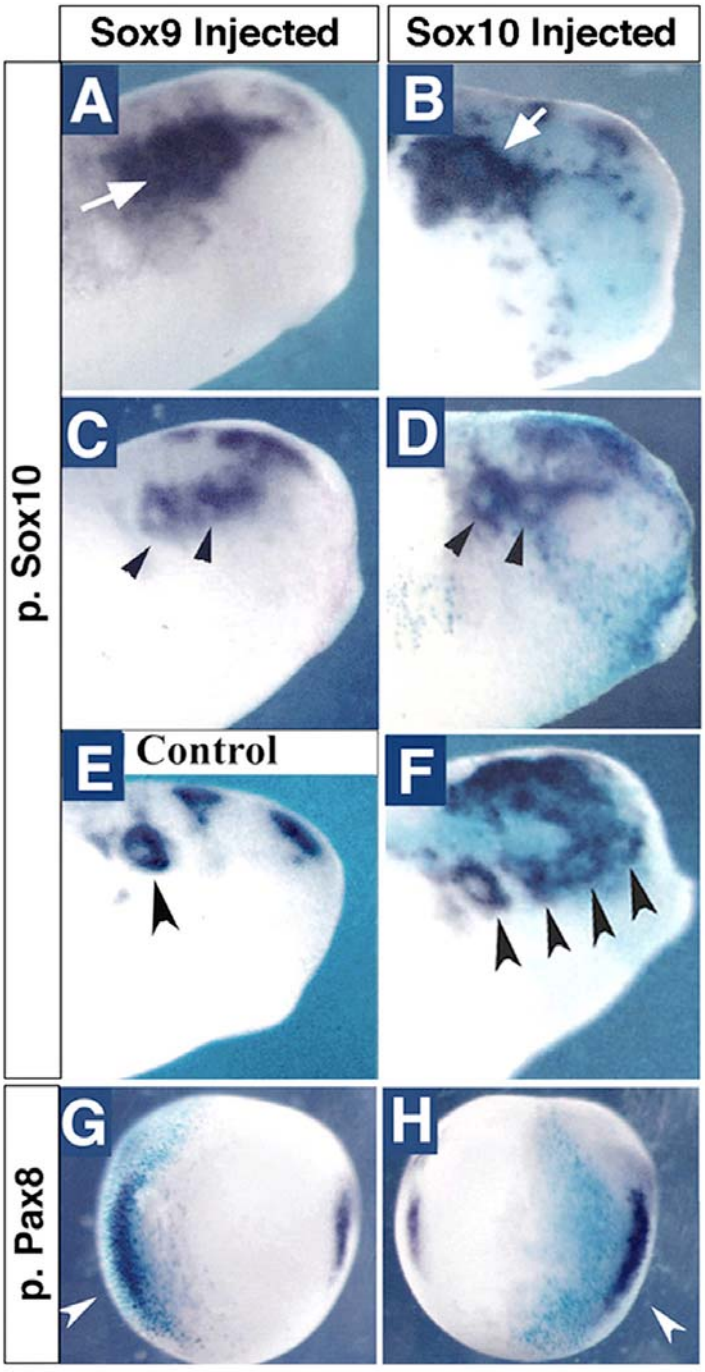

Control
Injected
Sox10MO Injected
Sox10MO Injected
p.Sox10
A
B
G
H
Sox10MO + Sox9 Injected
Sox10MO + Sox9 Injected
C
D
I
J
Sox10MO + Sox10 Injected
Sox10MO + Sox10 Injected
E
F
K
L

Figure 2.4: Sox9 and Sox10 induce enlarged and ectopic ear structures.

A-F) *In situ* hybridization showing *Sox10* expression in the ears of Stage 28 embryos injected with Sox9 (**A,C**) or Sox10 (**B,D,F**). Expression of either of these factors consistently leads to the formation of enlarged ears (**A,B**, white arrows) and/or to the formation of one or more ectopic ears (**C,D**,**F**, black arrowheads). **G,H**) Increased expression of *Pax8*, which marks the otic placode (white arrowhead) in Sox9 (**G**) or Sox10 (**H**) injected embryos at stage 13. Light blue or red stain is the lineage tracer β-gal.

Figure 2.4


Sox9 Injected
Sox10 Injected
A
B
C
D
E
Control
F
p. Sox10
G
H
p. Pax8

expression in the developing ear at stage 25. Importantly, however, Sox9 and Sox10 did not differ in their ability to induce the formation of enlarged or ectopic ears, providing further evidence that these factors function equivalently.

UBC9 and SUMO-1 are SoxE interacting factors

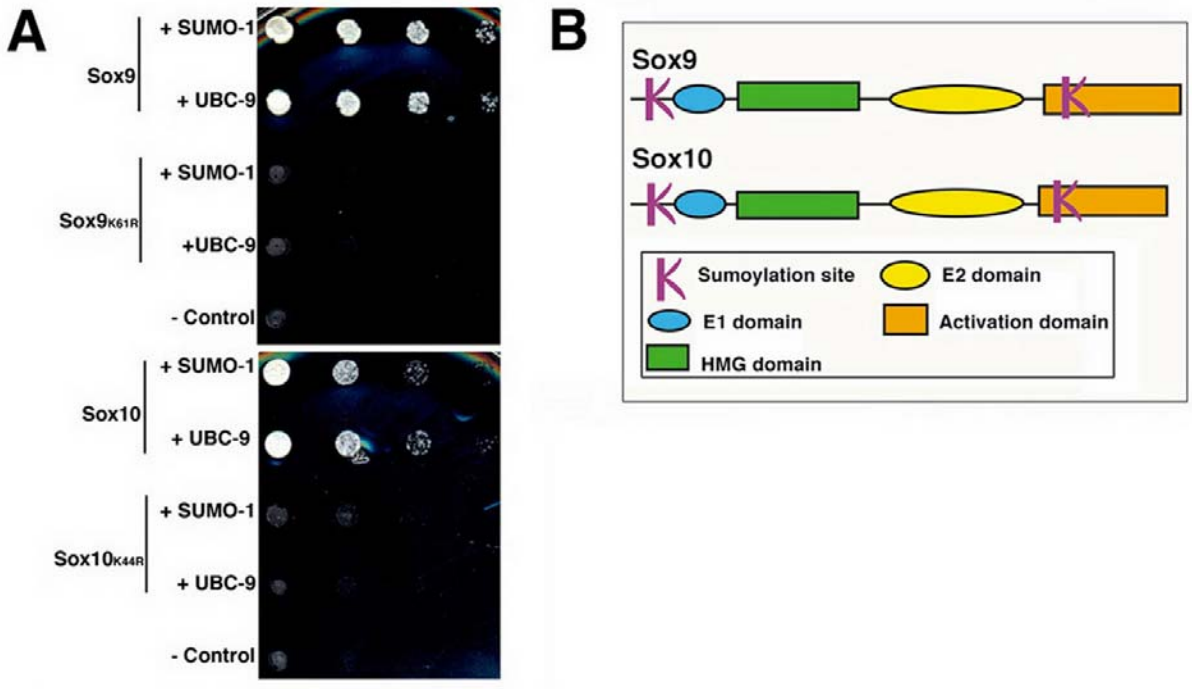
The above findings further highlight the question of how the activity of SoxE family proteins is regulated during the formation of diverse cell types such as the neural crest or otic placode. To address this, I sought to identify interacting proteins that might modify the function of these proteins during development. I carried out a yeast two-hybrid screen, using a Sox10 cDNA truncated before the activation domain as bait, and a *Xenopus* gastrula stage cDNA library as prey. This screen identified a number of Sox10 interacting proteins, among which were seven isolates of the small ubiquitin-like modifier SUMO-1, and twenty-seven isolates of the E2 SUMO conjugating enzyme UBC9, and these factors were also found to interact when the Sox9 N-terminus was used as bait (Figure 2.5A). Together, these findings suggested the intriguing possibility that SoxE activity might be regulated post-translationally via SUMOylation.

Examination of the *Xenopus* Sox9 and Sox10 sequences showed that they each possess two conserved SUMOylation consensus sites, ΨKXE (Gill, 2004). The first of these sites is located just N-terminal to the E1 domain, while the second site is located within the C-terminal activation domain (Figure 2.5B). Because not all proteins containing this motif are SUMOylated *in vivo* (Hay, 2005), I asked whether *Xenopus* Sox9 and Sox10 are SUMOylated in *Xenopus* embryos. When Sox9 or Sox10 were expressed in the presence or absence of epitope-tagged

Figure 2.5: Sox9 and Sox10 are SUMOylated

A) Growth of serially diluted cultures on selective media showing that both Sox9 and Sox10 (activation domain deleted) interact with UBC9 and SUMO-1 in a yeast two-hybrid assay. Mutation of the SUMOylation sites prevents interaction. **B)** Schematic of SoxE protein domains.

Figure 2.5


A
Sox9
+ SUMO-1
+ UBC-9
Sox9K61R
+ SUMO-1
+UBC-9
- Control
Sox10
+ SUMO-1
+ UBC-9
Sox10K44R
+ SUMO-1
+ UBC-9
- Control
B
Sox9
Sox10
Sumoylation site
E2 domain
E1 domain
Activation domain
HMG domain

SUMO-1, western blot analysis indicated that Sox9 and Sox10 could each be SUMOylated on two sites, and that each modification leads to a distinct and distinguishable shift in mobility on SDS-PAGE gels (Figure 2.6 A,B). Blotting against the myc tag on Sox9 or Sox10 following immunoprecipitation of SUMO-1 (flag) confirmed that the slower mobility SoxE isoforms represent SUMOylated products (Figure 2.6 C), and SUMO modification of Sox9 has also recently been observed in human embryonic kidney cells (Komatsu et al., 2004). I found that at gastrula stages Sox10 is SUMOylated at lysine 44 in the amino terminus as well as at lysine 333 in the activation domain. In Sox9, the predominant site of SUMOylation is lysine 365 in the C-terminus, although the protein can also be SUMOylated on lysine 61. The identities of these modified residues were confirmed by mutating one or both reactive lysines in the hypothesized SUMOylation sites to arginine in order to block their SUMOylation. Experiments in which Sox9 and Sox10 isoforms carrying these lysine mutations were co-expressed with SUMO-1 in early embryos confirmed that these were the only SUMOylated sites in these proteins (Figure 2.7A,B). Consistent with this, Sox9 and Sox10 proteins lacking these lysine residues no longer interact with SUMO-1 or UBC9 in yeast (Figure 2.5 A). Interestingly, when only a single modified lysine in Sox10 was mutated, only one of the two slower migrating Sox10 species was lost (Figure 2.7 B). These findings indicate that the SDS-PAGE mobility differences reflect which site in the protein has been SUMOylated, rather than the number of SUMO moieties appended.

SUMOylation modulates SoxE function during neural crest development

More than half of all identified SUMO substrates are transcriptional regulatory proteins, and SUMOylation can either up- or down-regulate the activity of these factors (Girdwood et al., 2004). Having confirmed biochemically that SoxE proteins are SUMOylated in *Xenopus*

Figure 2.6: Sox9 and Sox10 are SUMOylated

A, B) Western blot of lysates prepared from embryos injected as noted showing modification of Sox9 or Sox10 with SUMO-1. Arrows mark reduced mobility forms resulting from conjugation to one or more SUMO moieties. Note that Sox9 has one predominate site of SUMOylation, indicated by the black arrow. **C)** Lysates from embryos injected with Sox9 or Sox10 alone, or together with SUMO-1, were immunoprecipitated (IP) with antibodies against the epitope tag in SUMO-1 (flag) and then immunoblotted (IB) using antibodies against the epitope tag on the SoxE factors (myc), confirming that more slowly migrating SoxE species are SUMOylated. Direct immunoblotting of the crude lysate with either myc or flag antibodies served as in input control (bottom panels).

Figure 2.6

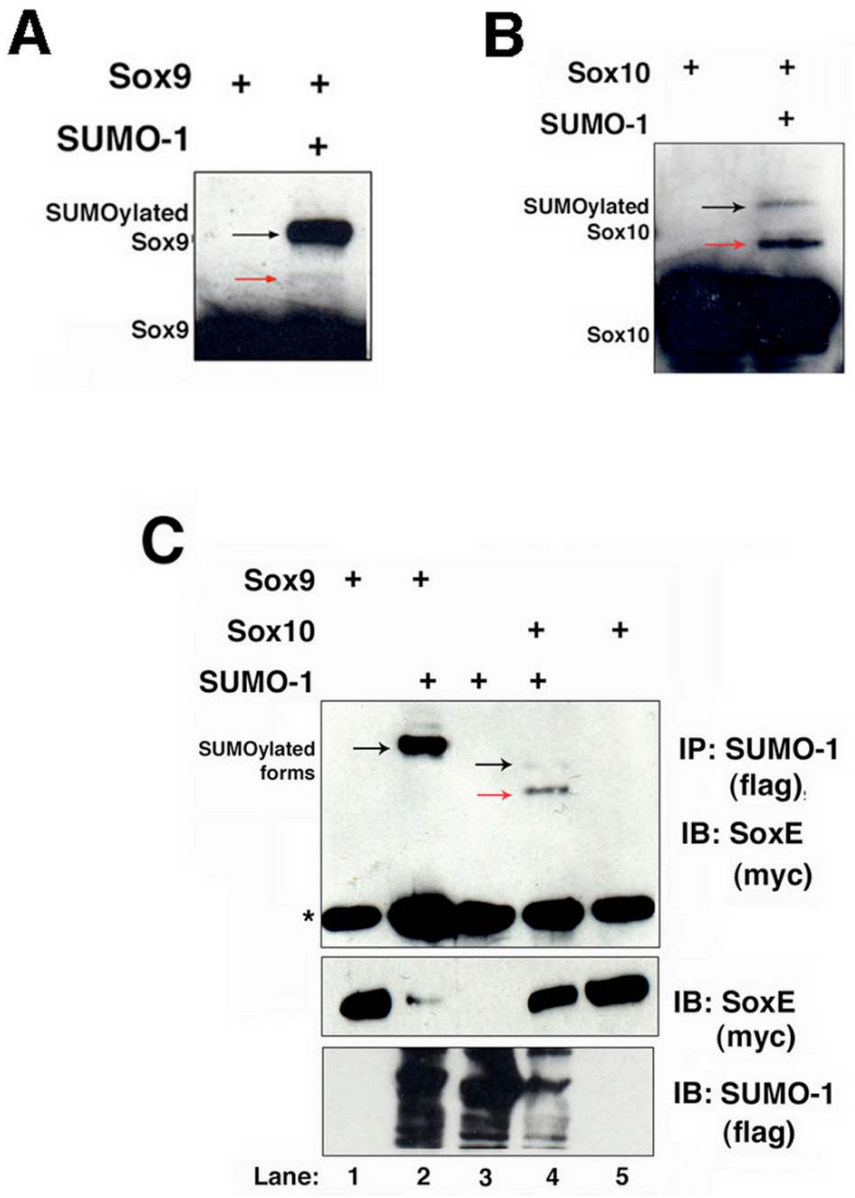
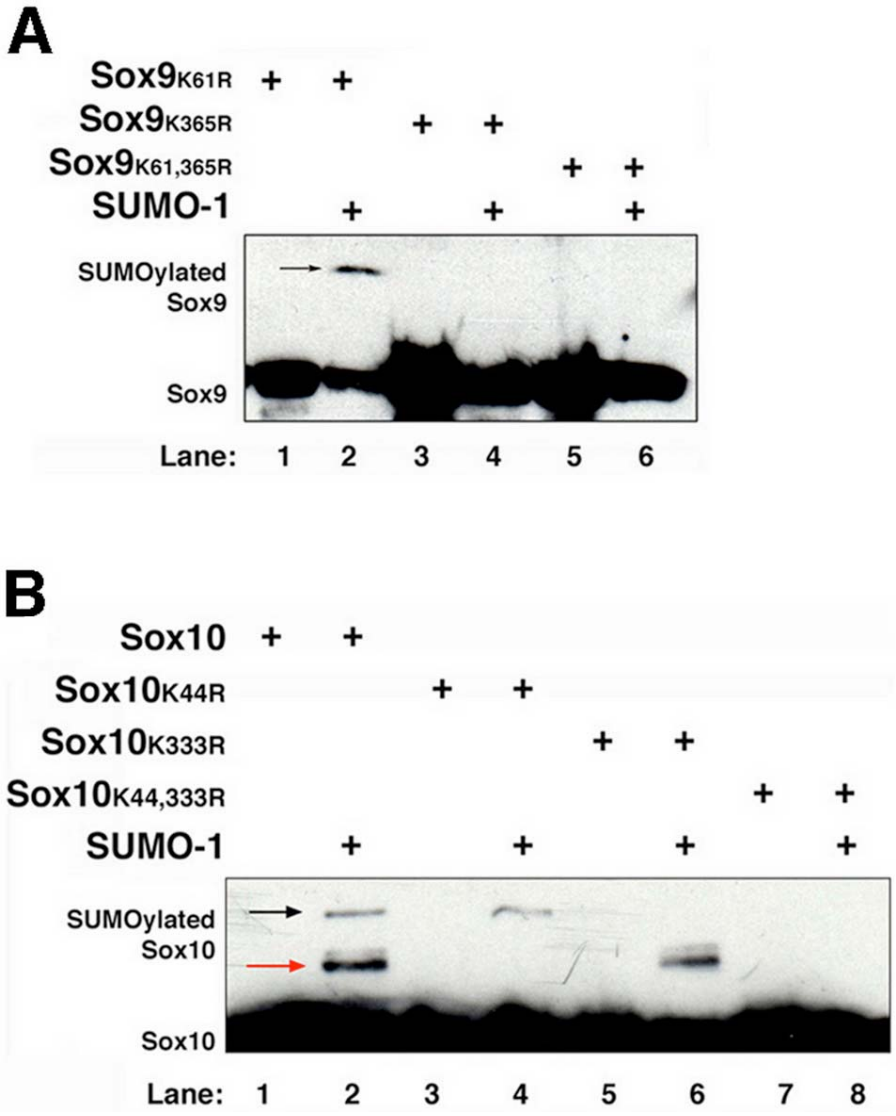

A
Sox9 + +
SUMO-1 +
SUMOylated Sox9
Sox9
B
Sox10 + +
SUMO-1 +
SUMOylated Sox10
Sox10
C
Sox9 + +
Sox10 + +
SUMO-1 + + +
SUMOylated forms
*
IP: SUMO-1 (flag)
IB: SoxE (myc)
IB: SoxE (myc)
IB: SUMO-1 (flag)
Lane: 1 2 3 4 5

Figure 2.7: Identification of SoxE SUMOylation sites

A) Mutation of the reactive lysine in the C-terminal Sox9 SUMOylation site eliminates the major modified species. **B)** Western blot of lysates prepared from embryos expressing wildtype or lysine mutant Sox10 proteins. Two SUMO-modified forms of Sox10 of different mobility are noted (Black and Red arrows). Each single lysine mutation eliminates only a single one of these species.

Figure 2.7


A
Sox9K61R + +
Sox9K365R + +
Sox9K61,365R + +
SUMO-1 + + +
SUMOylated Sox9
Sox9
Lane: 1 2 3 4 5 6
B
Sox10 + +
Sox10K44R + +
Sox10K333R + +
Sox10K44,333R + +
SUMO-1 + + + +
SUMOylated Sox10
Sox10
Lane: 1 2 3 4 5 6 7 8

embryos, I sought to better understand how SUMOylation modulates SoxE function during neural crest development. I initially focused these studies on Sox9, as this protein displayed only one predominant site of SUMO incorporation. In addition to our loss-of-SUMOylation mutants, I generated a form of Sox9 which had SUMO-1 fused in frame to its C-terminus (Figure 2.8). Such fusions have been found to mimic the constitutively SUMOylated state of a protein and have proven particularly useful when the native SUMOylation site occurs near the terminus of a protein of interest, as it is in Sox9 (Holmstrom et al., 2003; Long et al., 2004; Ross et al., 2002). In order to facilitate interpretation of these experiments, I ensured that the fused SUMO moiety would represent the only SUMOylation of the protein by appending it to the double lysine mutant to create $Sox9^{K61,365R}$/SUMO-1.

I first wished to ascertain whether the SUMOylation mutants affected Sox9 protein stability, as SUMOylation of targeted lysines in some proteins regulated by ubiquitin-dependant proteolysis results in stabilization of those proteins (Hay, 2005). I did not expect this to be the case for Sox9 and Sox10, however, as I have found these proteins to be very stable when expressed in *Xenopus*, and have been unable to detect their ubiquitination. Nevertheless, to directly ask if blocking SUMOylation alters Sox9 stability, I expressed wildtype Sox9 and $Sox9^{K61,365R}$ such that equivalent protein levels were detected at blastula stages, and then compared the expression levels of these proteins over time. I detected no difference in protein stability between wildtype Sox9 and the double lysine mutant (Figure 2.9); making it unlikely that SUMOylation regulates SoxE stability in this system.

To examine the effects of Sox9 SUMOylation on neural crest development, mRNA encoding wildtype Sox9, $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO-1 was injected into one cell of 2-

Figure 2.8

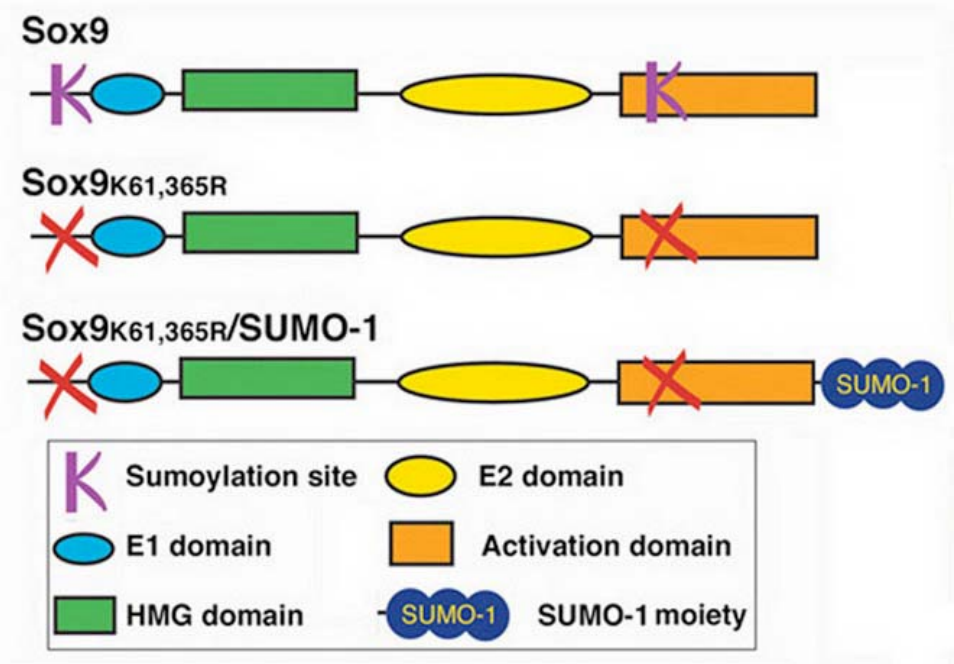


Figure 2.8: Schematic depicting expressed Sox9 isoforms

Figure 2.9

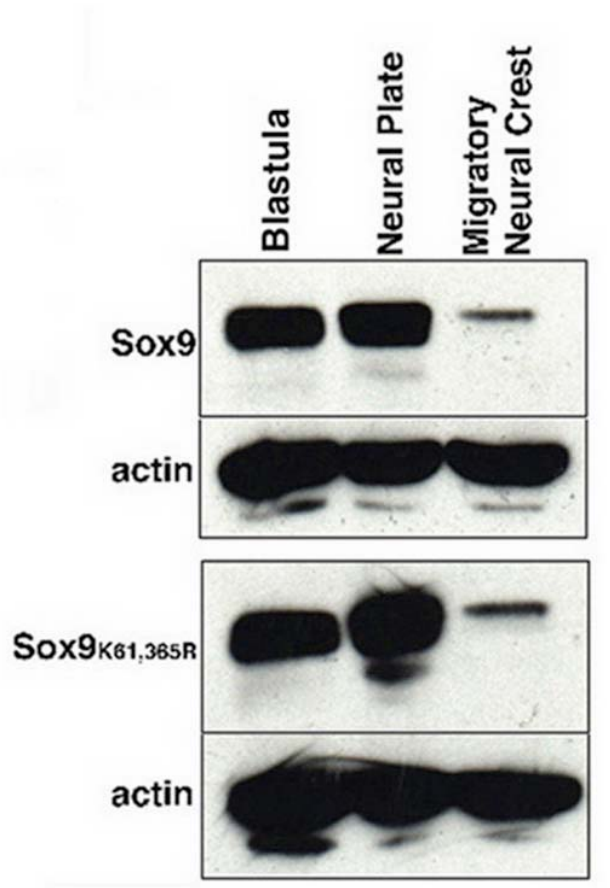


Figure 2.9: SUMOylation of Sox9 does not affect Sox9 protein stability

Western blot showing that mutation of SUMOylation sites does not affect Sox 9 protein accumulation.

cell stage embryos and effects on neural crest precursor cells were assayed by *in situ* hybridization at neurula stages. These proteins were epitope-tagged and were expressed at equivalent levels as determined by western blot. When the expression of markers such as *Slug* and *Sox10* were examined in these embryos, I found that Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 had dramatically different effects on neural crest precursor formation. For example, when expressed at levels at which the wildtype protein mediates a modest increase in *Sox10* expression, Sox9^{K61,365R} consistently induced a dramatic increase in *Sox10* expression (97% increased, N=81). Conversely, Sox9^{K61,365R}/SUMO-1 strongly inhibited *Sox10* expression (97% inhibited, N=82) (Figure 2.10 A). To confirm that my findings with Sox9 SUMOylation mutants were generally applicable to SoxE factors, I made an analogous set of mutations in Sox10 to create Sox10^{K44,333R} and Sox10^{K44,333R}/SUMO-1. I found that the effects of overexpressing these Sox10 SUMOylation mutants closely mimicked the effects of their Sox9 counterparts (Figure 2.10 B). Given that wildtype SoxE proteins can both positively and negatively influence the formation of neural crest progenitors (Figure 2.1), these results suggest the SUMOylation state of the expressed protein may be an important determinant of these different outcomes.

Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 were also found to differentially affect the formation of neural crest derivatives. For example, as with wildtype Sox9, embryos injected with Sox9^{K61,365R} developed numerous scattered Sox10-expressing melanoblasts on the injected side of the embryo (92%, N=121). By contrast, Sox9^{K61,365R}/SUMO-1 was unable to induce ectopic melanoblasts (0%, N=162) (Figure 2.11 A, black arrows). Similarly, both wildtype Sox9 and Sox9^{K61,365R} mediate the formation of supernumerary differentiated melanocytes on the injected side of the embryo, while Sox9^{K61,365R}/SUMO-1 does not (Figure 2.11 C). Again, I found

Figure 2.10: Regulation of Sox9 and Sox10 activity in neural crest precursors by SUMOylation

A) *In situ* hybridization showing neural crest expression of *Sox10* in Sox9 (i), Sox9^{K61,365R} (ii), and Sox9^{K61,365R}/SUMO-1 (iii) injected embryos. **B)** *In situ* hybridization showing *Sox10* expression in Sox10 (i), Sox10^{K44,333R} (ii), and Sox10^{K44,333R}/SUMO-1 (iii) injected embryos.

Figure 2.10

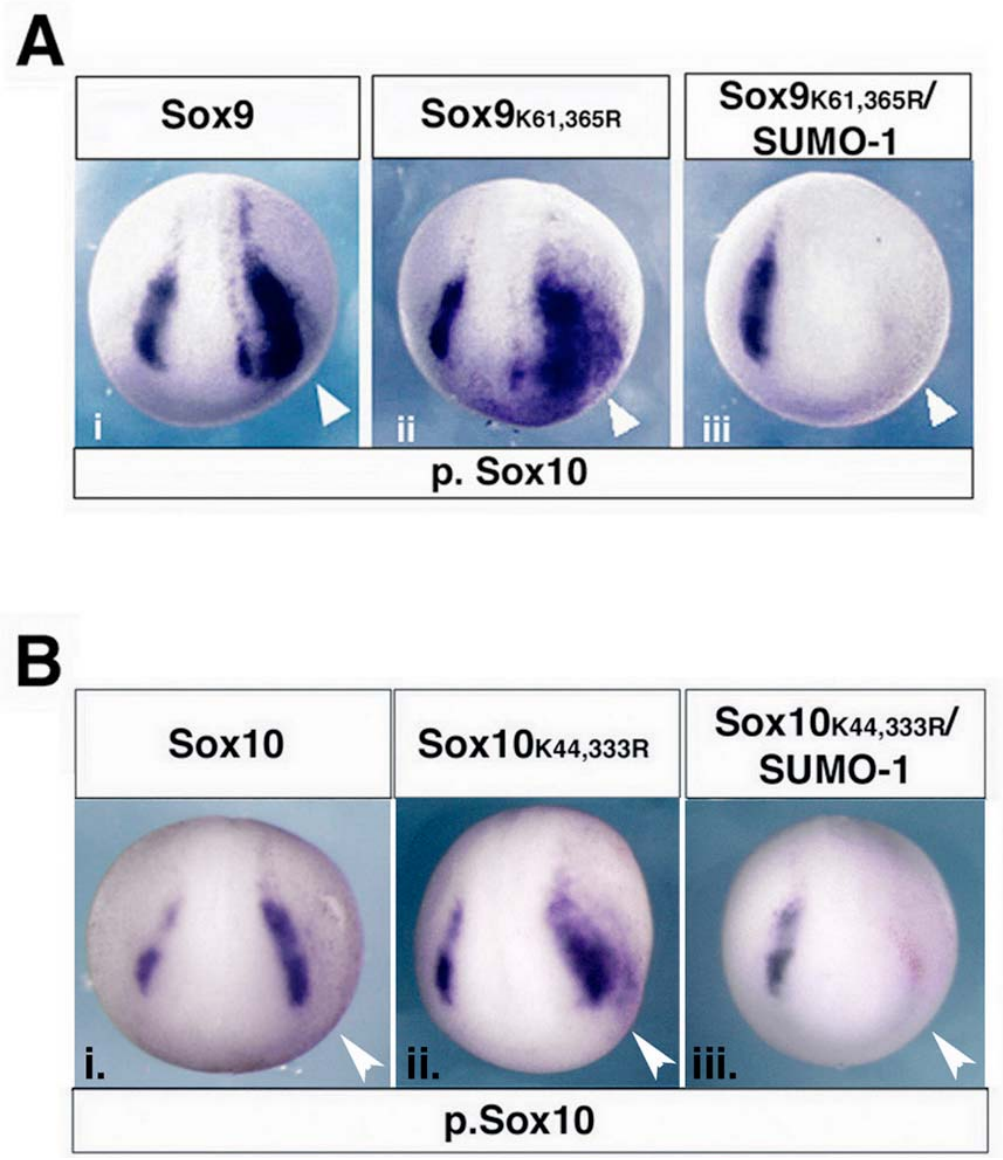
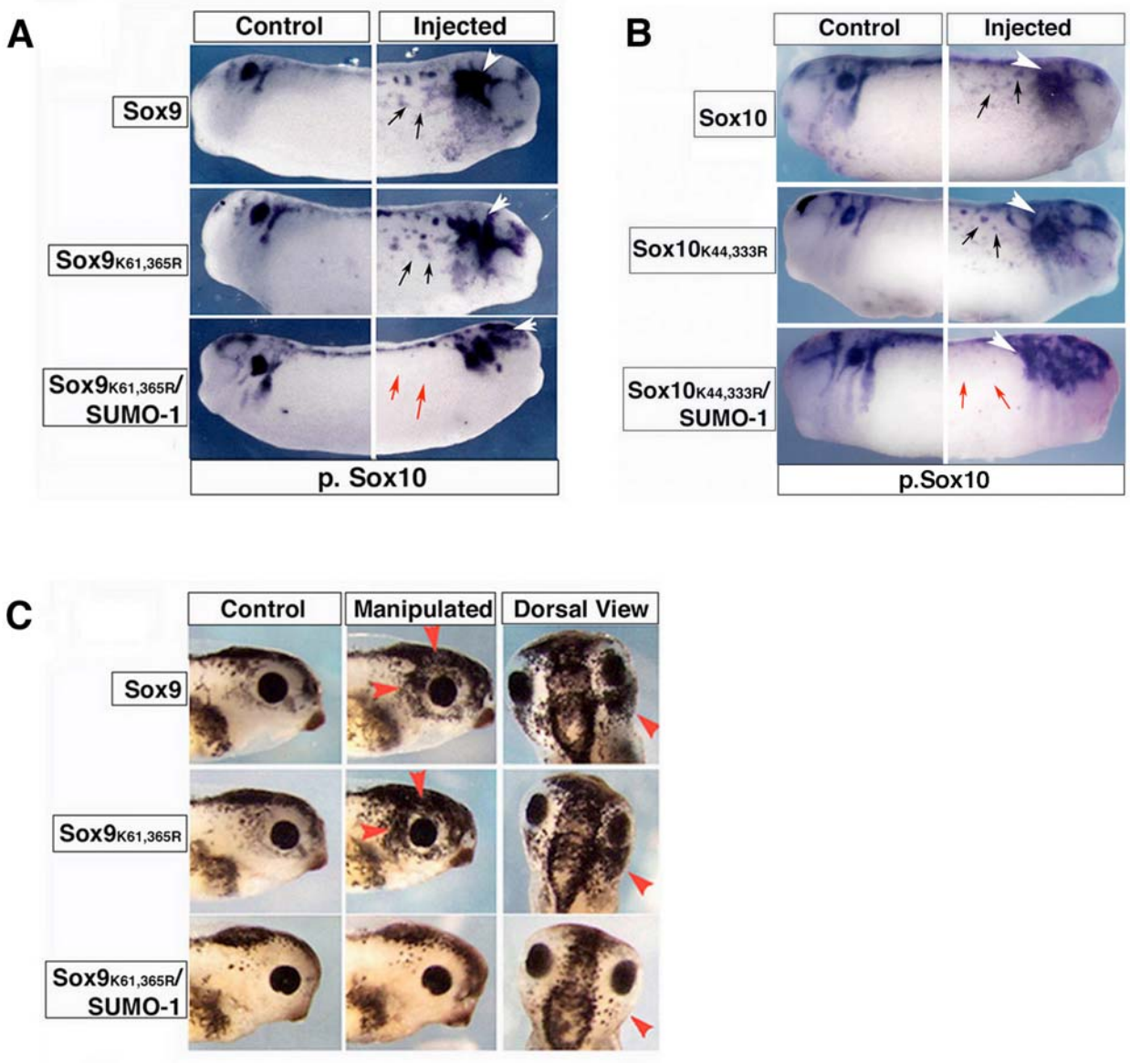

A
Sox9
Sox9K61,365R
Sox9K61,365R/
SUMO-1
i
ii
iii
p. Sox10
B
Sox10
Sox10K44,333R
Sox10K44,333R/
SUMO-1
i.
ii.
iii.
p.Sox10

Figure 2.11: Regulation of Sox9 and Sox10 activity at neural crest differentiation stages by SUMOylation

A) *In situ* hybridization showing ectopic *Sox10* expressing melanoblasts (black arrows) in stage 28 embryos injected with wildtype Sox9, $Sox9^{K61,365R}$. $Sox9^{K61,365R}$/SUMO-injected embryos never develop ectopic melanoblasts (note absence of these cells in region of red arrows) but do show increased *Sox10* expression in cranial regions (white arrowheads, compare injected and control sides of same embryos). **B)** *In situ* hybridization showing ectopic *Sox10*-expressing melanoblasts (black arrows) in stage 28 embryos injected with wildtype Sox10, $Sox10^{K44,333R}$. $Sox10^{K44,333R}$ /SUMO-injected embryos never develop ectopic melanoblasts (note absence of these cells in region of red arrows) but do show increased *Sox10* expression in cranial regions (white arrowheads, compare injected and control sides of same embryos). Red stain (including three cells visible in flank of embryo in bottom right panel) is the lineage tracer beta-gal. **C**) Close ups of the heads of the swimming tadpoles injected with wildtype Sox9, $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO. Both wildtype Sox9 and $Sox9^{K61,365R}$ mediate formation of supernumerary differentiated melanocytes on the injected sides of the embryos (red arrowheads), while $Sox9^{K61,365R}$/SUMO is unable to do so.

Figure 2.11


A
Control
Injected
Sox9
Sox9K61,365R
Sox9K61,365R/
SUMO-1
p. Sox10
B
Control
Injected
Sox10
Sox10K44,333R
Sox10K44,333R/
SUMO-1
p.Sox10
C
Control
Manipulated
Dorsal View
Sox9
Sox9K61,365R
Sox9K61,365R/
SUMO-1

that the effects of overexpressing the Sox10 SUMOylation mutants closely mimicked the effects of their Sox9 counterparts (Figure 2.11 B). Also like wildtype Sox9, I found that Sox9^{K61,365R} could rescue neural crest development in Sox10-depleted embryos (Figure 2.12; Sox10MO: 93% inhibited, N=28; Sox10MO+Sox9^{K61,365R}: 29% inhibited, N=34).

Importantly, however, Sox9^{K61,365R}/SUMO-1 is not inactive in these assays, as it strongly induced ectopic Sox10 expression in cranial regions (Figure 2.11 A,B, white arrowheads; Figure 2.13). Some of this staining was reflective of enlarged otocysts, although staining corresponding to an increase in cranial glia was also apparent.

SUMOylation of SoxE proteins is important for inner ear development

Given our findings that SoxE proteins could mediate otic placode formation, I compared the ability of Sox9^{K61,365R} or Sox9^{K61,365R}/SUMO-1 to modulate development of this tissue. In contrast to its effects on neural crest precursor formation, I found that Sox9^{K61,365R}/SUMO-1 increased the size of the otic placode as evidenced by an expanded domain of *Pax8* expression (47% increased, N=44). Conversely, Sox9^{K61,365R} inhibited *Pax8* expression in placodal regions (61% decreased, N=55) (Figure 2.14 A). Consistent with this, when I examined expression of *Dlx3* in the otic vesicle at tailbud stages I found that Sox9^{K61,365R} inhibited its expression (50% decreased, N=51), whereas Sox9^{K61,365R}/SUMO-1 injected embryos frequently showed expanded regions of *Dlx3* expression (55% increased, N=47) (Figure 2.14 B). These findings underscored that the constitutively SUMOylated form of Sox9 has activities that are distinct from those possessed by the unmodified protein.

Figure 2.12

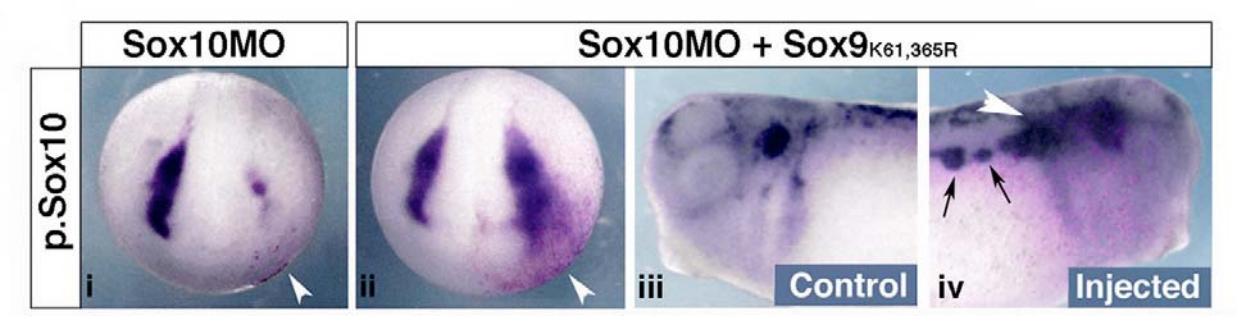


Figure 2.12: Sox9^{K61,365R} can rescue Sox10 morpholino-mediated depletion.

Sox9^{K61,365R} can rescue early (ii) and late (iv) aspects of neural crest formation in Sox10MO-injected embryos (white arrowheads), and retains its ability to induce ectopic melanoblasts (iv, black arrows).

Figure 2.13: Sox9^{K61,365R}/SUMO-1 has significant positive effects in embryos

Close ups of cranial regions of embryos injected with Sox9^{K61,365R} or Sox9^{K61,365R}/SUMO-1 and processed for *in situ* hybridization for *Sox10* at stage 28. Positive effects of Sox9^{K61,365R}/SUMO-1 include enlarged ears on the injected side (middle panels, red arrowhead) versus control side (black arrowhead), as well as increased glial staining (bottom panels). Red stain is the lineage tracer beta-gal.

Figure 2.13

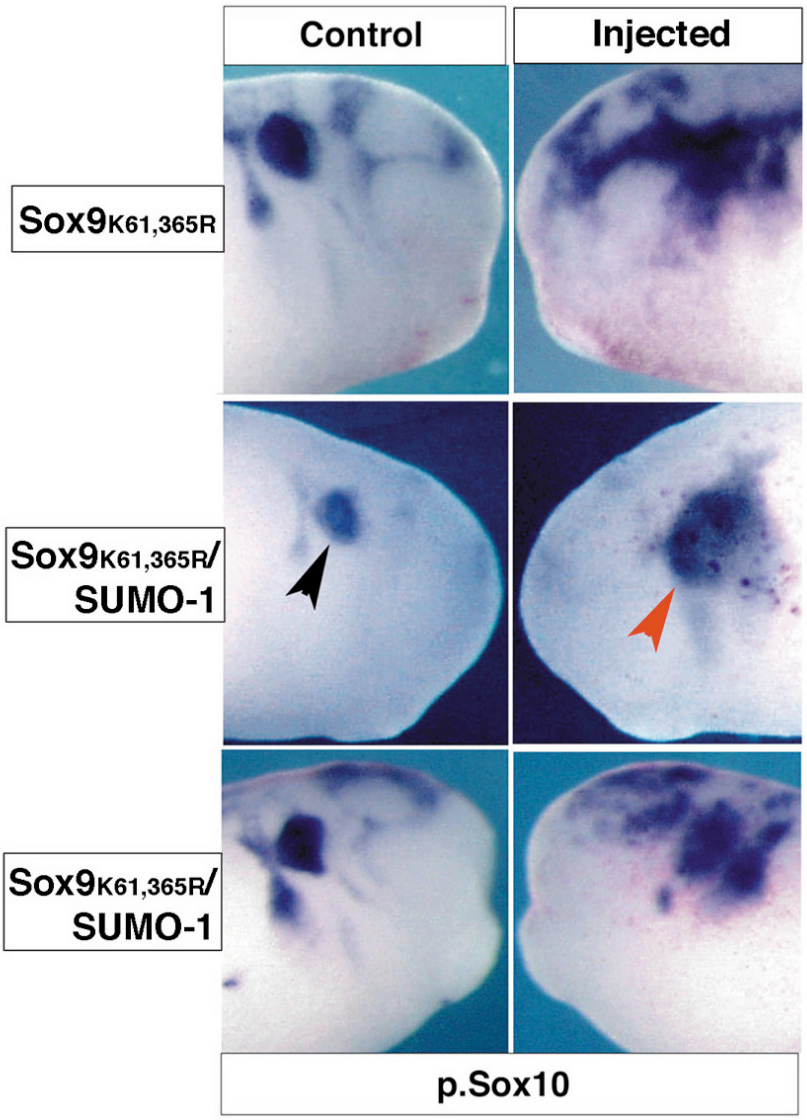

Control
Injected
Sox9K61,365R
Sox9K61,365R/
SUMO-1
Sox9K61,365R/
SUMO-1
p.Sox10

Figure 2.14

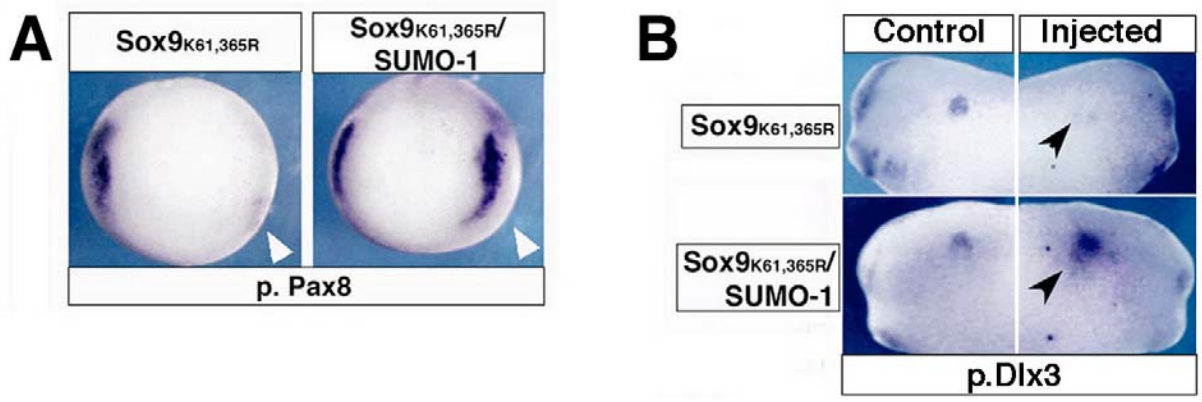


Figure 2.14: The effects of Sox9 SUMOylation on inner ear development

A) *Pax8* expression in the otic placode of stage 13 embryos previously injected with $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO. **B)** *Dlx3* expression, which marks the otocyst, in stage 28 embryos previously injected with $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO.

Moreover, my results strongly suggest that the SUMOylation state of SoxE factors plays a central role in determining whether these factors will mediate neural crest or otic placode formation, most likely by regulating their choice of partner interactions.

Discussion

SoxE factors are involved in early neural crest specification, neural crest derivative formation, and inner ear development. While Sox9 and Sox10 have overlapping expression patterns early on during neural crest precursor formation, the patterns diverge during neural crest differentiation stages. I became interested in this phenomenon upon taking a closer look at the protein structure of Sox9 and Sox10, which is highly homologous. It was curious that these factors would have different activities since they looked so physically similar, and perhaps it was their expression patterns that had evolved over time and not their function. As shown in the data above, I have provided evidence indicating that Sox9 and Sox10 can indeed perform equivalently in functional assays. This is perhaps most striking given that Sox9 can fully rescue Sox10 depleted embryos at both early and late developmental stages. Clearly, however, in unaltered developmental events, these proteins do contribute to the formation of different cell types, perhaps through the use of different cell-type specific partner proteins or by being post-translationally modified.

The yeast two-hybrid screen used to uncover novel Sox10 interactors provided me with the interactors SUMO-1 and UBC9. The identification of these two factors allowed me to uncover the evidence presented in the second half of this chapter. To summarize, the absence of SoxE SUMOylation enhances the formation of melanocytes and neural crest precursors, while the presence of SUMOylation inhibits the formation of neural crest precursors and does not lead

to supernumerary melanocyte formation. Conversely, with respect to inner ear development, the opposite occurred. The absence of SUMOylation inhibited formation of the ear, while the constitutive presence of a SUMO moiety enhanced the formation of this cell type.

The findings presented in this chapter demonstrate the first example of the SUMOylation state of a protein altering cell fate determination. This is perhaps how SoxE factors (and most likely many other regulatory factors) are able to simultaneously play different roles in cell types located within extremely close proximity to one another. The SUMOylation state may also be a contributing reason as to how the same factors can be used for different developmental events many times over throughout the time course of development.

The next chapter in this thesis will narrow in on the formation of one specific cell type, the melanocyte. Given that the data presented in this chapter shows that the absence of SUMOylation promotes melanocyte formation, I wanted to investigate the mechanism by which this scenario may unfold. Specifically, I performed experiments using the melanocyte-specific promoter, Dct, to achieve this goal.

CHAPTER 3

A NOVEL MECHANISM BY WHICH SOXE SUMOYLATION CONTROLS TRANSCRIPTIONAL REGULATION

Introduction

As demonstrated in Chapter 2, misexpressing forms of Sox9 or Sox10 that cannot be SUMOylated gives rise to ectopic melanocytes during *Xenopus* development. As previously described, melanocytes give rise to the pigmentation found in the hair and skin and are a neural crest derivative. There are a number of regulatory genes involved in melanocyte formation. MitF, known as the master regulator of melanocyte formation, controls the expression of several downstream melanocyte-specific factors, such as Dct. Interestingly, data obtained by several groups has shown MitF to cooperate with one of the SoxE factors, Sox10, on the Dct promoter (Jiao et al., 2004; Ludwig et al., 2004). Recently, MitF has been shown to be modified by SUMOylation at both amino acids 182 and 316 (Murakami and Arnheiter, 2005). It was of immediate interest that both SoxE factors and MitF are SUMOylated proteins. Given my previous results indicating that the SUMOylation state of SoxE proteins is important for melanocyte development and results regarding MitF SUMOylation obtained by fellow researchers, I hypothesized that perhaps SUMOylation of both of these factors could be a potential trancriptional regulatory mechanism for the Dct promoter.

The impact of SoxE SUMOylation was discussed in Chapter 2 of this thesis. Briefly, I found that constitutively SUMOylated SoxE factors had a positive effect on inner ear formation, but an inhibitory effect on neural crest precursor formation. While the SUMOylation of SoxE factors did not inhibit the formation of melanocytes, it did not allow the ectopic melanocyte formation that I saw when misexpressing unSUMOylated or wild-type versions of SoxE factors. A few additional groups have published their findings on the SUMOylation of Sox9 and Sox10. Girard and Goossens found that SUMOylation had no effect on subcellular localization or DNA binding; however, SUMOylation of Sox10 did repress transcriptional activity on the GJB(I) and

MitF promoters (Girard and Goossens, 2006). Sox9 was originally shown to be SUMOylated by Komatsu and colleagues. These authors demonstrated that mutation of the C-terminal Sox9 SUMOylation site, as well as the SUMOylation sites on Ad4BP/SF1, led to increased synergistic activation of the mouse Mis promoter (Komatsu et al., 2004). Sox9 SUMOylation has also been demonstrated by Oh and colleagues, where the mutation of the C-terminal SUMOylation site in Sox9 led to greater activation of the Col11a2 promoter, while covalently attaching a SUMO moiety to Sox9 reduced this activation (Oh et al., 2007).

The SUMOylation of MitF has also been examined in several publications. Miller and colleagues found that the SUMOylation of MitF affected transcription, but not dimerization, DNA binding, stability or nuclear localization (Miller et al., 2005). This group demonstrated that SUMOylation only had an effect on transcription when there were multiple MitF binding sites present (Miller et al., 2005), presenting an argument for the synergy control model (Iniguez-Lluhi and Pearce, 2000). In this study, Miller and colleagues used the TRPM1 promoter, which contains three natural MitF binding sites, and upon mutation of two of these sites, they saw that SUMO could no longer modulate activity (Miller et al., 2005). However, these results are context dependent to this particular promoter and cannot be recognized as a generalized mechanism for MitF regulation. Murakami and Arnheiter also examined the role of MitF SUMOylation. They found that SUMOylation does not appear to alter the DNA binding ability of MitF and that in comparing the wildtype MitF to the unSUMOylated form of MitF, there was no difference in subcellular localization. However, they did find differences in the ability of MitF to regulate transcriptional events when SUMOylation was involved. They tested the ability of wildtype MitF and unSUMOylated MitF to activate four MitF-responsive promoters, tyrosinase, tyrp-1, Dct and cathepsin K and found that the unSUMOylated form of MitF could

activate transcription to higher levels than wildtype and when a promoter contained more MitF binding sites, this activation was more potent. They then went on to demonstrate that on the Dct promoter, the combinatorial effects of Sox10 and the unSUMOylated form of MitF could synergistically activate transcription to a level much higher than wildtype MitF and Sox10 (Murakami and Arnheiter, 2005).

To begin addressing my hypothesis that the SUMOylation state of both SoxE and MitF will impact melanocyte development, I will perform embryological-based assays as well as luciferase assays using the melanocyte-specific promoter, Dct.

The goals of this chapter are as follows. First, I will examine the functions of MitF and the MitF SUMOylation mutants during melanocyte development in *Xenopus* embryos. Then, I will investigate the consequences of misexpressing the SUMOylation mutants of either Sox9 + MitF or Sox10 + MitF during melanocyte formation in *Xenopus* embryos. Next, I will assay the impact that the Sox9 and MitF SUMOylation mutants have on the Dct promoter by performing luciferase assays. Finally, I will determine the mechanism by which these SUMOylation mutants are regulating the Dct promoter. To achieve these goals, I will be using both *Xenopus* embryo-based methods, as well as a melanoma cell line.

Methods

DNA Constructs, Embryological Methods, and Cell culture

XSox9 was isolated from stage 17 cDNA using low copy number PCR and a high-fidelity polymerase (Tgo, Roche, Indianapolis, IN). *mmMitF* (gift of D. Lang) and *XSox9* were cloned into a pCS2 variant that adds either five N- or C-terminal myc tags (gift of R. Davis and D. Turner) or a pCS2 variant that adds three N-terminal Flag tags. All constructs confirmed by

sequencing. The Sox9 K61R and K365R mutations and MitF K182R and K316R mutations were generated using the Quick Change method (Stratagene, La Jolla, CA). $Sox9^{K61,365R}$/SUMO-1 and $MitF^{K182,316R}$/SUMO-1 were created by ligating SUMO-1 in-frame C-terminal to the full-length $Sox9^{K61,365R}$ or $MitF^{K182,316R}$ mutants using PCR methods, inserting a proline and a glycine between the two sequences. The fusions proteins were N-terminally epitope tagged by insertion into vector pCS2-Myc (gift of D. Turner). The linked versions of these constructs were created by similar PCR-based methods and inserted into the pCS2 N-terminal myc tagged vector. The linker region was inserted in frame between constructs and is a glycine-rich-repeat. The portion of the linker construct which has SUMO-1 fused upstream of $MitF^{K182,316R}$ was created in an analogous manner to the $MitF^{K182,316R}$/SUMO-1 construct but with SUMO-1 ligated to the N-terminus of $MitF^{K182,316R}$. The cMyc construct is myc epitope tagged and was created as described (Bellmeyer et al., 2003). *Xenopus* E12 was a generous gift from NIBB. E12 was cloned into the pCS2 variant with six C-terminal myc tags using PCR based methods. *Xenopus* Groucho4 was a generous gift from Hans Clevers. Groucho4 was subcloned into the pCS2 variant with three N-terminal flag tags using PCR based methods. All constructs were confirmed by sequencing. All results shown are representative of at least two independent experiments. Animal cap explants were harvested from stage 9 embryos and cultured in 1XMMR containing 50μg/ml gentamycin until sibling embryos reached the noted stages (LaBonne and Bronner-Fraser, 1998b). Additional embryological methods, including *in situ* hybridization and injection protocols, can be found in the methods section of Chapter 2 of this thesis. The cell line used in this paper is the human melanoma cell line, C8161 (gift of M. Hendrix). This line is characterized as a highly aggressive melanoma line. C8161 cells were maintained in RPMI supplemented with 10% fetal bovine serum (Mediatech, Inc., Herndon, VA).

Luciferase (and Trichostatin A, Valproic acid) Assays

The luciferase constructs, pGL2-Basic-DCT and pGL2-DCT2, were a gift from D. Lang and the Renilla construct was a gift of K. Horvath. The form of Renilla used is a null construct. The promoter has been taken out of the construct through the use of restriction enzymes so as to eliminate extremely high background readings. The full-length Dct promoter contains the proximal 3.2 kB mouse Dct promoter and Dct2 contains 350 bp proximal to the mRNA start of mouse Dct. Briefly, the luciferase construct and renilla constructs (DNA) were either injected alone or in combination with RNA into both cells of a two-celled *Xenopus* embryo. The embryos were cultured until stage 17 when they were collected in ten embryo sets and lysed in 500 uL of Passive Lysis Buffer using methods described in the Dual Luciferase Kit (Promega; Madison, Wisconsin). Cell transfection experiments were performed with 800 ng of DNA total per well transfected, 100 ng of each reporter and 300 ng of each construct. Those wells that did not contain experimental constructs were transfected with the appropriate amount of empty vector. Cells were transfected using the Lipofectamine Plus system (Invitrogen, Carlsbad, CA). Assays were performed using a Turner 20/20 luminometer or the Turner Glomax luminometer (Turner Biosystems, Sunnyvale, CA). Trichostatin A experiments were performed in *Xenopus* embryos by adding Trichostatin A (Sigma-Aldrich, St. Louis, MO) at a final dilution of 1:25,000 of a 2 mg/mL stock solution at stage 11 and were then cultured until stage 17. The control embryos were treated with similar amounts of ethanol. The Trichostatin A cell transfection experiments were stopped at transfected hour six, allowed to recover for two hours, and TSA was added at a final dilution of 1;15,000 of a 2 mg/mL stock solution. The cells were then cultured overnight and stopped at the 24-hour mark (TSA treatment for a total of 16 hours).

Control wells were treated with ethanol. Valproic acid treatments were performed in *Xenopus* embryos by adding Valproic acid at a final concentration of 50 mM at stage 11. Embryos were then cultured until stage 17. Control embryos were treated with water. All results shown are representative of at least two independent experiments.

Western blot analysis and co-Immunoprecipitation Assays

For western blots, one cell of a two-celled embryo was injected, harvested at stage 8, and lysed in Lysis Buffer (1 x PBS + 1% NP40), supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. For co-immunoprecipitation assays, embryos were injected into both cells of a two-celled embryo, collected at stage eight, and lysed as above. The immunoprecipitation, or "pull" antibody was added to the lysate at a dilution of 1:250 (CBP and p300 IPs) or 1:500 (all other IPs). Also added to the lysate was RIPA buffer (50 mM Tris HCl [ph 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS) supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. The antibody/lysate mixture was incubated by rocking at 4˚C for two hours (only CBP and p300 IPs) or on ice for two hours (all other IPs), upon which 40 μL (CBP and p300 IPs) or 50 μL (all other IPs) of Protein A-Sepharose beads were added (Sigma-Aldrich, St. Louis, MO) and then rocked for an additional two hours at 4˚C (p300 (N-15): sc-584X and CBP (A-22): sc-369X antibodies, Santa Cruz Biotechnology, Santa Cruz, CA). The beads were then washed three times with RIPA Buffer, and resuspended in lysis buffer. All samples were resolved on SDS-PAGE and proteins were detected using antibodies against the epitope tags (Myc: 9E10; Santa Cruz Biotechnology, Santa Cruz, CA; Flag: Affinity Purified M2, Sigma-Aldrich, St. Louis, MO; or Actin; Sigma-Aldrich).

Antibody staining

C8161 cells were first seeded onto Fibronectin-coated coverslips (BD Biosciences; Bedford, MA) and allowed to grow on the coverslips in an overnight incubation. The following day, cells were transfected as described above with 500 ng of DNA. At hour 24 post-transfection, the cells were washed in PBS and fixed in 3% paraformaldehyde for 15 minutes at room temperature. Cells were then washed in PBS and blocked for one hour in 10% Heat-treated Sheep Serum at room temperature. Primary antibody was then added at a concentration of 1:1000 in 10% Heat-treated Sheep Serum overnight, rocking at 4˚C (Myc: 9E10; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed with PBS and a TRITC-labeled secondary antibody was added at a concentration of 1:250 in 10% Heat-treated Sheep Serum. At this time, DAPI was also added at a concentration of 1:500 in order to illuminate the cell nucleus, as was Phalloidin at a concentration of 1:100 to mark the cytoplasm (DAPI gift from Weiss Lab and Phalloidin gift from the Fölsch Lab). The incubation time was three hours at room temperature. The cells were then washed with PBS + 1% Tween-20 (PBST) for a total of 5 one hour washes. The coverslips were then mounted and visualized with a Zeiss 510 Laser Scanning Confocal microscope.

GST pull-down assays

hSUMO-1 was inserted downstream of the coding region for GST in the pGEX-6P-1 vector (GE Healthcare, Buckinghamshire, UK) by PCR based methods. This construct was verified by sequencing. hSUMO-1pGEX-6P-1 protein was produced in *E. coli* and induced by the addition of IPTG for three hours. The hSUMO-1 protein was then rocked at 4˚C for two hours to allow attachment to Glutathione beads (Sigma-Aldrich, St. Louis, MO. To verify protein production and bead attachment, samples were analyzed by SDS-PAGE and stained with Coomassie. Once

bead attachment was verified, immunoprecipitations were performed. Both cells of a two-celled *Xenopus* embryo was injected with the appropriate construct and collected at stage 8. Embryos were then lysed in Lysis Buffer (1xPBS + 1%NP40) supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. 30 μl of hSUMO-1 attached Glutathione beads were then added to the cleared lysates and rocked at 4˚C for two hours. The beads were then washed three times in Lysis Buffer. All samples were resolved on SDS-PAGE and proteins were detected using antibodies against the epitope tag (Flag: Affinity Purified M2, Sigma-Aldrich, St. Louis, MO).

Electrophoresis Mobility Shift Assay (EMSA)

The probe used for the EMSAs consists of the short oligo containing the S2 and M binding site on the mouse Dct promoter (UP: 5' CTTAGGGTCATGTGCTAACAAAGAGGATTTCTC 3'; DN: 5' GAGAAATCCTCTTTGTTAGCACATGACCCTAAG 3'). The probe was labeled using γ-ATP ^{32}P and purified using ProbeQuant™ G-50 Microcolumns (GE Healthcare, Buckinghamshire, UK). The RNA of interest was injected into both cells of a two-celled *Xenopus* embryo and collected in sets of five the next day for processing. Embryos were lysed in lysis buffer (see above) supplemented with phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. The lysates were incubated with poly dI/dCs or poly dG/dCs (Sigma-Aldrich, St. Louis, MO) and EMSA buffer (50% glycerol, 5 mM DTT, 0.5 mg/mL BSA, 10 mM $MgCl_2$, 375 mM NaCl, 100 mM HEPES, 50 μg/mL ssDNA) for five minutes at room temperature. Labeled probe was then added to the mixture and incubated for thirty minutes at room temperature. All samples were then resolved on a 5% TBE/acrylamide gel. Gel was imaged using autoradiography.

Results

Non- or constitutively SUMOylated forms of MitF have similar effects on melanocyte development as parallel Sox9 constructs and together the nonSUMOylated forms of SoxE and MitF factors promote a synergistic effect on melanocyte development in Xenopus.

In Chapter 2, I have shown that like Sox10^{K44,333R} misexpression, Sox9^{K61,365R} can give rise to ectopic melanocytes on the flank of the developing *Xenopus* embryo. These studies were done by *in situ* hybridization, looking at *Sox10* expression, as well as by examining the formation of pigment in swimming tadpoles. As mentioned, melanocyte development also depends on the SUMOylated transcription factor, MitF. In this chapter, I want to first look at what effects MitF has on developing melanocytes. Specifically, I wanted to investigate not only wild-type MitF, but whether the SUMOylation of this factor had any impact on melanocyte development. To begin to address this question, I generated two mutant forms of MitF that were analogous to my SoxE SUMOylation mutants. I created the loss-of-SUMOylation mutant, MitF^{K182,316R} by mutating the SUMOylated lysines to arginines, as well as a constitutively SUMOylated form of MitF, MitF^{K182,316R}/SUMO-1 (Figure 3.1). The constitutively SUMOylated mutant has a SUMO-1 moiety fused to the C-terminus of the double lysine mutant. By doing this, I have eliminated the possibility of complication by endogenous SUMOylation. Such fusions have been found to function as indicated and can be useful in studying the effects of SUMOylation on a particular protein (Holmstrom et al., 2003; Long et al., 2004; Ross et al., 2002; Taylor and Labonne, 2005).

In order to examine the effects of these three constructs, I expressed these factors in early Xenopus embryos and compared their effects on melanocyte development. To control for dosage-dependent effects, these constructs were epitope-tagged. Experiments directly

Figure 3.1

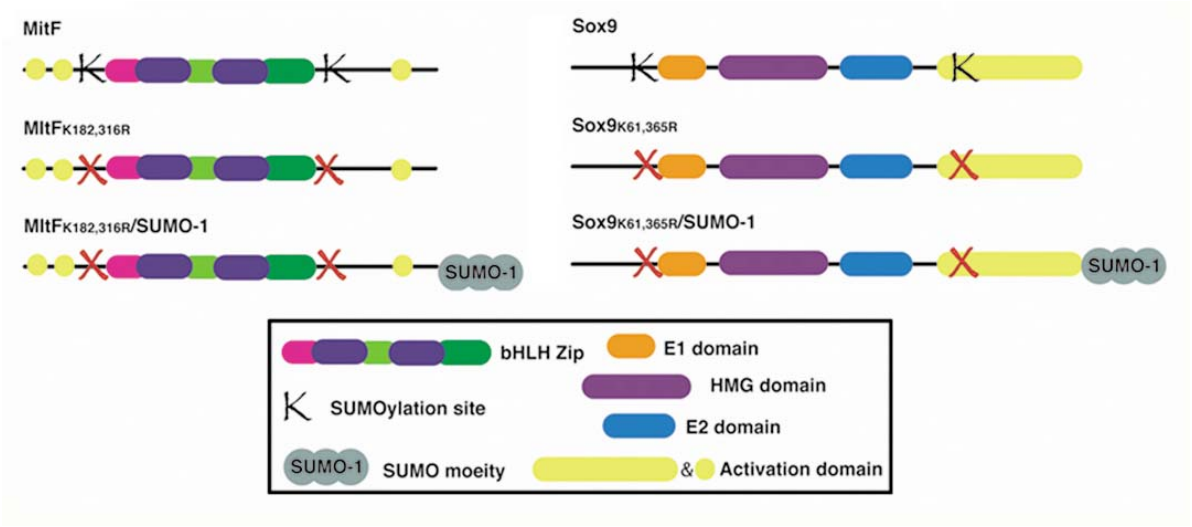


Figure 3.1: Schematics of MitF and Sox9 SUMOylation mutants

Wild-type MitF and Sox9 proteins were mutated to form $MitF^{K182,316R}$, $Sox9^{K61,365R}$, $MitF^{K182,316R}$/SUMO-1 and $Sox9^{K61,365R}$/SUMO-1.

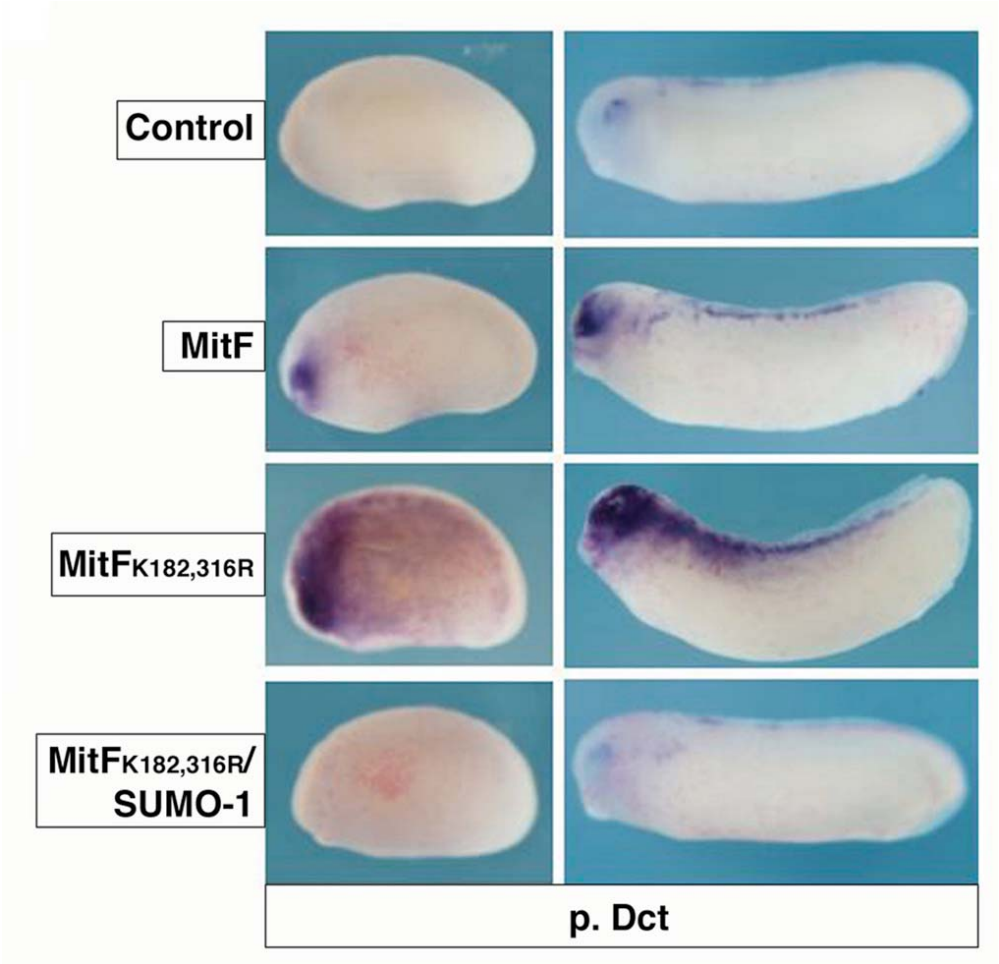
comparing MitF, MitF^{K182,316R}, and MitF^{K182,316R}/SUMO-1were carried out using the epitope-tagged factors, and protein levels were monitored by Western blots. mRNA encoding these epitope-tagged factors was injected into one cell of a two-cell stage *Xenopus* embryos, and the injected embryos were cultured until neural crest migratory stages, as well as neural crest differentiation stages, when the effects on melanoblast formation could by assayed by *in situ* hybridization, using the Dct probe. ß-galactosidase mRNA was co-injected to serve as a lineage tracer and the uninjected side of the embryo served as a control for normal development.

In control embryos, Dct is first expressed during neural crest differentiation stages when melanoblasts are being formed and this expression cannot be detected during neural crest migratory stages. However, I found that when misexpressed, both wildtype MitF and MitF^{K182,316R} could give rise to ectopic Dct staining early, during neural crest migratory stages (Figure 3.2). This suggested that the melanocyte differentiation program was being turned on prematurely and was done so more potently in MitF^{K182,316R}-injected embryos. However, misexpression of MitF^{K182,316R}/SUMO-1 did not lead to ectopic Dct expression during these migratory stages (Figure 3.2). At neural crest differentiation stages, once the wild-type melanocyte differentiation program has begun, I saw similar phenotypes with misexpression of wildtype MitF and MitF^{K182,316R} leading to ectopic Dct expression on the flanks of the embryos (Figure 3.2). Again, misexpression of MitF^{K182,316R} leads to a more penetrant phenotype. However, misexpression of MitF^{K182,316R}/SUMO-1 did not lead to ectopic Dct expression at this later stage (Figure 3.2). I therefore concluded that my MitF constructs behaved in a similar manner to my SoxE SUMOylation mutants. The inhibition of SUMOylation enhanced melanoblast formation while the presence of SUMO suppressed the formation of ectopic melanoblasts.

Figure 3.2: Effects of MitF, MitF^{K182,316R}, and MitF^{K182,316R}/SUMO-1 on Dct expression

In situ hybridization examining the expression of the melanocyte specific marker, *Dct* on Control, MitF-, MitF^{K182,316R}-, and MitF^{K182,316R}/SUMO-1-injected embryos at both neural crest migratory (left column) and neural crest differentiation (right column) stages. Injection of both MitF and MitF^{K182,316R} frequently leads to both early expression of *Dct* (left column), as well as an increase in *Dct* expression at later stages (right column). However, injection of MitF^{K182,316R}/SUMO-1 does not lead to an increase in *Dct* expression. Light red staining is indicative of beta-galactosidase, which is co-injected as a lineage tracer.

Figure 3.2



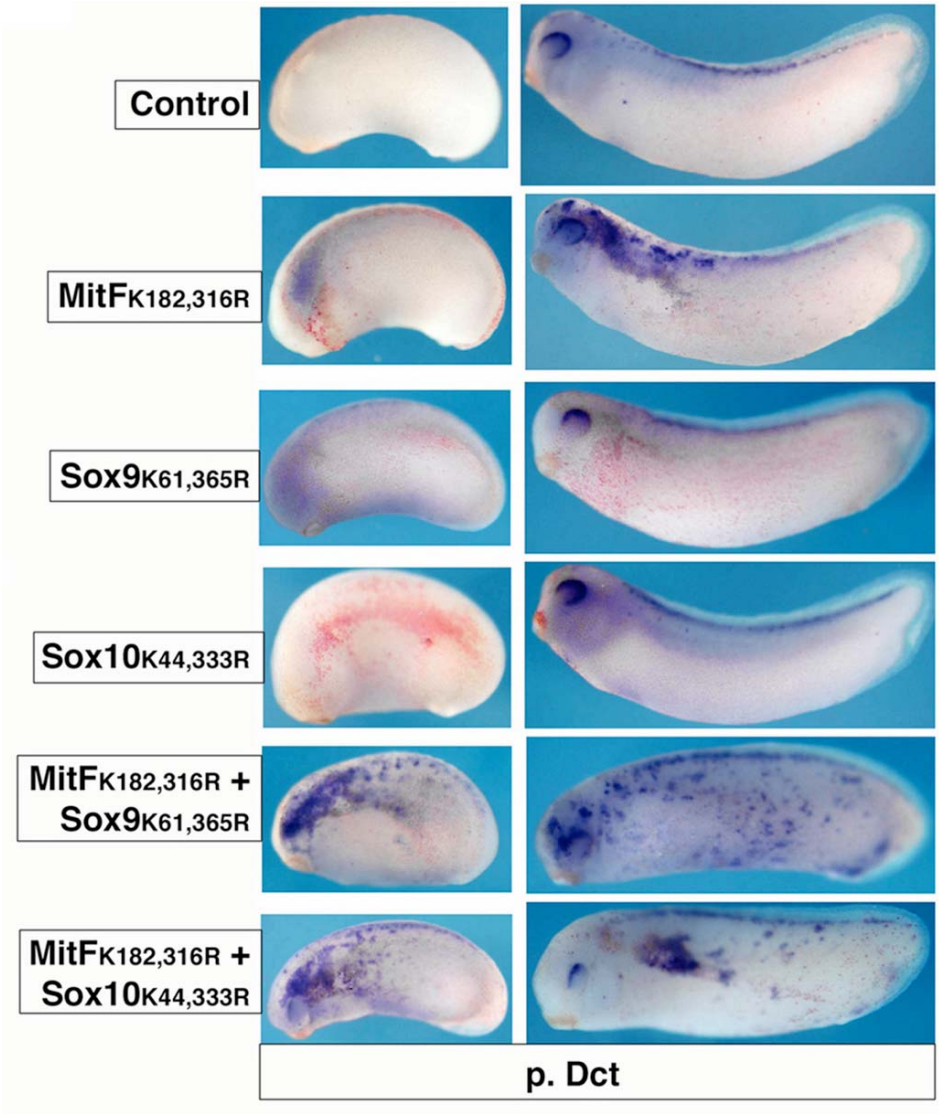
It was extremely interesting that misexpression of MitF^{K182,316R}, Sox9^{K61,365R}, or Sox10^{K44,333R} could give rise to ectopic melanoblasts. I next wanted to determine what the effects of misexpressing SoxE and MitF factors at the same time would be on melanoblast formation (Figure 3.3). Since the MitF^{K182,316R} misexpression phenotype enhanced ectopic Dct to such a large degree, I lowered, but kept equivalent, the amount of mRNA injected in order to observe any synergistic effects I might see with the two proteins together. Upon misexpression of these factors at lower protein levels, I observed faint ectopic Dct expression with MitF^{K182,316R} at neural crest migratory stages. At the levels expressed, I did not see ectopic staining with misexpression of either Sox9^{K61,365R} or Sox10^{K44,333R} at neural crest migratory stages. This suggests that although misexpression of these factors turns on ectopic *Sox10* expression (refer to Chapter 2), they cannot fully activate the melanocyte differentiation program, as Sox10 is a precursor to both *MitF* and *Dct* expression. However, dual misexpression of either MitF^{K182,316R} and Sox9^{K61,365R} or MitF^{K182,316R} and Sox10^{K44,333R} led to a vast increase in ectopic *Dct* expression at neural crest migratory stages. Because the protein levels were kept strictly equal, I could postulate that the unSUMOylated forms of MitF and SoxE were working in synergy to enhance ectopic *Dct* expression. This data also demonstrates a clear dependence on MitF to ensure proper melanocyte development. The same synergistic effects at neural crest migratory stages were also apparent at neural crest differentiation stages (Figure 3.3).

While this data is informative in telling me that MitF and SoxE factors can work together to produce melanoblasts in the whole embryo, there are many additional factors present in this area of the embryo, such as Wnt signaling, that could be playing a role in this process. To investigate whether MitF and Sox9 were sufficient to induce melanoblast formation, I next tested the misexpression of MitF and Sox9 SUMOylation mutants in a more isolated embryonic tissue

Figure 3.3: Effects of nonSUMOylated SoxE and MitF factors on Dct expression

In situ hybridization examining the expression of the melanocyte-specific marker, *Dct* in Control, MitF^{K182,316R}-, Sox9^{K61,365R}-, Sox10^{K44,333R}-, MitF^{K182,316R} + Sox9^{K61,365R}-, and MitF^{K182,316R} + Sox10^{K44,333R}-injected embryos. Injection of MitF^{K182,316R} leads to an increase in *Dct* expression at both neural crest migratory stages (left column) and neural crest differentiation stages (right column). At the doses presented here, Sox9^{K61,365R} and Sox10^{K44,333R} do not lead to ectopic Dct expression. However, the dual misexpression of either MitF^{K182,316R} + Sox9^{K61,365R} or MitF^{K182,316R} + Sox10^{K44,333R} leads to a synergistic increase in *Dct* expression at both neural crest migratory stages (left column) and neural crest differentiation stages (right column) with respect to misexpression of MitF^{K182,316R} alone. Light red staining is indicative of beta-galactosidase, which is co-injected as a lineage tracer.

Figure 3.3


Control
MitFK182,316R
Sox9K61,365R
Sox10K44,333R
MitFK182,316R + Sox9K61,365R
MitFK182,316R + Sox10K44,333R
p. Dct

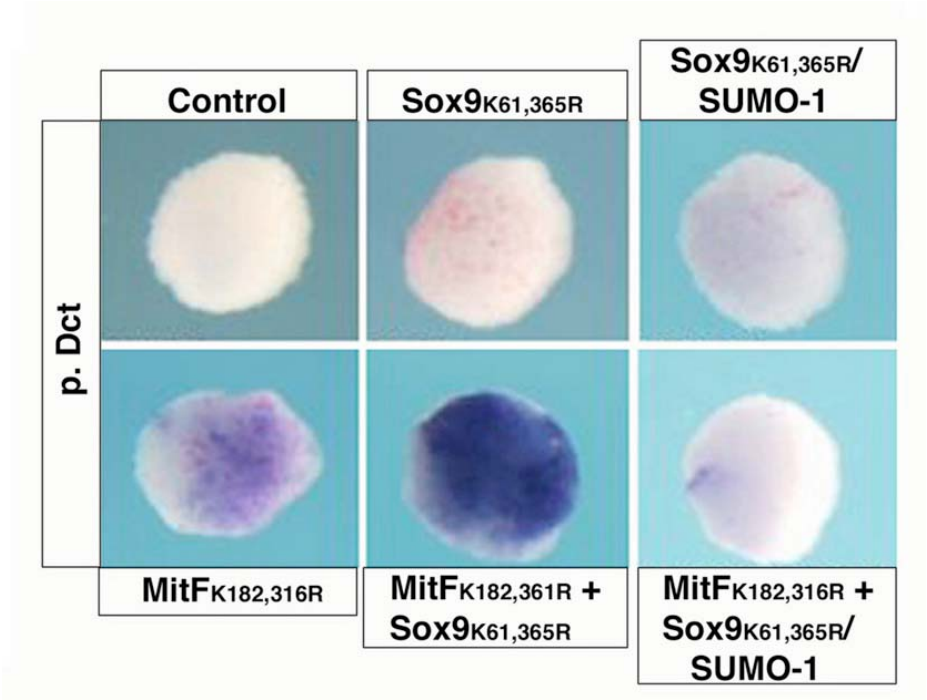
system. To do this, I used animal cap explants to eliminate any tissue-specific effects on the manipulations I have made to the embryo. Animal cap explants, if left undisturbed, turn into balls of epidermis, or skin. Without proper signaling factors, these explants can no longer differentiate into neural crest cells or neural plate cells. By misexpressing different factors into the embryo at the two-cell stage, targeting the area that will become the animal cap explant, and culturing the explants to later stages of development, the tissue the explants form can be heavily altered from the original epidermal fate. I wanted to determine if I could produce melanoblasts/melanocytes in these explants without the addition of extraneous factors.

To do this, I misexpressed equivalent levels of $Sox9^{K61,365R}$, $MitF^{K182,316R}$, $Sox9^{K61,365R}$/SUMO-1 or a combination of these factors in *Xenopus* embryos, dissected out the explants, and cultured them until neural crest differentiation stages, upon which I performed *in situ* hybridization on the explants, probing for *Dct* expression. Interestingly, I see the same results obtained in the whole embryo experiment repeat in this isolated animal cap system (Figure 3.4). While neither $Sox9^{K61,365R}$ nor $Sox9^{K61,365R}$/SUMO-1 alone was able to initiate *Dct* expression in animal caps, $MitF^{K182,316R}$ was able to do so moderately. This piece of data confirmed that $MitF^{K182,316R}$ was sufficient to form melanocyte precursors. Excitingly, the dual misexpression of $MitF^{K182,316R}$ and $Sox9^{K61,365R}$ was able to synergistically activate *Dct* expression in animal cap explants, confirming that these two factors alone were sufficient to promote the synergy seen in the whole embryo. Interestingly, the dual misexpression of $MitF^{K182,316R}$ and $Sox9^{K61,365R}$/SUMO-1 disrupted the ability to induce *Dct* expression in animal cap explants (Figure 3.4). While previously I had observed that embryos injected with $Sox9^{K61,365R}$/SUMO-1 could not form ectopic melanocytes, it now appeared as though the presence of SUMO was blocking the ability of $MitF^{K182,316R}$ to propagate *Dct* expression in the animal cap system. And,

Figure 3.4: Effects of Sox9^{K61,365R}, MitF^{K182,316R}, Sox9^{K61,365R}/SUMO-1, and MitF^{K182,316R}/SUMO-1 in animal cap explants

In situ hybridization examining the expression of the melanocyte-specific marker *Dct* at neural differentiation stage (stage 28) in animal cap explants. Neither Sox9^{K61,365R}- or Sox9^{K61,365R}/SUMO-1-injected embryos can lead to *Dct* expression in animal cap explants. MitF^{K182,316R}-injected embryos can give rise to ectopic *Dct* expression in animal cap explants and Sox9^{K61,365R} + MitF^{K182,316R}-injected embryos produce synergistic ectopic *Dct* expression in animal cap explants. However, Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R}-injected embryos can no longer produce ectopic *Dct* expression. Light red staining is indicative of beta-galactosidase, which is co-injected as a lineage tracer.

Figure 3.4


Control
Sox9K61,365R
Sox9K61,365R/ SUMO-1
p. Dct
MitFK182,316R
MitFK182,361R + Sox9K61,365R
MitFK182,316R + Sox9K61,365R/ SUMO-1

important to note is that while these factors could lead to ectopic Dct expression, they could not lead to actual pigmentation in these animal caps (data not shown). This suggested that additional factors were necessary to complete the melanocyte differentiation program. The above findings indicated that misexpression of MitF and MitF^{K182,316R} could lead to ectopic *Dct* expression at both neural crest migratory stages and neural crest differentiation stages, with the misexpression of MitF^{K182,316R} leading to a more penetrant phenotype. However, misexpression of the constitutively SUMOylated form of MitF could not give rise to ectopic *Dct* expression. Furthermore, when the unSUMOylated forms of MitF and SoxE were misexpressed in combination, a synergistic effect was observed with respect to ectopic *Dct* expression. These results were then recapitulated in an animal cap explant system, indicating that the role of these factors in turning on *Dct* expression was, in fact, direct.

MitF^{K182,316R} and Sox9^{K61,365R} have a synergistic effect on the Dct promoter, while the SUMOylated forms of these proteins inhibits this synergy.

While I have shown the effects that MitF and SoxE SUMOylation mutants have on *Dct* expression in the embryo, I wanted to gain a better understanding of what was actually occurring on the Dct promoter. To do this, I obtained the Dct promoter in the luciferase vector, pGL2. The luciferase gene is located just downstream of the Dct promoter (courtesy of D. Lang). This 3.2 kB promoter contains six Sox10 binding sites (including site 4, which also contains a 4' binding site), one MitF binding site and one Lef-1 binding site (Figure 3.5). I was also able to obtain a shorter, 350 bp version of this construct, Dct2, which only contains five of the Sox10 sites (including the 4' site), the MitF site and the Lef-1 site. The shorter version of this promoter will allow me to observe whether the activation trends seen on the full-length promoter are

Figure 3.5

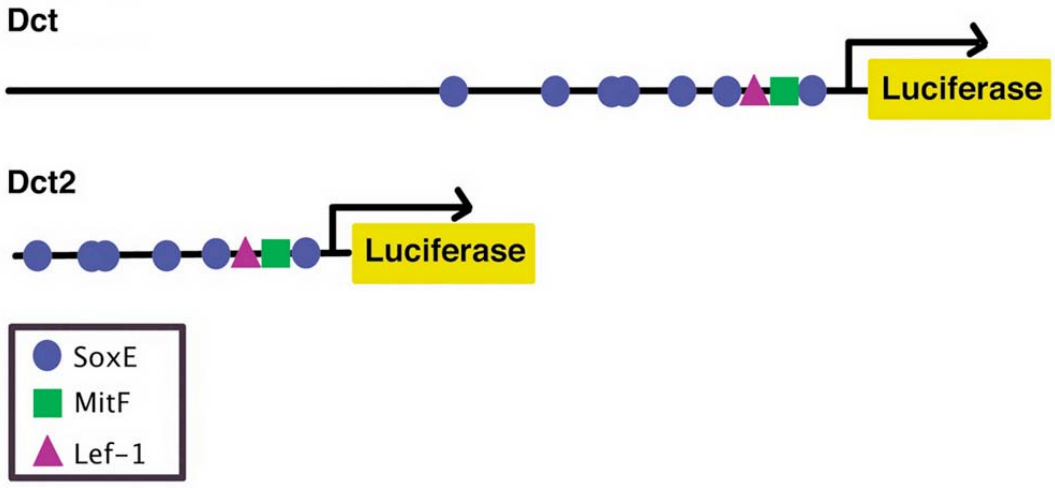


Figure 3.5: Schematic of the Dct promoter

The Dct promoter is comprised of approximately 3.2 kB upstream of the transcription initiation site. It contains six SoxE binding sites (site 4 contains a 4' site as well), one Lef-1 binding site and one MitF binding site. The Dct2 promoter contains the first 350 bp upstream of the initiation site. This shortened version contains five SoxE binding sites, the MitF binding site, and the Lef-1 binding site. Both of these promoters have luciferase fused in frame just downstream to them.

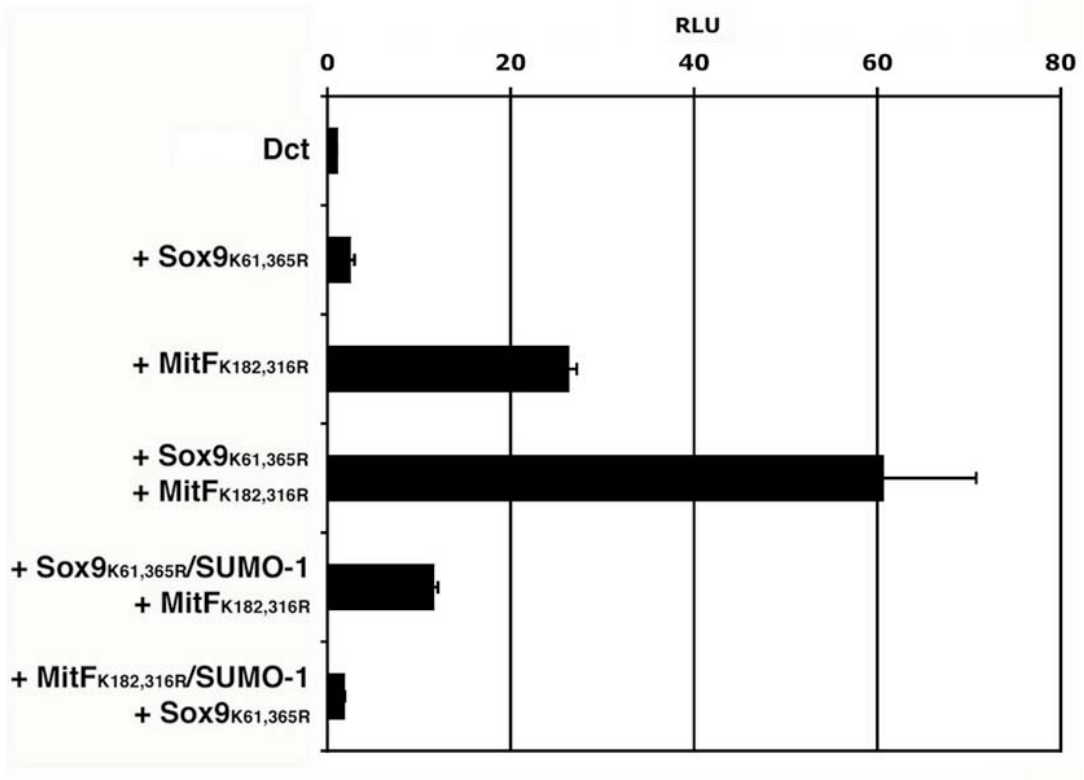
similar to the trends seen on the shorter, Dct2 promoter. I expected that the trends would be similar because the Dct2 version contains all but one SoxE binding site, as well as the MitF site.

I was first interested in examining the effects of my SUMOylation constructs on the full-length Dct promoter (Figure 3.6). To do this, I misexpressed mRNA encoding MitF^{K182,316R}, Sox9^{K61,365R}, MitF^{K182,316R} + Sox9^{K61,365R}, Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R}, or Sox9^{K61,365R} + MitF^{K182,316R}/SUMO-1, alongside of DNA encoding the Dct promoter and DNA for the Renilla control promoter in *Xenopus* embryos by injecting into both cells of a two-cell embryo. The Renilla was used in the luciferase assays to serve as a normalization promoter so that all wells could be compared directly as a ratio measurement. The injected embryos were cultured until late neurula stages, collected in sets of ten, lysed, and processed. I performed these assays at neurula stages to avoid complication by the wild-type melanocyte program, which turns on in late neural crest migratory stages. Basal levels, which were a measure of only embryos injected with the luciferase and renilla promoters, were normalized to one and all other results are presented as fold-activation to this base level of one. The following results are shown in Figure 3.6. As postulated, due to its weak transcriptional activation ability, misexpression of Sox9^{K61,365R} alone led to only a two-fold induction above basal levels, thus confirming my *in situ* hybridization results. Misexpression of MitF^{K182,316R} alone was able to activate the Dct promoter approximately 27-fold above basal. This result coincided nicely with the *in situ* hybridization data. Interestingly, when combined, misexpression of MitF^{K182,316R} + Sox9^{K61,365R} gave rise to synergistic activation on the Dct promoter, leading to a 61-fold activation. This clearly paralleled the previously demonstrated embryonic data. However, as the animal cap explant data suggested, when either Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R} or Sox9^{K61,365R} + MitF^{K182,316R}/SUMO-1 was misexpressed in the embryo, the synergy previously observed was abolished, reducing

Figure 3.6: Effects of Sox9^{K61,365R}, MitF^{K182,316R}, Sox9^{K61,365R}/SUMO-1 and MitF^{K182,316R}/ SUMO-1 on the full-length Dct promoter in *Xenopus* embryos.

Relative luciferase activities were calculated using the activity of Dct promoter alone in *Xenopus* embryos as a control. Misexpression of Sox9^{K61,365R} alone does not activate the Dct promoter much above basal levels. MitF^{K182,316R} alone activates the Dct promoter approximately 27-fold above basal levels. However, when Sox9^{K61,365R} and MitF^{K182,316R} are misexpressed in combination, the Dct promoter is activated 61-fold above basal, demonstrating synergistic activation on this promoter. When MitF^{K182,316R} and Sox9^{K61,365R}/SUMO-1 are misexpressed in combination, however, this synergy is inhibited, only activating the promoter ~11-fold above basal. The dual misexpression of Sox9^{K61,365R} and MitF^{K182,316R}/SUMO-1 also inhibits synergy, reducing activation to ~2-fold above basal.

Figure 3.6


RLU
0
20
40
60
80
Dct
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316R/SUMO-1
+ Sox9K61,365R

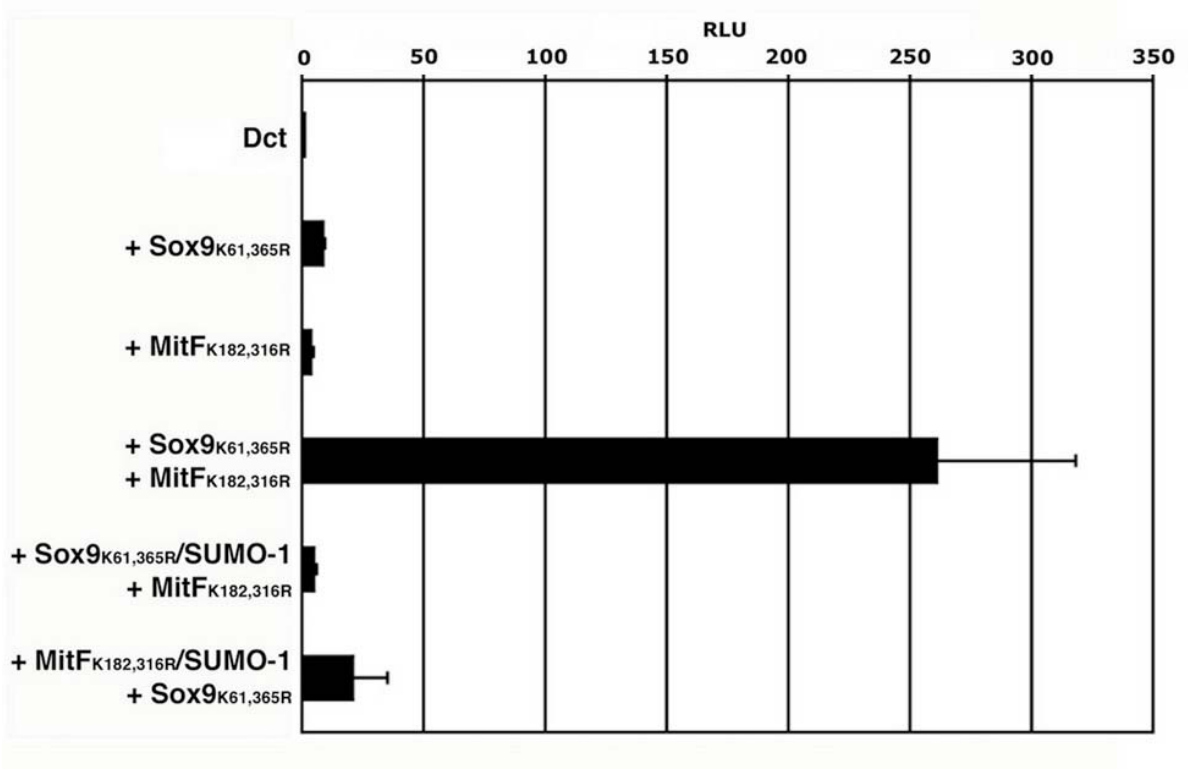
activation levels to 11-fold or 2-fold activation on the Dct promoter, respectively. Again, I saw that the presence of SUMO-1 led to an inhibition of the transcriptional synergy observed with the misexpression of $MitF^{K182,316R}$ + $Sox9^{K61,365R}$ on the melanocyte-specific promoter, Dct.

Although I performed these experiments at stages when melanocytes are not present, there was always the possibility that other factors were present during the culturing of the injected embryos that could alter the results seen in the luciferase assays. I therefore wanted to try the same experiment just described in a more homogenous cell population. To do this, I used the human metastatic melanoma cell line, C8161 (courtesy of M. Hendrix). I performed my cell line experiments by transfecting DNA from my MitF and Sox9 SUMOylation constructs into the C8161 cell line. I transfected in 100 ng of each reporter construct (Dct-Luc and Renilla), as well as 300 ng of each experimental construct, such as $MitF^{K182,316R}$. The total amount of DNA transfected was always kept at a standard 800 ng. If one particular well had less than the required DNA transfected, the extra amount was made by the transfection of empty vector DNA. The luciferase assays were performed after a total of 24 hours, post-transfection, including a six-hour transfection period and an additional 18 hours of recovery. I transfected DNA encoding $MitF^{K182,316R}$, $Sox9^{K61,365R}$, $MitF^{K182,316R}$ + $Sox9^{K61,365R}$, $Sox9^{K61,365R}$/SUMO-1 + $MitF^{K182,316R}$, or $Sox9^{K61,365R}$ + $MitF^{K182,316R}$/SUMO-1, alongside of DNA encoding the Dct promoter and as well as the Renilla control promoter into the cells. Again, basal levels, which were a measure of cells only transfected with the luciferase and Renilla promoters, were normalized to one and all other results are presented as fold-activation to this base level. Excitingly, I saw similar results in this homogenous cell population as I did with misexpression of these factors in *Xenopus* embryos (Figure 3.7). Neither the transfection of $MitF^{K182,316R}$ or $Sox9^{K61,365R}$ alone could give rise to much (~5-10 fold) transcriptional activity on the Dct promoter in the C8161 cell line. However,

Figure 3.7: Effects of Sox9^{K61,365R}, MitF^{K182,316R}, Sox9^{K61,365R}/SUMO-1 and MitF^{K182,316R}/ SUMO-1 on the full-length Dct promoter in the melanoma cell line, C8161.

Relative luciferase activities were calculated using the activity of Dct alone in the melanoma cell line, C8161, as a control. Transfection of Sox9^{K61,365R} alone activates the Dct promoter ~10-fold above basal levels. Transfection of MitF^{K182,316R} alone activates the Dct promoter ~5-fold above basal levels. However, when Sox9^{K61,365R} and MitF^{K182,316R} are transfected in combination, the Dct promoter is activated 260-fold above basal, demonstrating clear synergistic activation on this promoter. When MitF^{K182,316R} and Sox9^{K61,365R}/SUMO-1 are transfected in combination, however, this synergy is inhibited, only activating the promoter ~5-fold above basal. Transfecting both Sox9^{K61,365R} and MitF^{K182,316R}/SUMO-1 also inhibits synergy, reducing activation to ~20-fold above basal.

Figure 3.7


RLU
0
50
100
150
200
250
300
350
Dct
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316R/SUMO-1
+ Sox9K61,365R

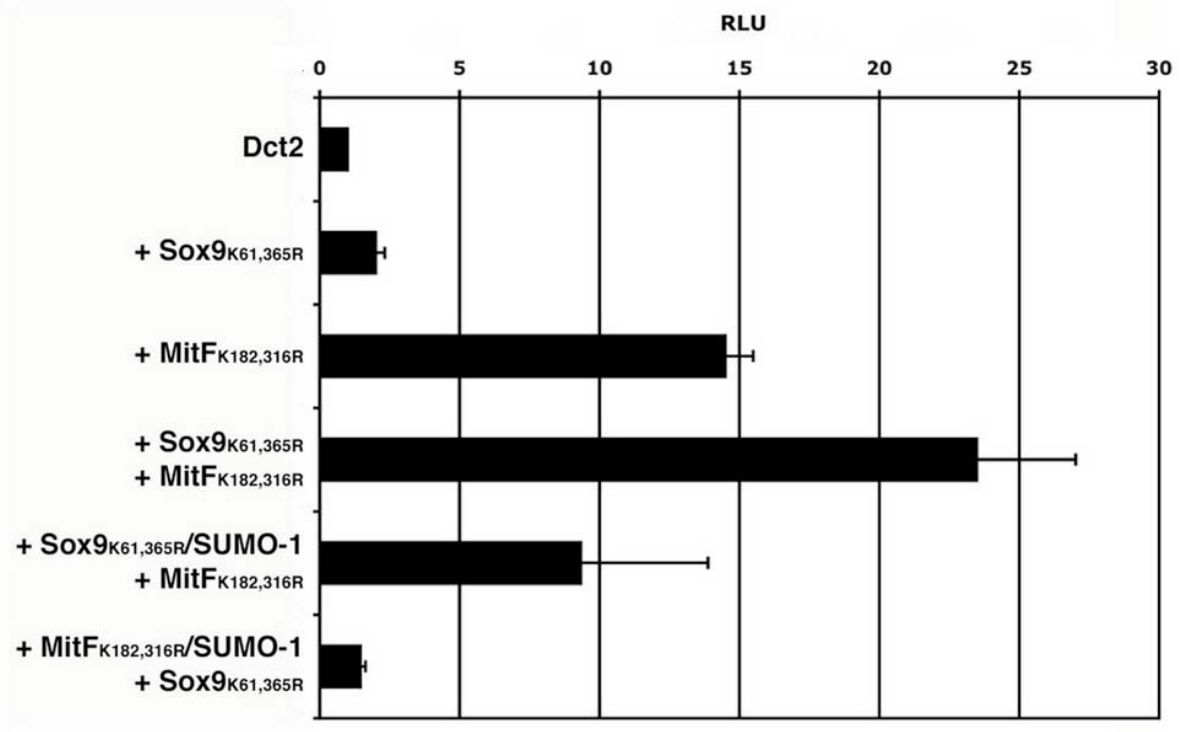
co-transfection of MitF^{K182,316R} + Sox9^{K61,365R} synergistically activated the Dct promoter approximately 260-fold to basal. This result clearly demonstrated the ability of these two factors to work together on the Dct promoter in a melanoma cell line. Again, as in the embryos, co-transfection of Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R} or Sox9^{K61,365R} + MitF^{K182,316R}/SUMO-1 into the C8161 cell line inhibited the synergistic activation of the Dct promoter, going from a 250-fold induction to a 5- to 20-fold induction (respectively). This helped confirm that it was the presence of SUMO-1 that was inhibiting the synergistic ability of MitF and Sox9.

As mentioned, I also obtained a shortened version of the Dct promoter, Dct2, so I next verified the full-length reporter results on the shortened form. To do this, I misexpressed mRNA encoding MitF^{K182,316R}, Sox9^{K61,365R}, MitF^{K182,316R} + Sox9^{K61,365R}, Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R}, or Sox9^{K61,365R} + MitF^{K182,316R}/SUMO-1, alongside of DNA encoding the Dct promoter and DNA for the Renilla control promoter in *Xenopus* embryos by injecting into both cells of a two-cell embryo. The injected embryos were cultured until late neurula stages, collected in sets of ten, lysed, and processed. Basal levels, which were a measure of only embryos injected with the luciferase and Renilla promoters, were normalized to one and all other results are presented as fold-activation to this base level. The following results are shown in Figure 3.8. I saw the same trend occurring using the Dct2 promoter, the fold activation, however, was less pronounced. This made it apparent that there were components present in the rest of the Dct promoter that led to a more robust activation when using the full-length promoter. Misexpression of MitF^{K182,316R} alone led to a 14-fold increase in activation and Sox9^{K61,365R} misexpression still led to a much lower, 2-fold activation. However, dual misexpression of MitF^{K182,316R} + Sox9^{K61,365R} led to a 23-fold synergistic increase in activation. This data indicated that the combinatorial effects of the MitF and Sox9 double lysine mutants could still produce

Figure 3.8: Effects of Sox9^{K61,365R}, MitF^{K182,316R}, Sox9^{K61,365R}/SUMO-1 and MitF^{K182,316R}/ SUMO-1 on the 350 bp Dct2 promoter in *Xenopus* embryos.

Relative luciferase activities were calculated using the activity of Dct2 alone in *Xenopus* embryos as a control. Misexpression of Sox9^{K61,365R} alone activates the Dct2 promoter ~2-fold above basal levels. Misexpression of MitF^{K182,316R} alone activates the Dct2 promoter ~14-fold above basal levels. However, when Sox9^{K61,365R} and MitF^{K182,316R} are misexpressed in combination, the Dct2 promoter is activated 23-fold above basal, demonstrating clear synergistic activation on this promoter. When MitF^{K182,316R} and Sox9^{K61,365R}/SUMO-1 are misexpressed in combination, however, this synergy is inhibited, only activating the promoter ~9-fold above basal. Misexpression of both Sox9^{K61,365R} and MitF^{K182,316R}/SUMO-1 also inhibits synergy, reducing activation to ~1.5-fold above basal.

Figure 3.8


RLU
0
5
10
15
20
25
30
Dct2
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316R/SUMO-1
+ Sox9K61,365R

synergy on the shortened form of the Dct promoter. Still, misexpression of $Sox9^{K61,365R}$/SUMO-1 + $MitF^{K182,316R}$ or $Sox9^{K61,365R}$ + $MitF^{K182,316R}$/SUMO-1 led to an inhibition of the synergy observed with the misexpression of the double lysine mutants. Misexpression of $Sox9^{K61,365R}$/SUMO-1 + $MitF^{K182,316R}$ led to only a 9-fold activation and $Sox9^{K61,365R}$ + $MitF^{K182,316R}$/SUMO-1 only led to a 1.5-fold activation of the Dct2 promoter. This data indicates that while the misexpression of the double lysine mutants could not give rise to the same levels of synergistic activation on the Dct2 promoter compared with the full-length promoter, comparing a 61-fold induction on the full-length to a 23-fold on the shortened version, I still got synergistic activation of these promoters. This data also demonstrates that the presence of SUMO-1 has an inhibitory effect on the synergy produced when neither factor is able to be SUMOylated. The cell line data also supports the notion that these same transcriptional events are able to happen in a more homogenous cell population, so my whole embryo data is applicable specifically to the melanocyte lineage. Given the data presented thus far regarding the impact that a SUMO moiety has on the transcriptional activity of these factors, I next wanted to uncover the mechanism underlying this inhibition.

The inhibitory effects of SUMO-1 are not due to steric interference

There are many well-characterized mechanisms by which SUMOylation can inhibit the transcriptional activity of a protein. One such mechanism is by physically blocking the ability of a transcription factor to bind to the target DNA. To determine if SUMOylation of MitF or Sox9 was sterically interfering with the ability of these factors to properly bind to the Dct promoter, I created several more constructs, termed “linkers” (Figure 3.9). The concept behind these factors was to create isoforms of these proteins that could not be physically separated from one another.

Figure 3.9

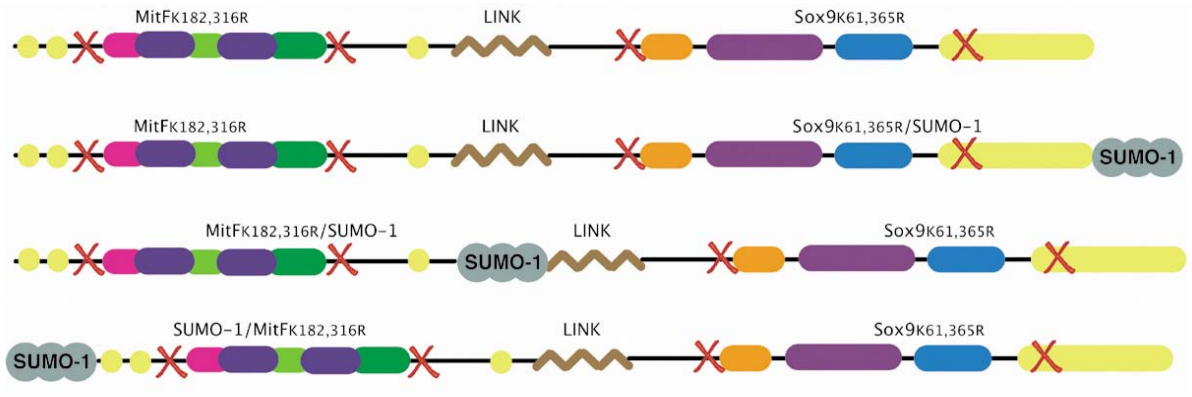


Figure 3.9: Schematic depicting the different MitF-SoxE linker constructs.

Therefore, if a double lysine protein is unable to bind DNA when linked to a constitutively SUMOylated form of a protein, then I would see absolutely no transcriptional activity on the Dct promoter in luciferase assays, not just a reduction in synergy. I created these epitope-tagged factors by tethering together the different MitF and Sox9 SUMOylation mutants with a low-complexity, glycine-rich-region. I first created a linked form of the double lysine mutants, MitF^{K182,316R}LINK Sox9^{K61,365R}, to use as a positive control to ensure that these constructs would perform similarly to the dual misexpression of the unlinked forms in *Xenopus* embryos. Then, in order to test my hypothesis that SUMO was physically blocking DNA binding, I created several other linker constructs, including MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1, MitF^{K182,316R}/SUMO-1 LINKSox9^{K61,365R}, and finally, a form in which the SUMO-1 moiety was linked at the very N-terminus of the linker to form SUMO-1/MitF^{K182,316R}LINKSox9^{K61,365R}. I created this last construct to ensure that the location of the SUMO-1 moiety itself was not altering my data.

I first wanted to test the functionality of my linker constructs in *Xenopus* embryos through *in situ* hybridization before looking at luciferase assays. I did this by comparing the misexpression of mRNA encoding for MitF^{K182,316R}LINKSox9^{K61,365R} to the dual misexpression of MitF^{K182,316R} + Sox9^{K61,365R} and then comparing the effects of misexpressing the unlinked forms of MitF^{K182,316R} + Sox9^{K61,365R}/SUMO-1 to the linked version, MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1. I cultured the injected embryos to neural crest differentiation stages and performed *in situ* hybridization on these embryos, looking for changes in *Dct* expression. Due to severe toxicity, the linker constructs had to be injected at a much lower dose than the unlinked counterparts, as demonstrated by Western blot (Figure 3.10). However, the linked constructs, MitF^{K182,316R}LINKSox9^{K61,365R} versus MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1, were kept at equivalent expression levels so as to be directly compared, as were the unlinked constructs

Figure 3.10

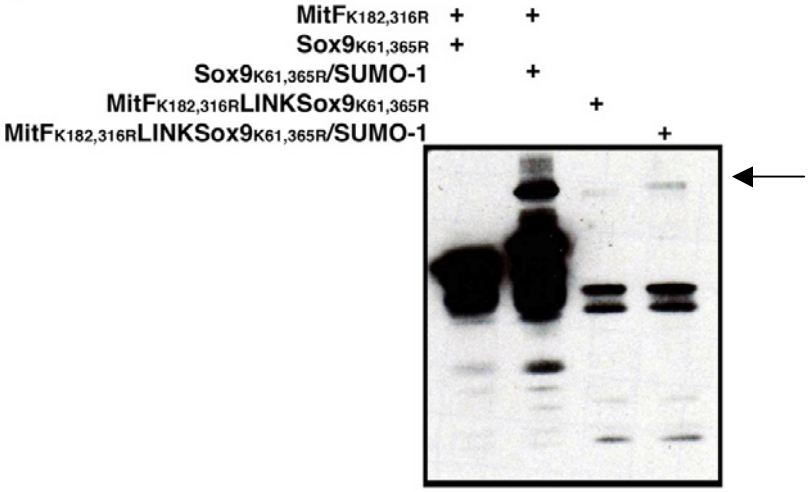


Figure 3.10: Expression levels of injected unlinked versus linked Sox9 and MitF proteins. The linked versions of Sox9 and MitF cannot be expressed at similar levels to the unlinked versions due to embryo toxicity. The linked versions are expressed at approximately 100-fold less than the unlinked counterparts.

MitF^{K182,316R} + Sox9^{K61,365R} versus MitF^{K182,316R} + Sox9^{K61,365R}/SUMO-1 (Figure 3.10). The following *in situ* hybridization data are shown in Figure 3.11. Interestingly, the misexpression of MitF^{K182,316R}LINKSox9^{K61,365R} gave rise to ectopic Dct expression on the flank of the embryos that looked almost equivalent to the dual misexpression of MitF^{K182,316R} + Sox9^{K61,365R}. This result was fascinating because the double lysine linker was expressed at a much lower level compared to the unlinked counterparts. This suggested the perhaps tethering the two proteins together enhanced the activation of the Dct promoter. It is this reason why I believe the linker constructs are so toxic to the embryos; they are simply overloading the system, which eventually causes cell death. Also very interesting was that upon misexpressing MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 in the embryo, I found that the phenotype mirrored what I saw with the misexpression of MitF^{K182,316R} + Sox9^{K61,365R}/SUMO-1 gave rise to - no ectopic *Dct* expression on the flank of the embryo. As these results demonstrated that the linked versions paralleled the results obtained from the unlinked injections, I assumed that these constructs were functioning appropriately in the embryo and next moved on to luciferase assays in order to determine whether the SUMO moiety was causing steric interference.

I misexpressed mRNA encoding for all of my unlinked and linked constructs alongside of DNA encoding the Renilla promoter and either the full-length Dct or Dct2 promoter and performed luciferase assays on these embryos in the same manner as described earlier in this chapter. Again, the basal level, Dct promoter + Renilla promoter only, was set to one and all other results are fold activation above this level. The results of this experiment are shown in Figure 3.12. As previously suspected, given the strong embryonic phenotype, misexpression of the double lysine linker gave rise to an approximate 460-fold synergistic activation of the Dct

Figure 3.11: Effects of Sox9^{K61,365R} + MitF^{K182,316R}, Sox9^{K61,365R}/SUMO-1 + MitF^{K182,316R}, MitF^{K182,316R}LINKSox9^{K61,365R}, MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 on *Dct* expression.

In situ hybridization examining the expression of the melanocyte-specific marker, *Dct* in Control, MitF^{K182,316R} + Sox9^{K61,365R}-, MitF^{K182,316R} + Sox9^{K61,365R}/SUMO-1-, MitF^{K182,316R}LINKSox9^{K61,365R}, and MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1-injected embryos. Misexpression of both MitF^{K182,316R} + Sox9^{K61,365R} leads to an increase in *Dct* expression at neural crest differentiation stages. MitF^{K182,316R} + Sox9^{K61,365R}/SUMO-1 does not lead to an increase in *Dct* expression at neural crest differentiation stages. Misexpression of MitF^{K182,316R}LINKSox9^{K61,365R} also leads to an expansion of the melanocyte-specific marker, *Dct*, while misexpression of MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 does not. Due to the toxicity of the linker constructs, the protein level at which they can be injected in comparison to the level at which the unlinked versions can be injected is approximately 100-fold less, as can be seen in Figure 3.11. Therefore, it can be concluded that the level to which *Dct* expression is up-regulated is far greater in the linker versions of these proteins. Light red staining is indicative of beta-galactosidase expression, which is co-injected as a lineage tracer.

Figure 3.11

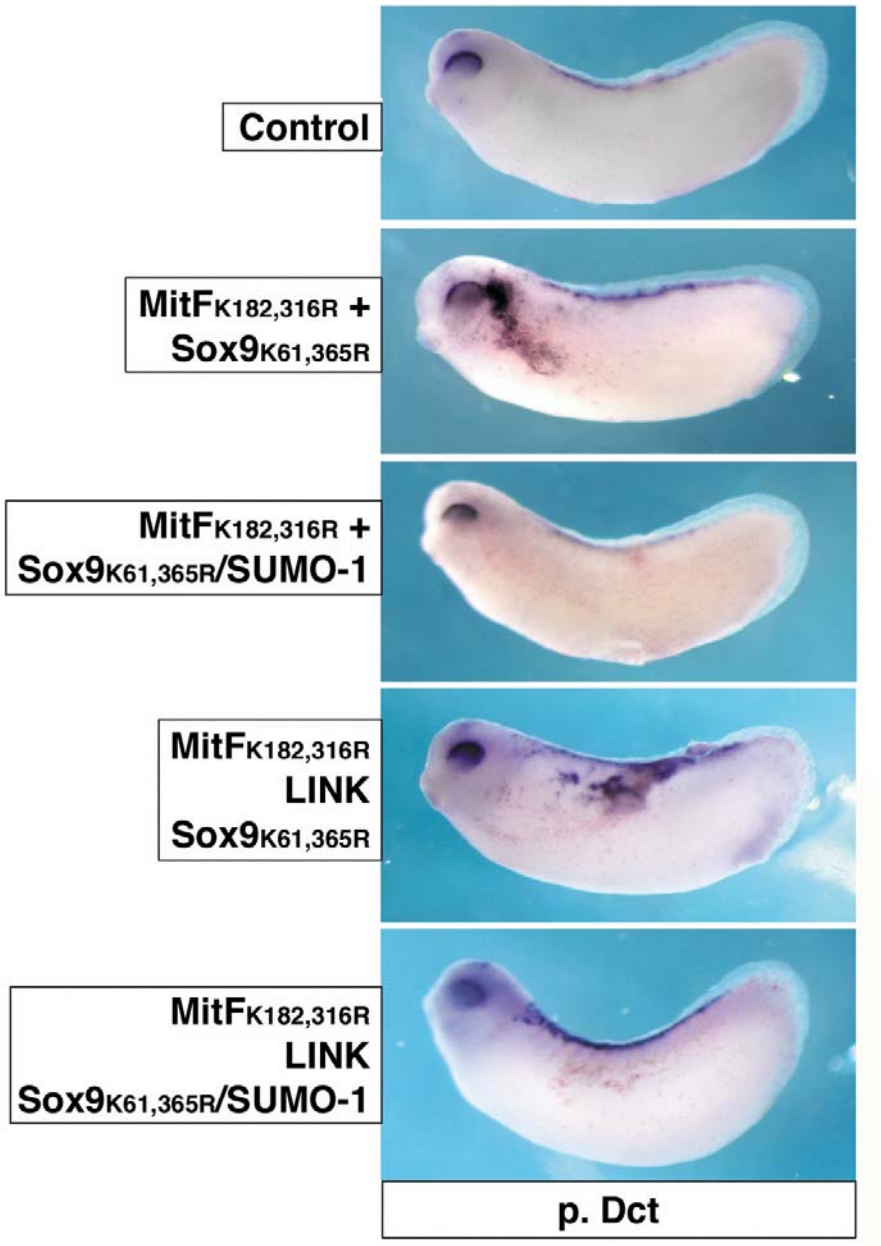
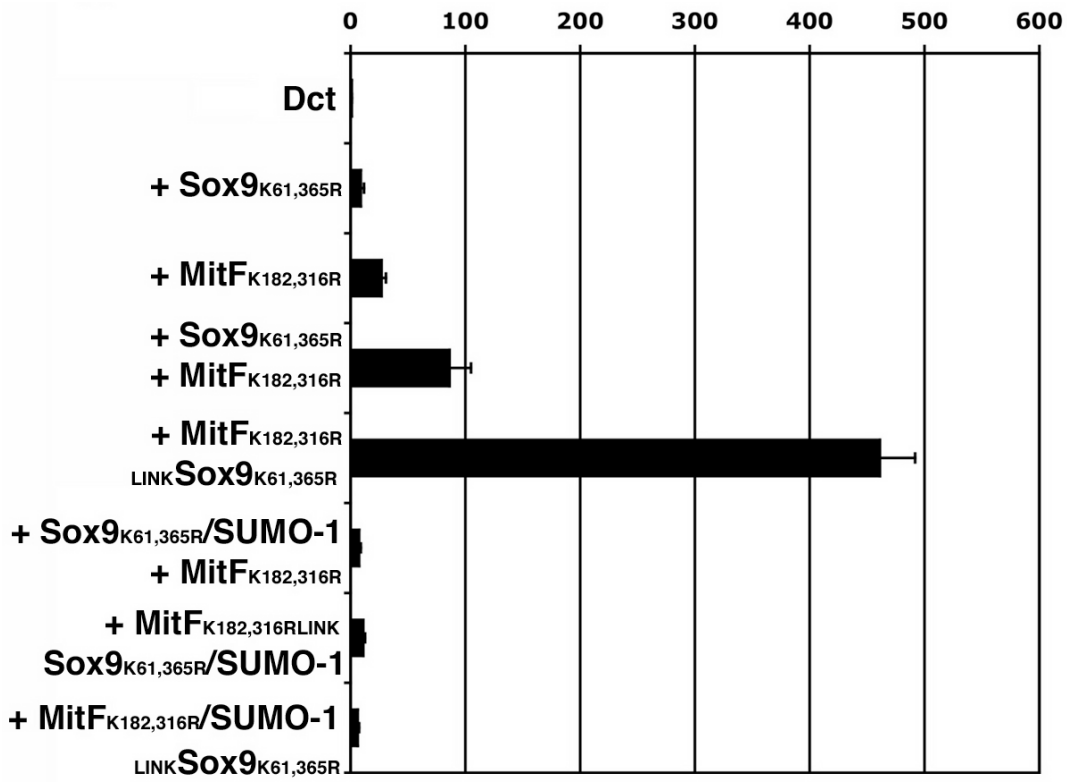

Control
MitF$_{K182,316R}$ + Sox9$_{K61,365R}$
MitF$_{K182,316R}$ + Sox9$_{K61,365R}$/SUMO-1
MitF$_{K182,316R}$ LINK Sox9$_{K61,365R}$
MitF$_{K182,316R}$ LINK Sox9$_{K61,365R}$/SUMO-1
p. Dct

Figure 3.12: Effects of linked versions of Sox9 and MitF on the full-length Dct promoter. Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. While the misexpression of unlinked $Sox9^{K61,365R}$ + $MitF^{K182,316R}$ still shows synergistic activation on the Dct promoter (~90-fold above basal), misexpression of the linked versions of these two factors, $MitF^{K182,316R}LINKSox9^{K61,365R}$, demonstrates a more potent synergistic activation on this promoter (460-fold above basal). Like the unlinked counterpart, $MitF^{K182,316R}LINKSox9^{K61,365R}$/SUMO-1 still shows an inhibition of synergy, with ~5-fold over basal activation. Importantly, linking MitF to a SUMOylated Sox9 shows some residual activity on the Dct promoter.

Figure 3.12


0
100
200
300
400
500
600
Dct
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ MitFK182,316R
LINKSox9K61,365R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316RLINK
Sox9K61,365R/SUMO-1
+ MitFK182,316R/SUMO-1
LINKSox9K61,365R

promoter. Misexpression of the unlinked counterparts of this linker still produced synergistic activation of the Dct promoter, but to a less extent (90-fold). This data showed that the double lysine linker had an activity approximately 4.5 times greater than that of the unlinked counterparts. However, to answer the question of whether the SUMO-1 moiety is interfering with synergistic activation of the Dct promoter by steric interference, I misexpressed MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 or MitF^{K182,316R}/SUMO-1LINKSox9^{K61,365R} in *Xenopus* embryos and compared their activities by luciferase assays on the Dct promoter. I found that both of these linker constructs had roughly the same activation potential as the unlinked counterparts on the Dct promoter, indicating that this was not the mechanism by which SUMO was inhibiting transcriptional synergy. Also, to be sure that the location of the SUMO-1 moiety was not altering my results in any way, I tested the SUMO-1/ MitF^{K182,316R}LINKSox9^{K61,365R} construct in luciferase assays (Figure 3.13). I found that this construct still had residual activation (approximately 10-fold) on the Dct promoter, just as what was seen with the other SUMO-1 linker constructs. I therefore assumed that it was the presence of the SUMO-1 moiety and not the location that created the inhibitory effects on transcriptional activation.

I also performed the same luciferase assays described above with the linker constructs on the Dct2 promoter. Again, I found similar, yet less potent trends with this promoter construct. The results from this experiment are shown in Figure 3.14. Misexpression of MitF^{K182,316R}LINKSox9^{K61,365R} demonstrated synergistic activation on the Dct2 promoter (125-fold above basal), while misexpression of the SUMO-1 linkers, MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 and MitF^{K182,316R}/SUMO-1LINKSox9^{K61,365R} still inhibited synergistic activation of the Dct2 promoter, yet retained some residual activation (20-fold and 5-fold, respectively). This data provided more evidence supporting the notion that the

Figure 3.13: Effects of altering the location of the SUMO-1 moiety has no effect on transcriptional activity on the Dct promoter.

Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. The misexpression of SUMO-1/MitF^{K182,316R}LINKSox9^{K61,365R} still shows an inhibition of synergy, but retains some residual activity with ~10-fold over basal activation.

Figure 3.13

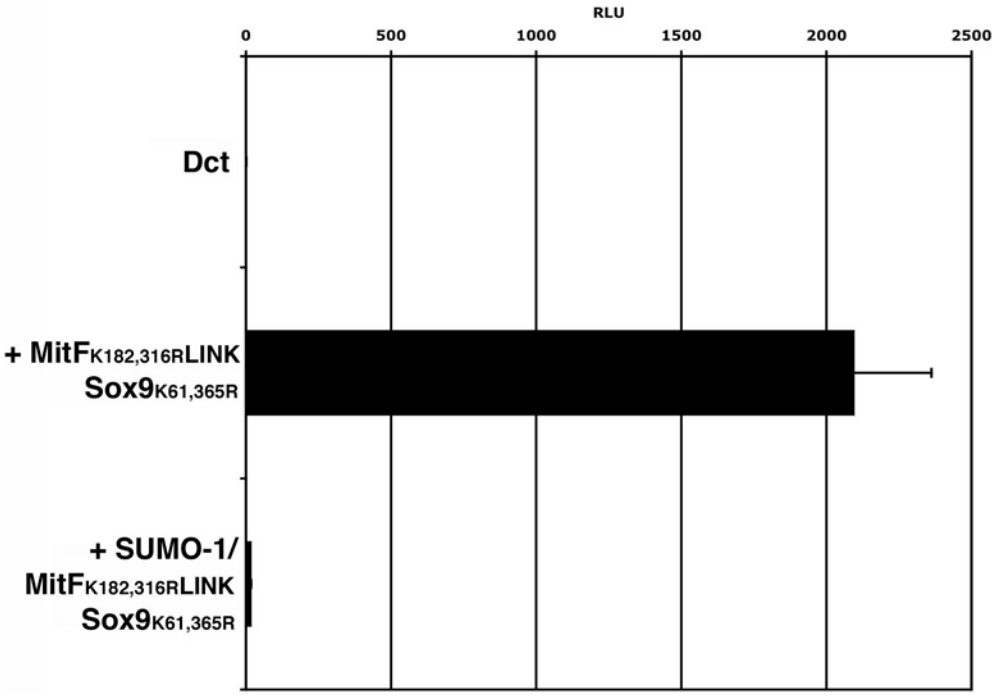
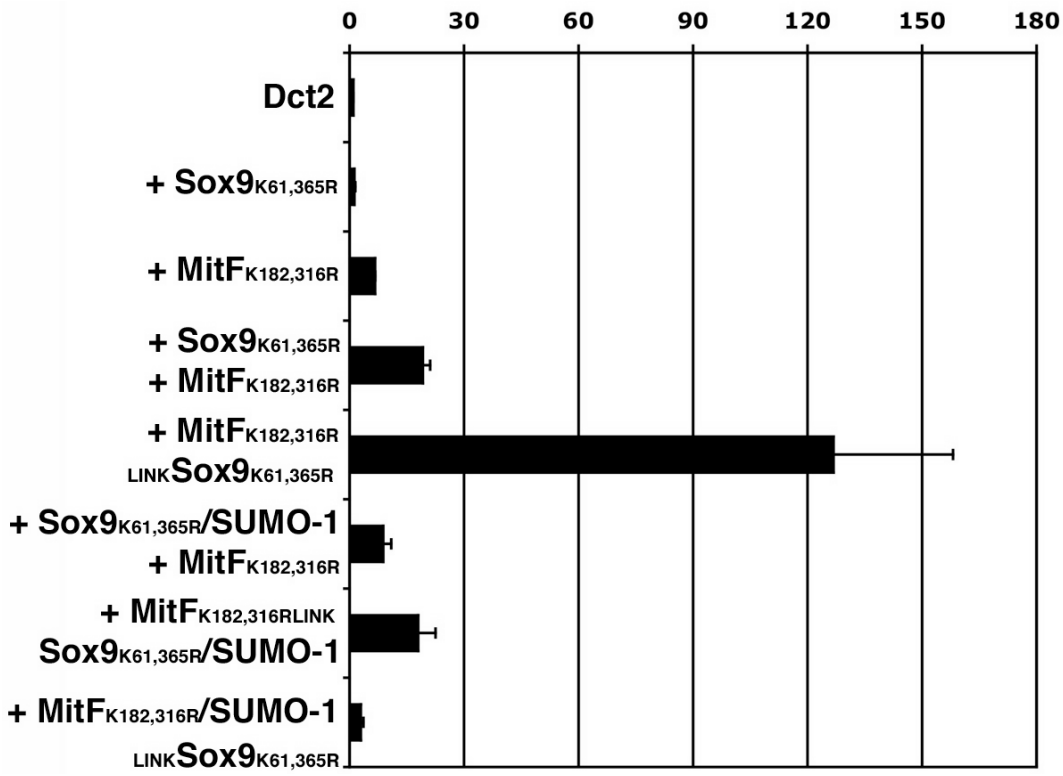

RLU
0
500
1000
1500
2000
2500
Dct
+ MitFK182,316RLINK
Sox9K61,365R
+ SUMO-1/
MitFK182,316RLINK
Sox9K61,365R

Figure 3.14: Effects of linked versions of Sox9 and MitF on the Dct2 promoter.

Relative luciferase activities were calculated using the activity of Dct2 alone in *Xenopus* embryos as a control. While the misexpression of unlinked $Sox9^{K61,365R}$ + $MitF^{K182,316R}$ still shows synergistic activation on the Dct2 promoter (~20-fold above basal), misexpression of the linked versions of these two factors, $MitF^{K182,316R}$LINK$Sox9^{K61,365R}$, demonstrates a more potent synergistic activation on this promoter (125-fold above basal). Like the unlinked counterpart, $MitF^{K182,316R}$LINK$Sox9^{K61,365R}$/SUMO-1 still shows an inhibition of synergy, with ~5-fold over basal activation. Importantly, linking MitF to a SUMOylated Sox9 still shows some residual activity on the Dct2 promoter.

Figure 3.14


0
30
60
90
120
150
180
Dct2
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ MitFK182,316R
LINKSox9K61,365R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316RLINK
Sox9K61,365R/SUMO-1
+ MitFK182,316R/SUMO-1
LINKSox9K61,365R

SUMO-1 moiety was not inhibiting synergy by physically blocking proper DNA-protein interactions on the Dct promoter.

Finally, I demonstrated that SUMO-1 was not working through steric interference on this promoter by performing gel shift assays. If this were the mechanism at hand, then the constitutively SUMOylated proteins should not be able to bind to DNA *in vitro*. However, $Sox9^{K61,365R}$, $Sox9^{K61,365R}$/SUMO-1, $MitF^{K182,316R}$ and $MitF^{K182,316R}$/SUMO-1 were all able to bind to a portion of the Dct promoter containing one SoxE and one MitF binding site (Figure 3.15 A,B). The double lysine linker and the constitutively SUMOylated linker were also able to bind to DNA through gel shift assays (Figure 3.15 B). The above results overwhelmingly demonstrate that steric interference is not the mechanism by which SUMO-1 is working to inhibit synergistic activation of the Dct promoter.

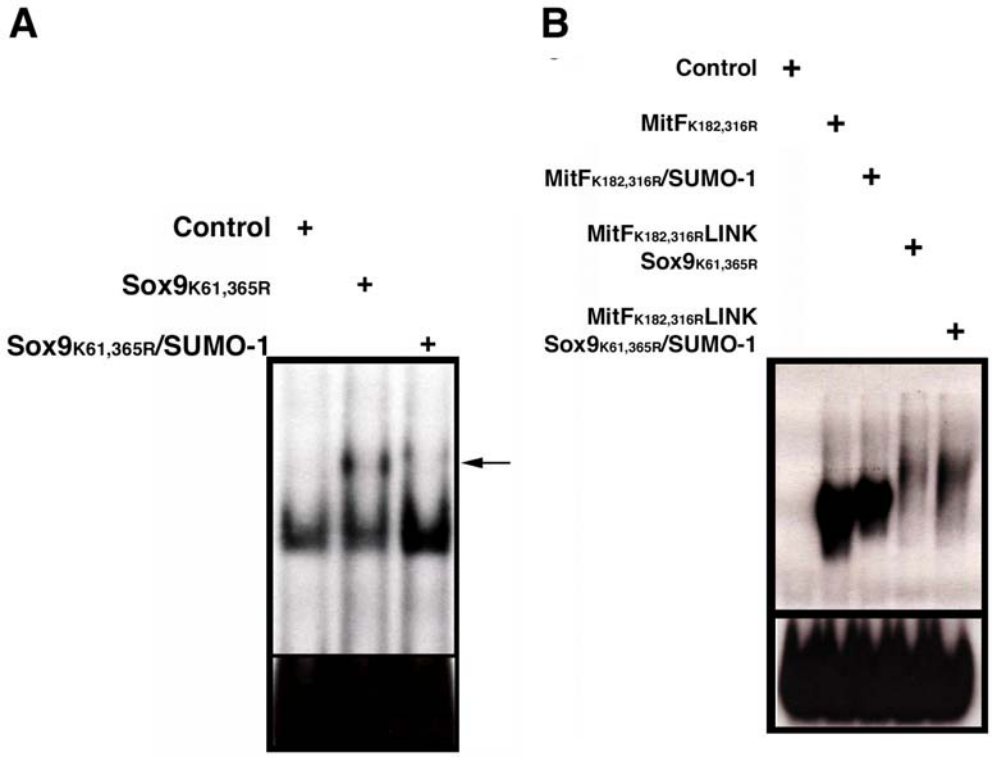
SUMOylation does not inhibit the synergistic activity between Sox9 and MitF through HDAC recruitment.

Yet another possible mechanism through which SUMO-1 acts to inhibit transcriptional activation is by the recruitment of histone deacetylase (HDAC) activity. HDAC recruitment leads to the removal of acetyl groups from histones and allows the histones to wrap tightly around a specific region of DNA, thereby blocking access of transcription factors to these regions. Due to the inability of factors to bind their target DNA, transcriptional activity is silenced. I felt that perhaps the addition of a SUMO-1 moiety to my proteins could be recruiting HDAC activity to the Dct promoter. Interestingly, there are compounds that can inhibit HDAC activity. Two specific HDAC inhibitors are Trichostatin A (TSA) and Valproic acid (VPA).

Figure 3.15: All Sox9 and MitF SUMOylation mutants, both unlinked and linked, can bind to the Dct promoter *in vitro*.

A) Gel shift assay demonstrating that *Xenopus* embryos injected with either $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO-1 can bind to a probe containing one MitF and one SoxE binding site from the Dct promoter. **B)** Gel shift assay demonstrating that *Xenopus* embryos injected with either $MitF^{K182,316R}$ or $MitF^{K182,316R}$/SUMO-1 can bind to a probe containing one MitF and one SoxE binding site from the Dct promoter. *Xenopus* embryos injected with either of the linked versions of these factors, $MitF^{K182,316R}$LINK$Sox9^{K61,365R}$ or $MitF^{K182,316R}$LINK$Sox9^{K61,365R}$/SUMO-1, can also bind to a probe containing one MitF and one SoxE binding site from the Dct promoter**.** Control lanes represent uninjected *Xenopus* embryos mixed with the labeled probe containing one MitF and one SoxE binding site from the Dct promoter**.**

Figure 3.15


A
Control +
Sox9K61,365R +
Sox9K61,365R/SUMO-1 +
B
Control +
MitFK182,316R +
MitFK182,316R/SUMO-1 +
MitFK182,316RLINK
Sox9K61,365R +
MitFK182,316RLINK
Sox9K61,365R/SUMO-1 +

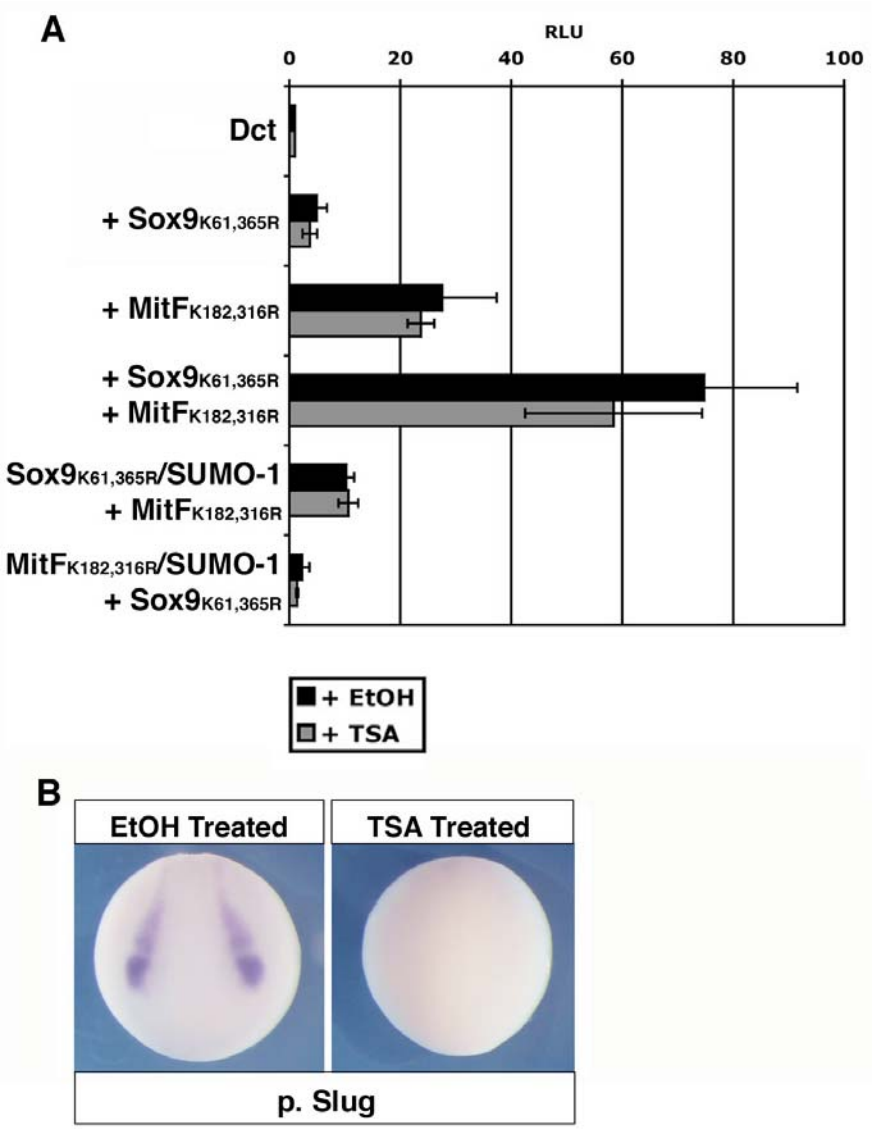
Each of these HDAC inhibitors can block the recruitment of different HDACs, so it was important to test both of these drugs. Both of these drugs work by blocking the removal of the acetyl groups from the histones. Then, because the histones are no longer able to tighten around the DNA, the DNA is always relaxed and open for binding by various transcription factors. Therefore, I can treat my embryos or cell lines with these drugs and if this is the mechanism by which SUMOylation works on the Dct promoter, I would expect to see a relief of the synergy inhibition that is usually present when a constitutively SUMOylated MitF or Sox9 factor is present.

To test this hypothesis, I performed luciferase assays in both *Xenopus* embryos and the C8161 cell line after treatment with TSA or VPA. I began these experiments in *Xenopus* embryos by misexpressing my constructs in the embryo for luciferase assays as described previously. However, while the embryos were being cultured to late neurula stages, I treated half of the embryos with TSA, while the other half received control treatment (ethanol, in this case). I then collected the embryos and processed them as described previously. I then directly compared the control versus treated lanes. As shown in Figure 3.16 A, focusing on the $MitF^{K182,316R}$ + $Sox9^{K61,365R}$/SUMO-1 and $MitF^{K182,316R}$/SUMO-1 + $Sox9^{K61,365R}$ lanes, treatment of *Xenopus* embryos with TSA did not lead to any inhibitory relief. HDAC recruitment, so far, did not appear to be the mechanism of choice for SUMO-1 inhibition of the Dct promoter. However, I had to be certain that my drug treatment was working properly and interestingly, the treatment of *Xenopus* embryos with both TSA and VPA had been shown to inhibit neural crest formation. Therefore, to make sure my TSA treatment was functioning appropriately, I performed *in situ* hybridization on uninjected, sister embryos that were cultured in either ethanol (control) or TSA. I looked for expression of the early neural crest marker Slug in both of these embryo sets. While

Figure 3.16: Treatment of *Xenopus* embryos with Trichostatin A has no impact on the synergy inhibition demonstrated by SUMOylated SoxE and/or MitF constructs.

A) Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. Luciferase assay demonstrating that when injected *Xenopus* embryos are treated with Trichostatin A, there are no statistically significant differences in transcriptional activation ability between treated and control embryos. The SUMOylated forms of MitF and Sox9 are not sensitive to this treatment. **B)** *In situ* hybridization performed on uninjected sister *Xenopus* embryos treated with ethanol alone (control) or Trichostatin A (TSA) examining expression of the early neural crest precursor marker, Slug. Treatment with ethanol does not impair proper neural crest precursor development, while treatment with TSA does, providing evidence that the TSA treatment is working properly.

Figure 3.16


A
RLU
0
20
40
60
80
100
Dct
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
Sox9K61,365R/SUMO-1
+ MitFK182,316R
MitFK182,316R/SUMO-1
+ Sox9K61,365R
+ EtOH
+ TSA
B
EtOH Treated
TSA Treated
p. Slug

the control embryos expressed Slug normally, the TSA-treated embryos lost expression of Slug, as shown in Figure 3.16 B. I concluded from this data that my drug treatments had worked and that HDAC recruitment did not appear to be the mechanism at hand. Still, I wanted to confirm that the TSA treatment did not have a different effect in the human cell line. I transfected in my constructs as previously described and treated the cells with either the control vehicle (ethanol) or TSA. As shown in Figure 3.17, by comparing the $MitF^{K182,316R}$ + $Sox9^{K61,365R}$/SUMO-1 and $MitF^{K182,316R}$/SUMO-1 + $Sox9^{K61,365R}$ lanes, treatment of the cells with TSA did not release synergy inhibition on the Dct promoter. These results mirrored the results obtained in the whole embryo experiment.

It is possible, however, that SUMO-1 could be acting through recruitment of HDACs that are not susceptible to TSA treatment. To address this possibility, I also used the HDAC-1-specific inhibitor, Valproic acid. I performed these treatments in *Xenopus* embryos in the same manner as the TSA treatments. However, the controls were treated with water instead of ethanol. Upon performing the luciferase assays on these embryos, I again saw no difference in synergy inhibition amongst treated versus untreated $MitF^{K182,316R}$ + $Sox9^{K61,365R}$/SUMO-1 and $MitF^{K182,316R}$/SUMO-1 + $Sox9^{K61,365R}$ lanes (Figure 3.18 A). To ensure treatment with VPA had worked properly, I performed *in situ* hybridization on uninjected, sister embryos and analyzed the ability of these embryos to produce the neural crest through Slug expression. The VPA-treated embryos did not give rise to any Slug expression, as shown in Figure 3.18 B. I therefore concluded that the VPA treatment was working properly in this experiment. The above data clearly demonstrates that SUMO-1 is not recruiting HDACs that are sensitive to TSA or VPA to the Dct promoter to achieve synergy inhibition.

Figure 3.17: Treatment of the melanoma cell line C8161 with Trichostatin A has no impact on the synergy inhibition demonstrated by transfected SUMOylated SoxE and/or MitF constructs.

Relative luciferase activities were calculated using the activity of Dct alone in transfected C8161 cells. Luciferase assay demonstrating that when transfected C8161 cells are treated with Trichostatin A, there are no statistically significant differences in transcriptional activation ability between treated- and control-transfected cells. The SUMOylated forms of MitF and Sox9 are not sensitive to this treatment.

Figure 3.17

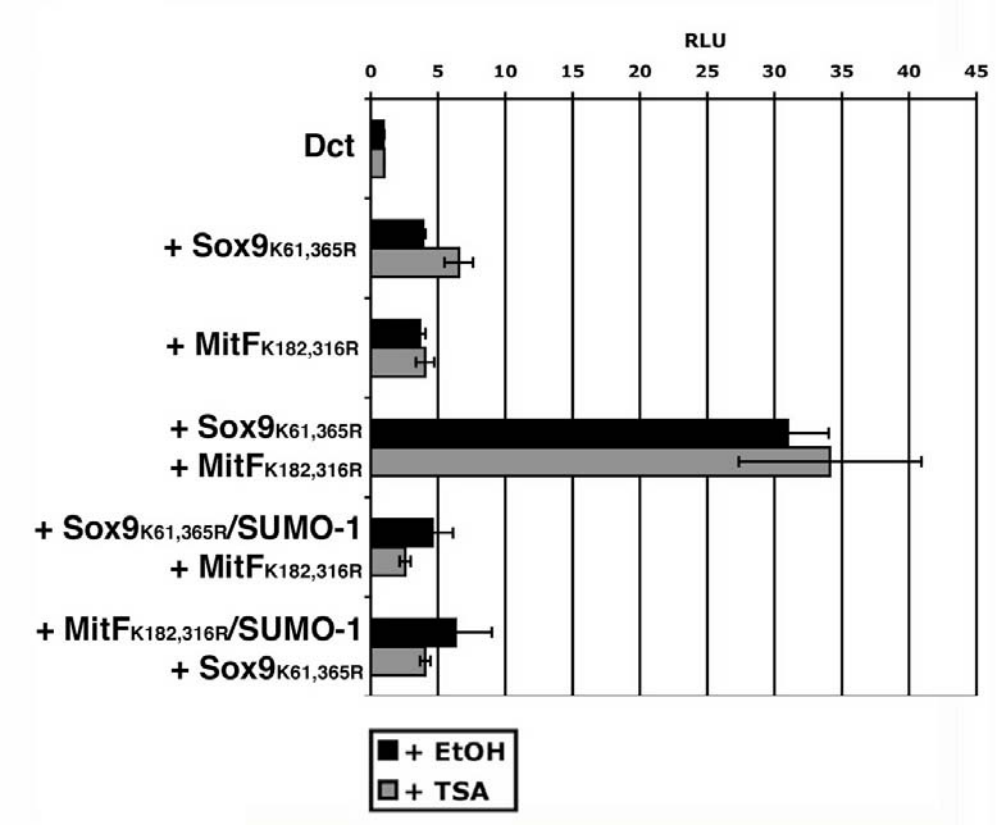
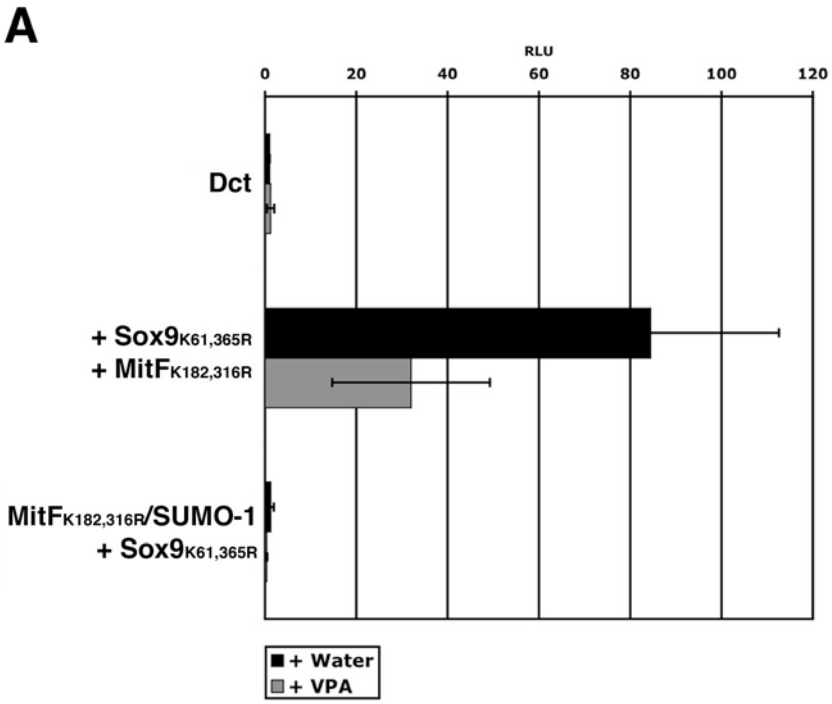
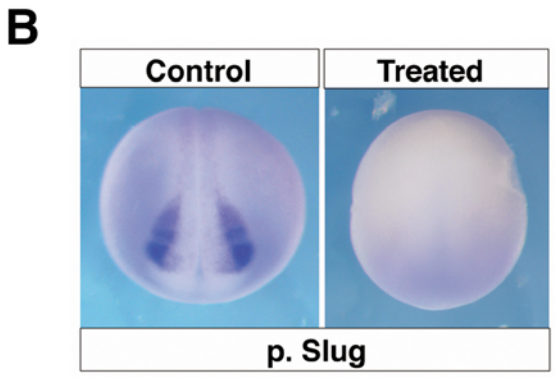

RLU
0
5
10
15
20
25
30
35
40
45
Dct
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R
+ MitFK182,316R
+ Sox9K61,365R/SUMO-1
+ MitFK182,316R
+ MitFK182,316R/SUMO-1
+ Sox9K61,365R
+ EtOH
+ TSA

Figure 3.18: Treatment of *Xenopus* embryos with Valproic acid does not relieve the synergy inhibition demonstrated by SUMOylated SoxE and/or MitF constructs.

A) Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. Luciferase assay demonstrating that when injected *Xenopus* embryos are treated with Valproic acid, there is no relief of the synergy inhibition seen with the SUMOylated forms of Sox9 and MitF. **B)** *In situ* hybridization performed on uninjected sister *Xenopus* embryos treated with water alone (control) or Valproic acid (VPA) examining expression of the early neural crest precursor marker, Slug. Treatment with water does not impair proper neural crest precursor development, while treatment with VPA does, providing evidence that the VPA treatment is working properly.

Figure 3.18


A
RLU
0
20
40
60
80
100
120
Dct
+ Sox9K61,365R
+ MitFK182,316R
MitFK182,316R/SUMO-1
+ Sox9K61,365R
+ Water
+ VPA


B
Control
Treated
p. Slug

Thus far, I have demonstrated that SUMO-1 was not inhibiting synergy on the Dct promoter through steric interference or HDAC recruitment. This left me with two alternative possibilities. The first was that the presence of SUMO-1 was altering subcellular localization of these factors and the second was that the presence of SUMO-1 was either recruiting a co-repressor to the promoter or inhibiting the recruitment of a co-activator to the Dct promoter.

Subnuclear localization of Sox9 SUMOylation mutants and linker constructs is altered in the melanoma cell line, C8161

As previously discussed, differences in subcellular localization have not been found in MitF SUMOylation mutants. Miller and colleagues examined subcellular localization of wild-type MitF, MitF^{K182,316R} and a SUMO-MitF construct and found no difference amongst these constructs (Miller et al., 2005). However, with regard to SoxE SUMOylation, conflicting reports have arisen with respect to subcellular localization. Girard and Goossens only examined whether Sox10 had to be SUMOylated to enter the nucleus and found no difference between these constructs (Girard and Goossens, 2006). Unfortunately, this group only looked briefly for nuclear entry by comparing the ability of a Sox10 lysine mutant versus wild-type Sox10 to enter the nucleus. Oh and colleagues found that unSUMOylated Sox9 and a constitutively SUMOylated form of Sox9 had different nuclear localization patterns from one another. The SUMOylated form had more distinct dots within the nucleus (Oh et al., 2007). Therefore, it was possible that the constitutively SUMOylated form of SoxE could impact subcellular localization and more specifically, subnuclear localization. Also, while I would not expect to see any differences in using Sox9 SUMOylation mutants compared to Sox10 SUMOylation mutants

given all of the data presented thus far showing that these two proteins act analogous to one another, this too was another variable.

As mentioned, I wanted to look for not only subcellular localization differences amongst my constructs, but also subtle differences in subnuclear localization. Interestingly, one mechanism that SUMO uses to repress transcription is the sub-compartmentalization of SUMO-modified proteins. These proteins are sequestered in promyelocytic leukemia (PML) nuclear bodies, which are repressive environments found within the nucleus, so that they may not contribute to normal transcriptional activities (reviewed in (Hay, 2005)). One additional sub-compartment of the nucleus is the newly discovered SUMO-1 nuclear bodies (SNBs) (Navascues et al., 2007). Different from PML nuclear bodies in that they do not contain the PML protein, which is standard to all PML bodies, the SNBs are also much larger than PML nuclear bodies. It is also thought that these SNBs may be sites of SUMOylation, as overexpression of the active form of SUMO-1 resulted in the formation of these SNBs (Navascues et al., 2007). Thus, it was possible that Sox9^{K61,365R}/SUMO-1 was being recruited to one of these subnuclear bodies as a mechanism used to inhibit synergy on the Dct promoter.

To perform the subcellular localization experiments, I first seeded C8161 cells onto fibronectin-coated coverslips and then transfected the myc-epitope tagged DNA of Sox9^{K61,365R}, Sox9^{K61,365R}/SUMO-1, MitF^{K182,316R}LINKSox9^{K61,365R}, or MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 into the seeded cells. Post-transfection, I fixed the cells, performed antibody staining on the cell-coated coverslips, and visualized my results using confocal microscopy. The results I obtained were extremely interesting, but appeared quite complicated. The images from the Sox9^{K61,365R}- and MitF^{K182,316R}LINKSox9^{K61,365R}-transfected cells looked similar to one another, as did the results from the Sox9^{K61,365R}/SUMO-1- and MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1-transfected

cells. I therefore concluded that tethering MitF^{K182,316R} to the Sox9 mutants had no impact on their subcellular localization. I saw both sets of proteins shuttling in and out of the nucleus, as Sox9 has been shown to do. However, once inside the nucleus, Sox9^{K61,365R} and MitF^{K182,316R}LINKSox9^{K61,365R} had small punctate staining, which was indicative of subnuclear compartmentalization (Figure 3.19 A,C). I could not, however, discern what compartments these factors were being sent to in the cell. Interestingly, the staining pattern of Sox9^{K61,365R}/SUMO-1 and MitF^{K182,316R}LINKSox9^{K61,365R}/SUMO-1 looked somewhat similar in that it was very punctate staining, but I noticed a few much larger compartments lighting up, perhaps indicative of SNBs (Figure 3.19 B,D). While this data appeared to be extremely interesting, I did not have the appropriate tools to examine in what compartments these factors were located. This would be potentially useful information to gather and I would suggest examining this in the future. As the subcellular localization data did not provide any clear-cut answers, I next turned to the possibility that SUMOylation was altering co-factor recruitment. Prior to investigating this hypothesis, however, I wanted to first examine the physical interactions between the factors I already knew were present, Sox9 and MitF.

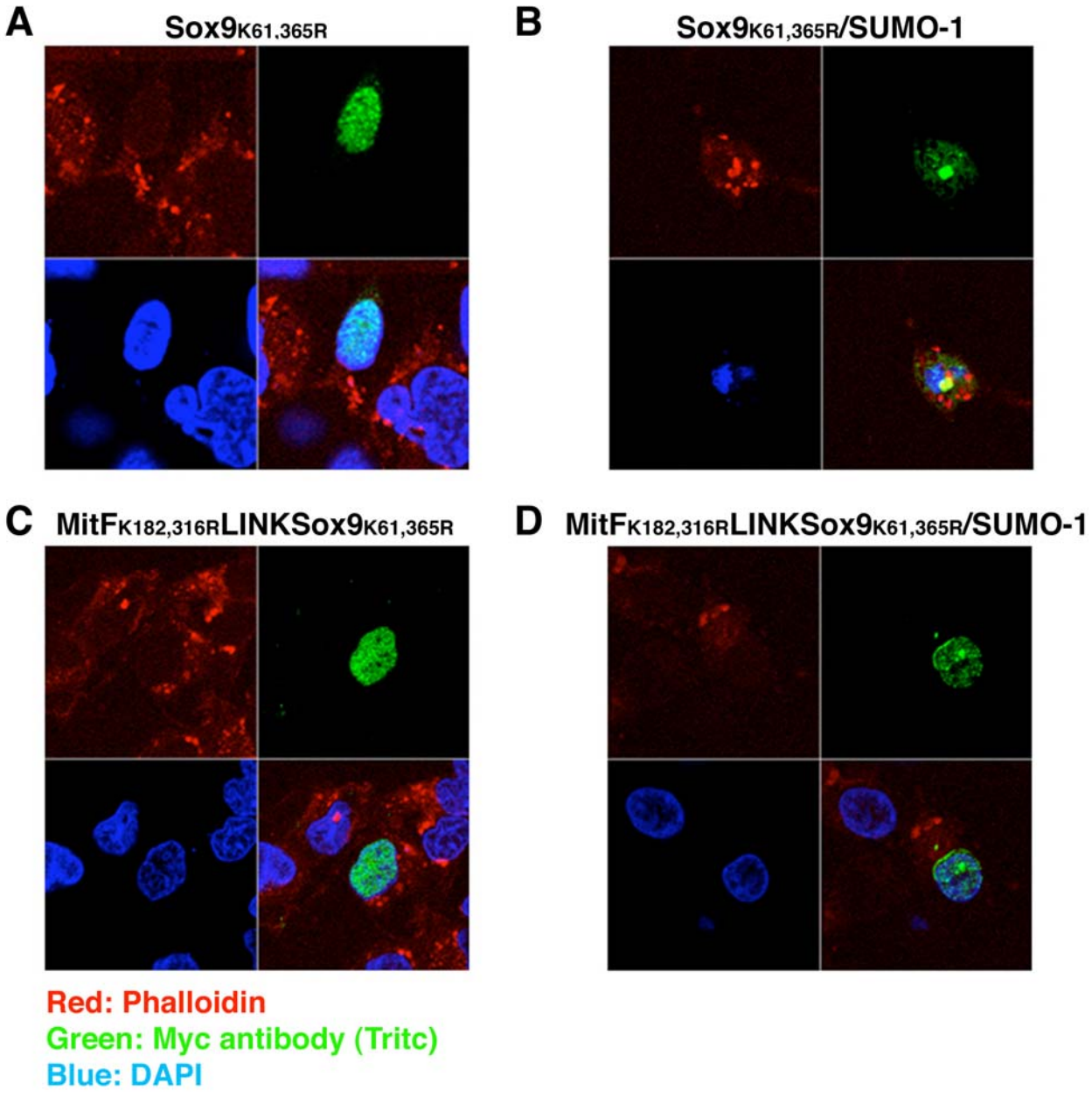
MitF can only physically interact with Sox9^{K61,365R}/SUMO-1

Although SoxE factors and MitF have been shown to work together in the context of the Dct promoter, the physical interactions that occur between these factors has never been examined in previous publications. It is interesting because the binding sites of these two factors are located adjacent to one another on the mouse Dct promoter. I therefore postulated that because Sox9^{K61,365R} and MitF^{K182,316R} worked together to create synergistic activation of the Dct promoter, these two constructs would be likely to physically interact. To investigate this hypothesis, I used

Figure 3.19: Subnuclear localization of transfected Sox9 SUMOylation mutants is altered in C8161 melanoma cells.

A) Nuclear staining of $Sox9^{K61,365R}$ appears concentrated in a small, punctate pattern. **B)** Nuclear staining of $Sox9^{K61,365R}$/SUMO-1 appears to also be sequestered in small, punctate sections of the nucleus, but also in much larger subnuclear bodies. **C)** Nuclear staining of $MitF^{K182,316R}LINKSox9^{K61,365R}$ does not appear to deviate from $Sox9^{K61,365R}$ alone, with it being concentrated in small, punctate areas of the nucleus. **D)** Nuclear staining of $MitF^{K182,316R}LINKSox9^{K61,365R}$/SUMO-1 does not appear to deviate from $Sox9^{K61,365R}$/SUMO-1 alone in that it is located in smaller, punctate nuclear compartments, but also larger nuclear compartments. Red staining represents Phalloidin, which marks the cytoskeleton. The Phalloidin staining in this experiment did not work well. Blue staining represents DAPI, which denotes the nucleus. Green staining is the visualization of the myc epitope-tagged construct using TRITC.

Figure 3.19


A
Sox9K61,365R
B
Sox9K61,365R/SUMO-1
C
MitFK182,316RLINKSox9K61,365R
D
MitFK182,316RLINKSox9K61,365R/SUMO-1
Red: Phalloidin
Green: Myc antibody (Tritc)
Blue: DAPI

co-immunoprecipitation assays. I co-injected mRNA encoding one (control) or two (to test interactions) different constructs into *Xenopus* embryos, each epitope-tagged differently (flag or myc tagged) into both cells of a two-celled embryo. Then, after lysing the embryos, I pulled down one of the factors with the α-flag antibody; the principle being that if two factors are physically bound to one another, both of these factors will be pulled down with the one antibody. I then added protein A-Sepharose beads to attract the antibody, washed the beads with stringent detergent conditions and processed the embryos via western blot. To detect whether two factors interacted, I then visualized for the α-myc antibody via chemiluminescence. If the myc-tagged factor interacted with the flag-tagged factor, then it will be visible on the blot. If no interaction occurred, then the myc-tagged factor would not have been pulled down in the first step of this experiment and would not be seen on the blot.

I first decided to look at the interactions of my different Sox9 and MitF SUMOylation constructs. Much to my surprise, I only saw a consistent, strong interaction between $Sox9^{K61,365R}$/SUMO-1 and $MitF^{K182,316R}$/SUMO-1 and not Sox9 + MitF or $Sox9^{K61,365R}$ + $MitF^{K182,316R}$ (Figure 3.20 A). This result suggested that SUMO-1 was important for a strong interaction between these proteins. I next asked whether both MitF and Sox9 needed to be constitutively SUMOylated or just one. To do this, I again used co-immunoprecipitation. My findings indicated that while wildtype MitF and $MitF^{K182,316R}$/SUMO-1 could interact with $Sox9^{K61,365R}$/SUMO-1, Sox9 could not interact with $MitF^{K182,316R}$/SUMO-1 (Figure 3.20 B). This suggested that perhaps the SUMO-1 moiety on Sox9 was the important key to the strong interaction of Sox9 with MitF. Next, to confirm that MitF indeed needed the SUMO-1 moiety for an interaction with Sox9, I performed co-immunoprecipitations with MitF and the three Sox9 constructs (wildtype, double lysine and SUMO fusion). Upon doing this, I only saw an

Figure 3.20: Only Sox9 needs to be SUMOylated to physically interact with MitF.

A) Co-immunoprecipitation showing a lack of interaction between Sox9 + MitF or $Sox9^{K61,365R}$ + $MitF^{K182,316R}$. $Sox9^{K61,365R}$/SUMO-1 + $MitF^{K182,316R}$/SUMO-1, however, shows a strong physical interaction. The immunoprecipitation antibody was α-flag (MitF constructs were flag-tagged) and the immunoblot antibody was α-myc (Sox9 constructs were myc-tagged). The input levels, shown in the lower two blots, were monitored via Western blot using either the α-myc or α-flag antibodies. **B)** Co-immunoprecipitation showing that only Sox9 has to be SUMOylated in order to interact with MitF, but MitF did not need to be SUMOylated. $Sox9^{K61,365R}$/SUMO-1 showed physical interaction with both MitF and $MitF^{K182,316R}$/SUMO-1. However, Sox9 could not interact with $MitF^{K182,316R}$/SUMO-1. The immunoprecipitation antibody was α-flag (MitF constructs were flag-tagged) and the immunoblot antibody was α-myc (Sox9 constructs were myc-tagged). The input levels, shown in the lower two blots, were monitored via Western blot using either the α-myc or α-flag antibodies.

Figure 3.20

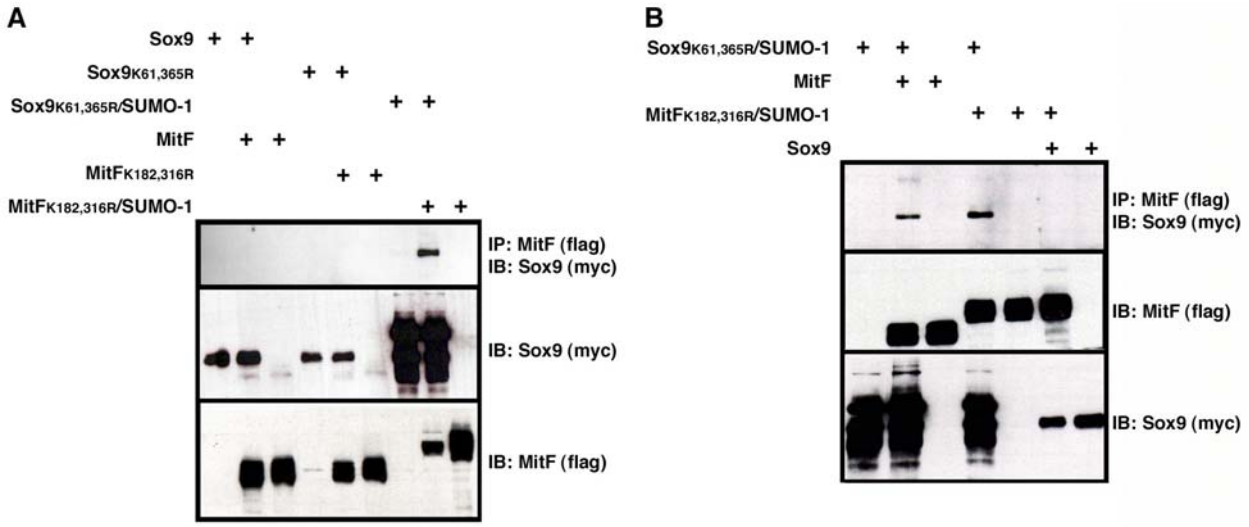
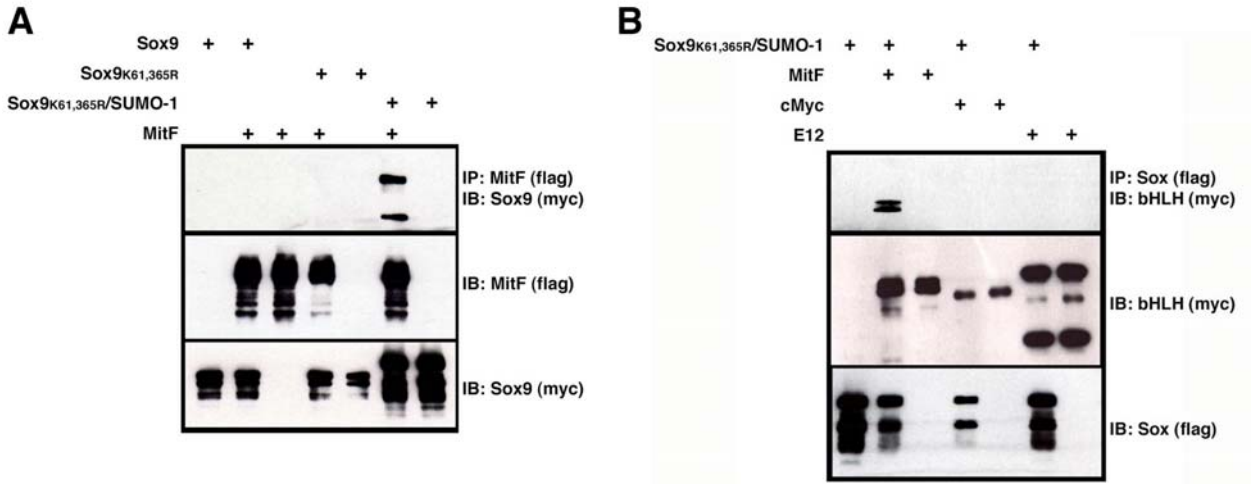

A
Sox9 + +
Sox9K61,365R + +
Sox9K61,365R/SUMO-1 + +
MitF + +
MitFK182,316R + +
MitFK182,316R/SUMO-1 + +
IP: MitF (flag)
IB: Sox9 (myc)
IB: Sox9 (myc)
IB: MitF (flag)
B
Sox9K61,365R/SUMO-1 + + +
MitF + +
MitFK182,316R/SUMO-1 + + +
Sox9 + +
IP: MitF (flag)
IB: Sox9 (myc)
IB: MitF (flag)
IB: Sox9 (myc)

Figure 3.21: Only Sox9^{K61,365R}/SUMO-1 can physically interact with MitF and this modification does not lead to a mechanism used by Sox9 to interact with other bHLH proteins.

A) Co-immunoprecipitation showing only Sox9 ^{K61,365R}/SUMO-1 could interact with MitF. Sox9 and Sox9^{K61,365R} could not interact with MitF in this assay. The immunoprecipitation antibody was α-flag (MitF was flag-tagged) and the immunoblot antibody was α-myc (Sox9 constructs were myc-tagged). The input levels, shown in the lower two blots, were monitored via Western blot using either the α-myc or α-flag antibodies. **B)** Co-immunoprecipitation showing that the SUMOylation of Sox9 was not a mechanism used by this protein to interact with other bHLH proteins. Sox9 ^{K61,365R}/SUMO-1 only showed an interaction with MitF and not other bHLH proteins, such as cMyc or E12. The immunoprecipitation antibody was α-flag (Sox9^{K61,365R}/SUMO-1 was flag-tagged) and the immunoblot antibody was α-myc (bHLH constructs were myc-tagged). The input levels, shown in the lower two blots, were monitored via Western blot using either the α-myc or α-flag antibodies.

Figure 3.21


A
Sox9
Sox9K61,365R
Sox9K61,365R/SUMO-1
MitF
IP: MitF (flag)
IB: Sox9 (myc)
IB: MitF (flag)
IB: Sox9 (myc)
B
Sox9K61,365R/SUMO-1
MitF
cMyc
E12
IP: Sox (flag)
IB: bHLH (myc)
IB: bHLH (myc)
IB: Sox (flag)

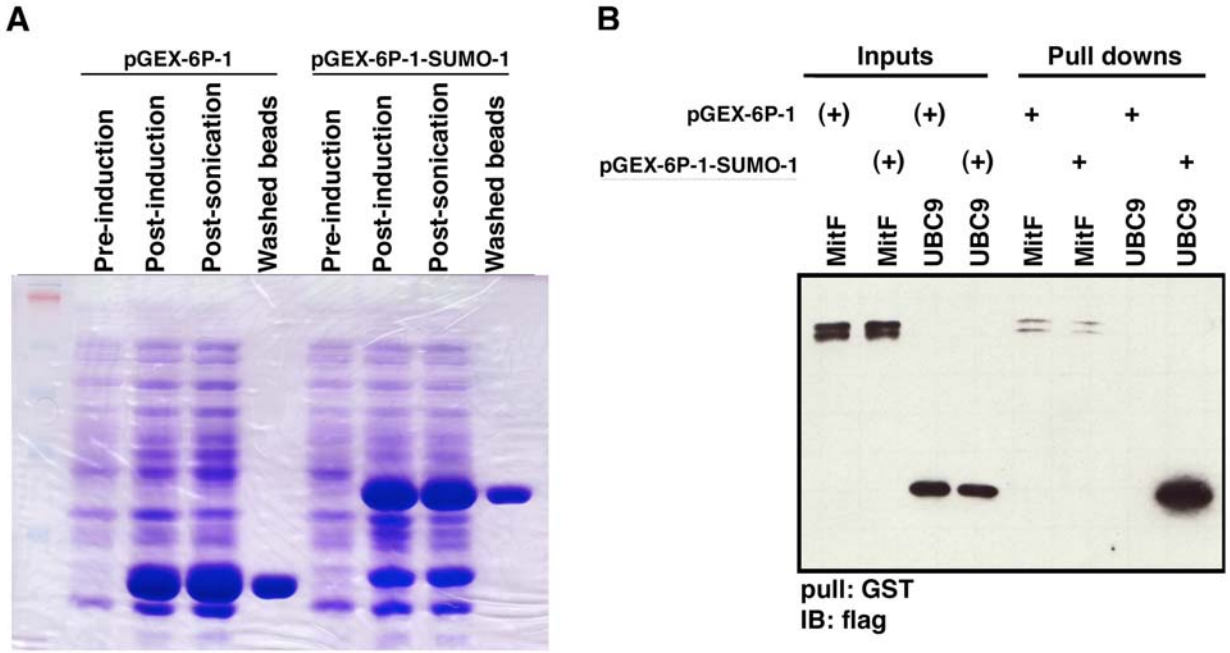
interaction with MitF and Sox9^{K61,365R}/SUMO-1 (Figure 3.21 A). This data indicated that perhaps MitF was actually binding to SUMO-1 and not to Sox9. In fact, there is precedence for a few factors that physically bind to a SUMO-1 moiety instead of being only modified by SUMO-1. These factors bind to SUMO via SUMO interacting motifs, or SIMs. Only in recent years have SIM consensus sequences been identified (reviewed in (Hecker et al., 2006)). However, before I investigated the MitF sequence for a potential SIM, I first wanted to determine whether this was a common mechanism that the SUMOylated form of Sox9 used to partner with different bHLH proteins. I looked at other bHLH proteins, such as cMyc and E12, and by using co-immunoprecipitation found that this was not a common mechanism used to partner with bHLH proteins, as only MitF showed an interaction with the constitutively SUMOylated form of Sox9 (Figure 3.21 B). So, I then turned back to the MitF sequence and could not identify any consensus SIM sites within the protein. However, this did not mean that MitF was not binding to SUMO. There was still the possibility of identifying a novel SIM.

First, I had to demonstrate that MitF could physically bind to SUMO-1. Due to the fact that MitF is a SUMOylated protein, I chose to do this experiment in a GST pull-down assay so that I could express the SUMO-1 protein in bacteria. This eliminated the presence of SUMOylation machinery that would be normally present in the embryo and I did not want to complicate matters by post-translationally SUMOylating MitF in the process of showing my interaction. Therefore, I constructed a GST fusion in which GST was fused upstream to the coding region of SUMO-1. I successfully expressed this construct in bacteria and attached the SUMO-1 protein to Glutathione beads (Figure 3.22 A). I then misexpressed epitope-tagged MitF or epitope-tagged UBC9 (which contains a well-characterized SIM) in *Xenopus* embryos, collected them and processed them in the same manner that I had done for other Western blot

Figure 3.22: MitF does not physically bind to SUMO-1.

A) Coomassie staining of an acrylamide gel depicting the protein purification of either pGEX-6P-1 vector alone (GST alone) or pGEX-6P-1-SUMO-1 (GST-SUMO-1 fusion). Pre-induction lanes showing no protein present, post-induction lanes show protein has been produced, post-sonication lanes show protein is still present after the cells have been broken open, and washed beads lanes show the attachment of either GST alone or GST-SUMO-1 to glutathione beads. **B)** GST pull-down assays demonstrating that MitF does not bind to SUMO-1. Input lanes show MitF and UBC9 protein is present by immunoblotting with α-flag antibody. The (+) symbol denotes which input samples were to have which beads applied to them, it does not indicate that beads were already added. Pull down lane proteins were incubated with GST alone (pGEX-6P-1) or GST-SUMO-1 (pGEX-6P-1-SUMO-1). Immunoblot was carried out with the α-flag antibody. UBC9 demonstrates a clear interaction with SUMO-1, as there is a band present in the + pGEX-6P-1-SUMO-1 lane, but not in the vector alone lane. MitF, however, pulled itself down equally as faint with both empty vector and pGEX-6P-1-SUMO-1.

Figure 3.22


A
pGEX-6P-1
pGEX-6P-1-SUMO-1
Pre-induction
Post-induction
Post-sonication
Washed beads
Pre-induction
Post-induction
Post-sonication
Washed beads
B
Inputs
Pull downs
pGEX-6P-1
pGEX-6P-1-SUMO-1
(+)
(+)
+
+
(+)
(+)
+
+
MitF
MitF
UBC9
UBC9
MitF
MitF
UBC9
UBC9
pull: GST
IB: flag

samples. I next performed GST pull down assays on these embryo lysates with the control, GST-alone beads or the GST-SUMO-1-attached beads. As shown in Figure 3.22 B, UBC9 binds to SUMO-1 through its SIM, but does not bind to the GST-alone beads. This told me that my experiment had worked appropriately. However, I did not see a physical interaction between SUMO-1 and MitF (Figure 3.22 B). This data then led me back to the possibility that the presence of SUMO-1 was attracting a co-repressor to the complex of factors on the Dct promoter or inhibiting the ability of a co-activator to be recruited to the complex. I had a short list of candidate factors that could potentially act as such a factor, including the CBP/p300 complex, Pax3 and Groucho4.

The SUMOylation status of Sox9 alters co-factor recruitment

I first wanted to look at potential interactions with the CBP/p300 complex. These are two histone acetyltransferases (HATs) and act as co-activators. Perhaps when $Sox9^{K61,365R}$/SUMO-1 is present on the Dct promoter, one or both of these HATs cannot properly bind to Sox9 or MitF to help synergistically activate transcription on the Dct promoter. The reason behind choosing these two factors was because they had both been shown to interact with MitF and Sox9, although in separate circumstances.

MitF has been shown to interact with CBP/p300 in both melanocytes and osteoclasts. However, the way in which this interaction mechanistically contributes to these cell types is poorly understood (reviewed in (Steingrimsson et al., 2004)). Interestingly, it was recently demonstrated that MitF mutants that could not bind to CBP/p300 were still able to activate transcription in melanoma cells (Vachtenheim et al., 2007).

Sox9 has been shown to interact with CBP/p300 as well. Furumatsu and colleagues found that Sox9 and CBP/p300 could interact in the chondrocyte lineage where this interaction was propagated by the presence of Smad3, leading to enhanced transcriptional activity (Furumatsu et al., 2005a; Tsuda et al., 2003). Furthermore, this group went on to suggest that the mechanism behind this transcriptional activation was due to chromatin modification (Furumatsu et al., 2005b).

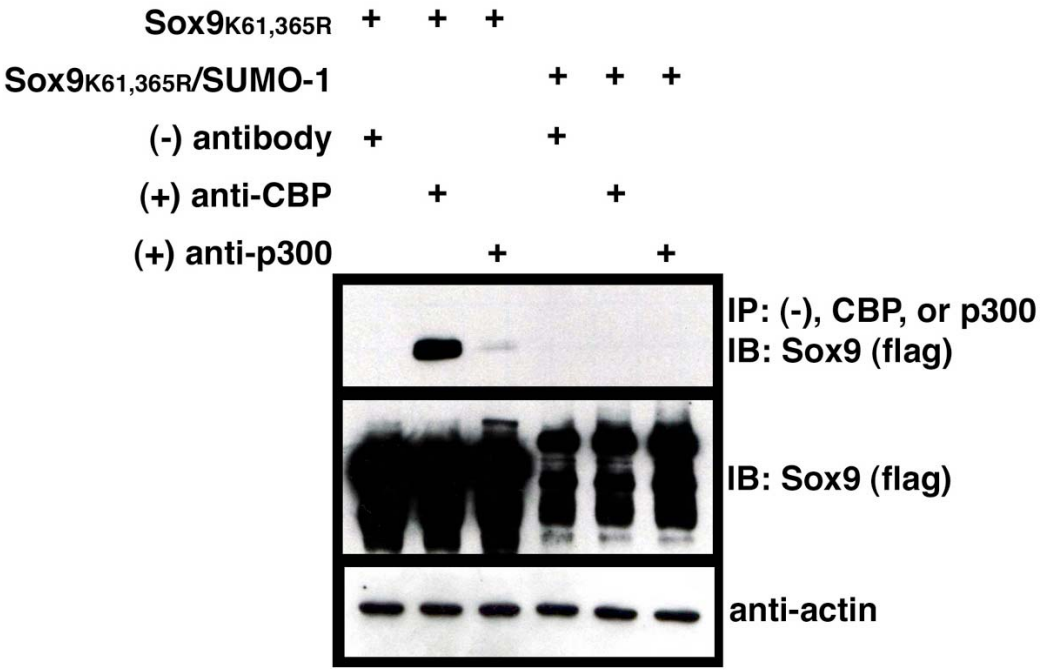
Given this data, I felt that CBP/p300 were good candidates to act as co-activators in my system. I obtained antibodies for endogenous CBP and p300 and performed co-immunoprecipitations, pulling with these new antibodies. I did these co-immunoprecipitations using *Xenopus* embryos injected with mRNA of my factor of interest (as previously described). I injected epitope-tagged $Sox9^{K61,365R}$ or $Sox9^{K61,365R}$/SUMO-1 and looked to see whether either of these mutants preferentially interacted with either of these two factors. Interestingly, I saw that only $Sox9^{K61,365R}$ could interact strongly with CBP and more weakly with p300. Important to note is that this faint interaction may be due to the antibody and not the actual strength of the interaction. $Sox9^{K61,365R}$/SUMO-1 interacted very faintly with CBP or did not do so at all (Figure 3.23). This data was extremely exciting, as it presents a model where the presence of a SUMO-1 moiety on Sox9 can alter the interaction of Sox9 with a co-activator. Perhaps when Sox9 is SUMOylated, it undergoes a conformational change that hides the binding site for CBP/p300. In fact, the binding site for CBP/p300 on Sox9 is located in the C-terminal activation domain, which is also near the predominate site of Sox9 SUMOylation (Tsuda et al., 2003).

I also performed parallel experiments looking for an interaction with MitF and CBP/p300, but could not detect any interactions between these factors in my system. This suggested that perhaps CBP/p300 was interacting with Sox9 alone as a co-activator.

Figure 3.23: CBP/p300 binds to Sox9^{K61,365R} but not to Sox9^{K61,365R}/SUMO-1 in *Xenopus* embryos.

Co-immunoprecipitation depicting interactions between CBP and Sox9^{K61,365R} and p300 and Sox9^{K61,365R}. Neither CBP nor p300 interact with Sox9^{K61,365R}/SUMO-1, however. The immunoprecipitation antibody was a Rabbit IgG negative control, CBP or p300. The immunoblot antibody was α-flag. Inputs were monitored via western blot, probed with the α-flag antibody. α-actin was used as a loading control. The inputs and loading controls are depicted in the lower two blots.

Figure 3.23


Sox9K61,365R + + +
Sox9K61,365R/SUMO-1 + + +
(-) antibody + +
(+) anti-CBP + +
(+) anti-p300 + +
IP: (-), CBP, or p300
IB: Sox9 (flag)
IB: Sox9 (flag)
anti-actin

However, I felt that the mere inhibition of co-activator recruitment could not inhibit synergy to such a great degree. So, I then turned to look for potential partner proteins that would lead to the inhibition of synergy on the Dct promoter. I started by examining interactions with the transcription factor Pax3, a paired domain protein, which plays a role in neural crest development. This was an attractive candidate for many reasons. First, Lang and Epstein demonstrated a physical interaction between Sox10 and Pax3 (Lang and Epstein, 2003). Then, Lang and colleagues demonstrated that Pax3 could compete with MitF for binding to the Dct promoter and subsequently inhibit synergistic activation of the Dct promoter. However, if a Wnt signal was present (ß-catenin), Pax3 no longer bound to the promoter and MitF was then able to occupy the Dct promoter and promote transcription (Lang et al., 2005).

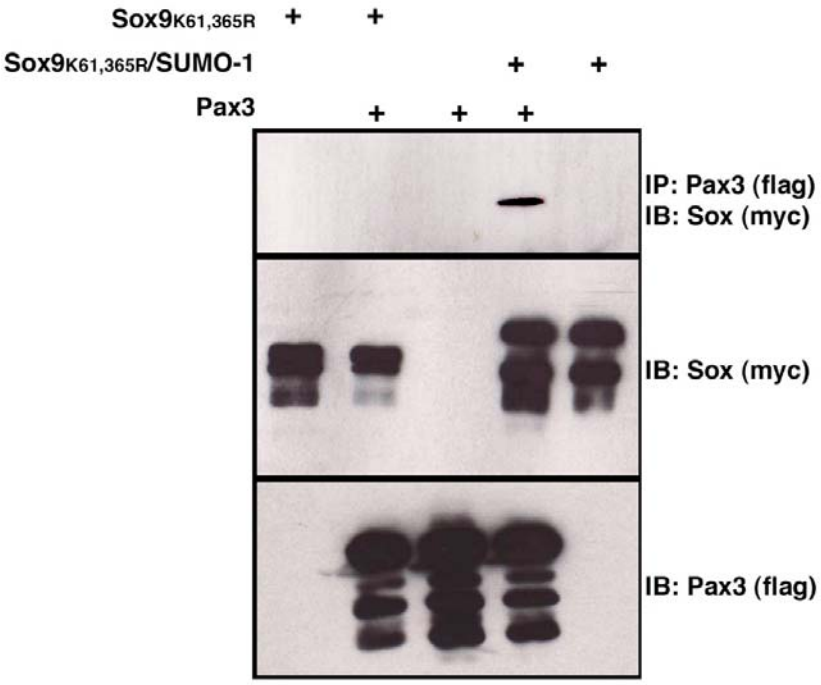
However, since the time in which the physical interaction between Sox10 and Pax3 was published in 2003, I have not been able to repeat this interaction with Sox10 or Sox9 by misexpressing these constructs in *Xenopus* embryos. Upon recent data showing that the SUMOylation state of Sox9 was important to the interaction with MitF and CBP/p300, I decided to investigate whether the SUMOylation status of Sox9 had an impact on the interaction with Pax3. In fact, it did. In performing the co-immunoprecipitation assays as described above, I saw that only the constitutively SUMOylated form of Sox9 could interact with Pax3, while $Sox9^{K61,365R}$ could not interact with Pax3 in *Xenopus* embryos (Figure 3.24).

However, this data did not fit in with previously published data. Thus far, I have shown that both MitF and Pax3 interact with $Sox9^{K61,365R}$/SUMO-1. However, Lang and colleagues showed in chromatin immunoprecipitation assays that when a Wnt signal is not present, Pax3 competes with MitF for binding to the Dct promoter (Lang et al., 2005). If Pax3 is located on the promoter and also binding to $Sox9^{K61,365R}$/SUMO-1 as I recently discovered, then my data, unlike

Figure 3.24: Pax3 cannot bind to Sox9^{K61,365R} but can bind to Sox9^{K61,365R}/SUMO-1 in *Xenopus* embryos.

Co-immunoprecipitation depicting an interaction between Pax3 and Sox9^{K61,365R}/SUMO-1. Sox9^{K61,365R}, however, could not interact with Pax3. The immunoprecipitation antibody was α-flag and the immunoblot antibody was α-myc. The input levels, shown in the lower two blots, were monitored by Western blot using either the α-myc or α-flag antibodies.

Figure 3.24


Sox9K61,365R + +
Sox9K61,365R/SUMO-1 + +
Pax3 + + +
IP: Pax3 (flag)
IB: Sox (myc)
IB: Sox (myc)
IB: Pax3 (flag)

the chromatin immunoprecipitation data from the Epstein lab, indicates that MitF may be present in this complex as well.

There are a few explanations for this. One potential problem with chromatin immunoprecipitation assays is the fixation time. If the cells are not fixed long enough, then factors that are associated with other factors that are doing the actual DNA binding can be washed away. Perhaps this could explain why I see MitF forming a proposed complex with Pax3 and Sox9^{K61,365R}/SUMO-1. It is possible that Pax3 displaces MitF, but MitF still physically associates with Sox9^{K61,365R}/SUMO-1. However, if this were the case, I would expect to see an interaction between Pax3 and MitF, unless however, my immunoprecipitation assays were too stringent to show multi-complex interactions. I have performed co-immunoprecipitation assays with these factors and found there to be an extremely weak interaction present (data not shown). To further examine the interactions with Pax3, MitF and Sox9^{K61,365R}/SUMO-1, I would need to perform chromatin immunoprecipitation (ChIP) assays. However, these assays are extremely difficult to perfect and I have thus far not been able to perform them on my Dct promoter. The alterative hypothesis, of course, is that these factors are interacting at a different time and place other than on the Dct promoter. The ChIP assays would resolve this question.

In a more complex twist to this mechanism, Lang and colleagues also found that Groucho4 played a role on this promoter. Groucho4 is a non-DNA binding co-repressor that binds to co-factors and can turn them from performing an activating role into a repressive role (Cai et al., 2003; Eberhard et al., 2000; Valentine et al., 1998). As depicted in Figure 3.5, the Dct promoter also contains a Wnt-responsive Lef binding site and this group found that when a Wnt signal is not present, Lef-1 and Pax3 both interact with and recruit Groucho4 to the Dct promoter and this binding resulted in an inhibition of Dct activation (Lang et al., 2005). They

went on to show that when a Wnt signal was present (ß-catenin), Groucho4 was displaced from the promoter, along with Pax3, thereby allowing proper MitF binding and henceforth Dct activation (Lang et al., 2005). I wanted to investigate whether the SUMOylation status of my Sox9 protein impacted an interaction with this co-repressor. This was a very interesting hypothesis, as SoxE factors have not been shown to associate with repressors.

In fact, I found that in performing the co-immunoprecipitation assays as described above, I saw that only the constitutively SUMOylated form of Sox9 could interact with Groucho4, while $Sox9^{K61,365R}$ could not interact with Groucho4 in *Xenopus* embryos (Figure 3.25). This data suggests that when a Wnt signal is not present, the SUMOylated form of Sox9 is recruited to the Dct promoter and forms a complex with Pax3, Groucho4, and presumably, Lef1. When a Wnt signal is present, then perhaps Pax3, Groucho4 and the SUMO-1 moiety are displaced from the Dct promoter. Then, MitF, Sox9, and CBP/p300 can bind to the Dct promoter and properly activate transcription.

I wanted to understand what made the constitutively SUMOylated form of Sox9 associate with Groucho4. I postulated that it was the presence of the SUMO moiety that was recruiting Groucho4 to the promoter. In order to test this hypothesis, I first wanted to know whether SUMO-1 physically interacted with Groucho4. I performed GST pull down assays in a manner analogous to that described above and found that Groucho4 could not associate with SUMO-1 alone (Figure 3.26). I then wanted to investigate whether the SUMOylated Sox9 was recruiting Groucho4 to the Dct promoter. Because I could not investigate this phenomenon through ChIP assays, I co-injected SUMO-1 alongside of either wildtype Sox9 or Sox9 without the DNA binding domain (Sox9ΔHMG) into *Xenopus* embryos and performed Western blot analysis on the samples. This would tell me if Sox9 needed to be bound to DNA in order to

Figure 3.25: Groucho4 physically interacts with Sox9^{K61,365R}/SUMO-1 but not to Sox9^{K61,365R} in *Xenopus* embryos.

Co-immunoprecipitation depicting interaction between Groucho4 and Sox9 ^{K61,365R}/SUMO-1. Groucho4 does not interact with Sox9^{K61,365R}, however. The immunoprecipitation antibody was a α-flag. The immunoblot antibody was α-myc. Inputs, pictured in the lower two blots, were monitored via western blot, probed with the α-flag or α-myc antibody.

Figure 3.25

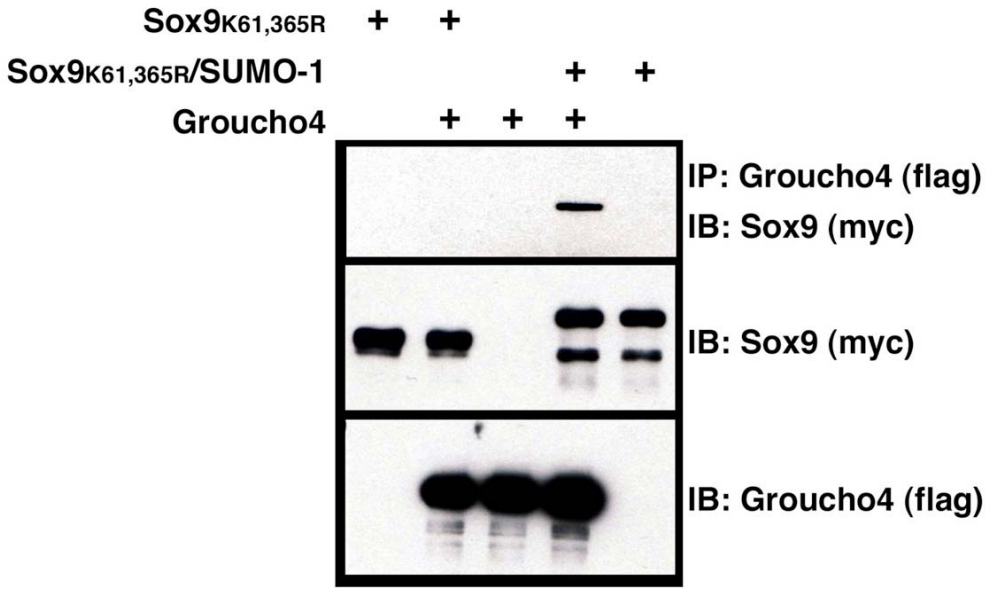
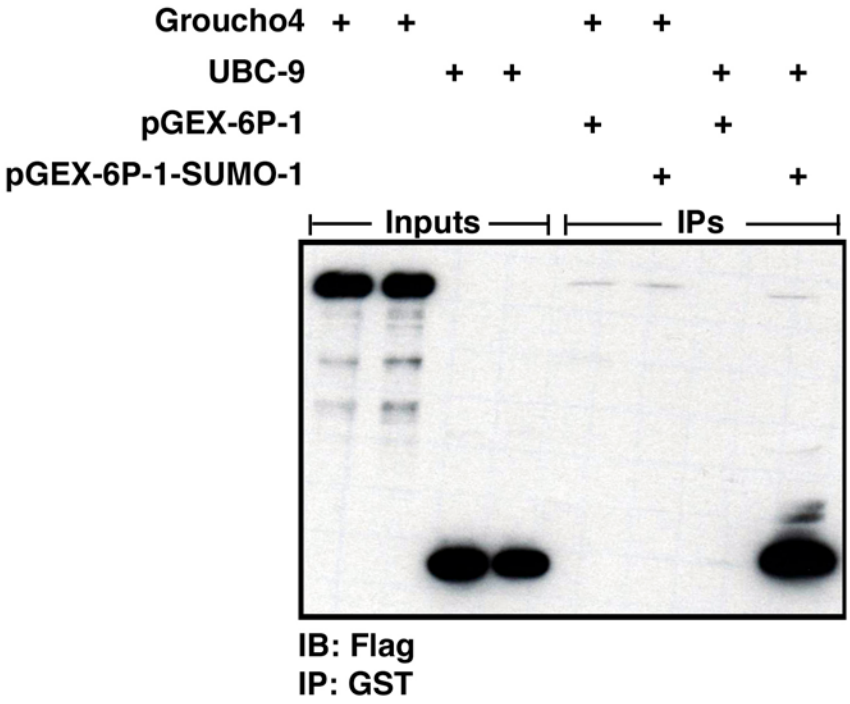

Sox9K61,365R + +
Sox9K61,365R/SUMO-1 + +
Groucho4 + + +
IP: Groucho4 (flag)
IB: Sox9 (myc)
IB: Sox9 (myc)
IB: Groucho4 (flag)

Figure 3.26: Groucho4 does not physically interact with SUMO-1.

GST pull-down assays demonstrating that Groucho4 does not bind to SUMO-1. Input lanes show Groucho4 and UBC9 protein is present by immunoblotting with α-flag antibody. Pull down lane proteins were incubated with GST alone (pGEX-6P-1) or GST-SUMO-1 (pGEX-6P-1-SUMO-1). Immunoblot was carried out with the α-flag antibody. UBC9 demonstrates a clear interaction with SUMO-1, as there is a band present in the + pGEX-6P-1-SUMO-1 lane, but not in the vector alone lane. Groucho4, however, does not interact with pGEX-6P-1-SUMO-1.

Figure 3.26


Groucho4 + + + +
UBC-9 + + + +
pGEX-6P-1 + +
pGEX-6P-1-SUMO-1 + +
Inputs
IPs
IB: Flag
IP: GST

become SUMOylated. The result of this experiment demonstrates that the amount of SUMOylated species in the wildtype + SUMO embryos is much higher than that of Sox9ΔHMG + SUMO embryos (Figure 3.27). This data suggests that the majority of SoxE factor SUMOylation occurs at the level of DNA binding and provides evidence that the recruitment of SUMO-1 by Sox9 on the DNA is a mechanism used by the protein to regulate transcriptional events. The mechanistic model encompassing all of these interactions is presented in Figure 3.28.

The above model is further supported by evidence I obtained in luciferase assays done with Pax3 and Groucho4. I misexpressed MitF^{K182,316R} and Sox9^{K61,365R} with or without Pax3 in *Xenopus* embryos and compared their activities by luciferase assays on the Dct promoter. I found that when Pax3 was introduced into the embryo with the double lysine mutants, the transcriptional synergy seen on the Dct promoter was reduced from a level of 45-fold activation to approximately 5-fold (Figure 3.29). I also wanted to confirm that the presence of Groucho4 would show a decrease in transcriptional synergy when present on the Dct promoter. I performed these assays in a similar manner to my other luciferase assays, but the graph reads as a percentage decrease from 100%. I made the synergy seen with Sox9 + MitF equal to 100% and then when Groucho4 was introduced, this synergy was inhibited, showing a 60% drop in activation of the Dct promoter (Figure 3.30). When Groucho4 was co-injected alongside of the Sox9 and MitF double lysine mutants, I did not see a significant drop in activation (Figure 3.30). If it is the SUMO moiety that is recruiting Groucho4 to the promoter, then this data makes sense because Sox9^{K61,365R} cannot be SUMOylated and would not be able to pull Groucho4 to the Dct promoter to create any inhibitory effects. Important to note is that the Groucho4 luciferase assays were performed prior to *Xenopus* gastrulation, as I wanted to eliminate as much

Figure 3.27: The amount of SUMOylated Sox9 decreases when the DNA binding domain is not present.

Either Sox9 + SUMO-1 or Sox9ΔHMG + SUMO-1 were co-injected into *Xenopus* embryos. Western blot analysis on these embryos shows that while the Sox9 and Sox9ΔHMG unmodified proteins are equivalently expressed, the amount of SUMOylated species present in the full-length Sox9 embryos is much greater compared to the embryos injected with Sox9 missing the DNA binding domain.

Figure 3.27

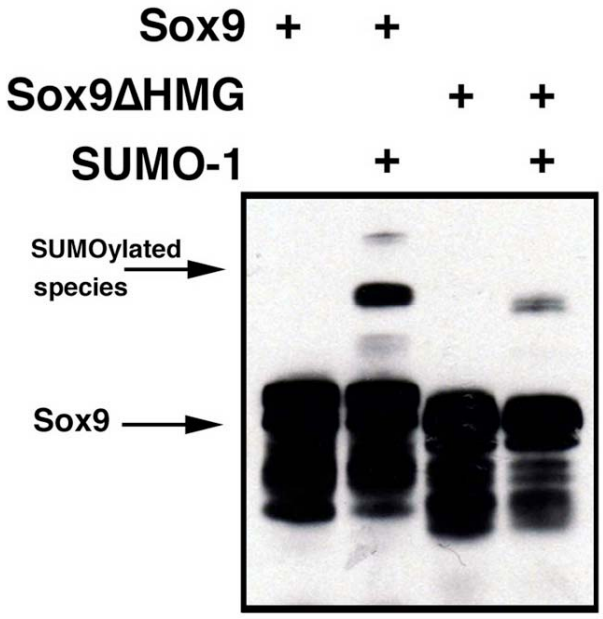


Figure 3.28: Mechanistic model showing transcriptional synergy and inhibition of transcriptional synergy on the Dct promoter.

Hypothetically model suggesting that when Sox9 is not SUMOylated and a Wnt signal is perhaps present, CBP/p300 can freely bind to Sox9 and contribute to the synergistic activation of the Dct promoter. However, when Sox9 is SUMOylated on this promoter and no Wnt signal is present, $Sox9^{K61,365R}$/SUMO-1 can no longer bind to CBP/p300 and instead recruits Groucho4 and Pax3 to the promoter which inhibits synergy on this promoter. The binding of $Sox9^{K61,365R}$/SUMO-1 and Pax3 may also displace MitF from the promoter. All of these changes lead to the inhibition of synergy between MitF and Sox9 to activate Dct transcription.

Figure 3.28

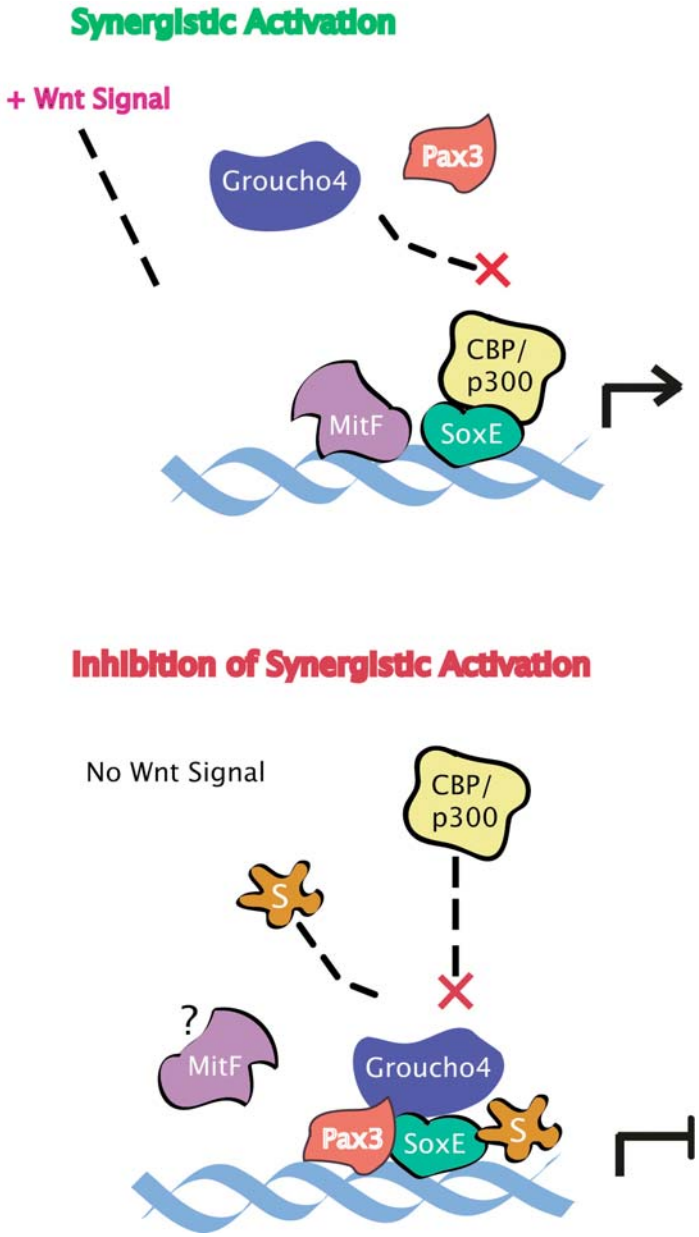

Synergistic Activation
+ Wnt Signal
Groucho4
Pax3
CBP/
p300
MitF
SoxE
Inhibition of Synergistic Activation
No Wnt Signal
CBP/
p300
S
?
MitF
Groucho4
Pax3
SoxE
S

Figure 3.29: Pax3 inhibits transcriptional synergy between Sox9^{K61,365R} and MitF^{K182,316R} on the Dct promoter.

Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. While the misexpression of unlinked Sox9^{K61,365R} + MitF^{K182,316R} shows synergistic activation on the Dct promoter (~45-fold above basal), misexpression of Pax3 with MitF^{K182,316R} + Sox9^{K61,365R} inhibits transcriptional synergy on the Dct promoter (~5-fold above basal).

Figure 3.29

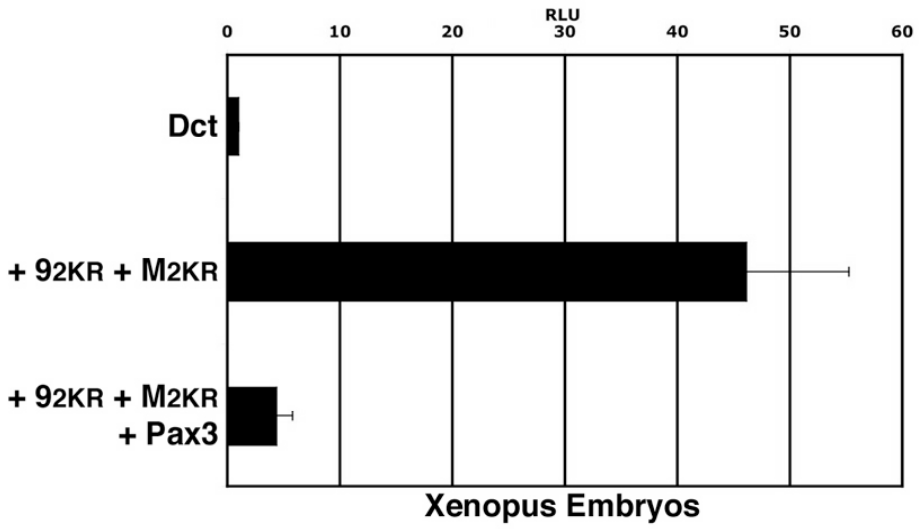
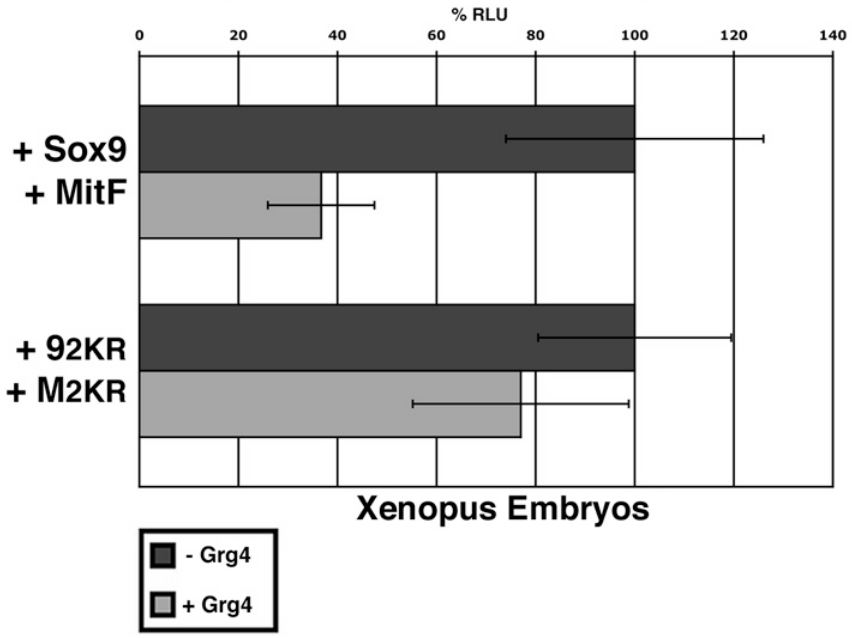

RLU
0
10
20
30
40
50
60
Dct
+ 92KR + M2KR
+ 92KR + M2KR
+ Pax3
Xenopus Embryos

Figure 3.30: Groucho4 inhibits transcriptional synergy between Sox9 and MitF, but not Sox9^{K61,365R} and MitF^{K182,316R} on the Dct promoter.

Relative luciferase activities were calculated using the activity of Dct alone in *Xenopus* embryos as a control. The synergy between Sox9 and MitF was then set to 100%. The co-expression of Groucho4 with Sox9 and MitF produced 60% less activation on the Dct promoter. The synergy between Sox9^{K61,365R} and MitF^{K182,316R} was also set to 100%. The co-expression of Groucho4 with MitF^{K182,316R} and Sox9^{K61,365R} produced ~20% less activation on the Dct promoter, however, the error bars produced demonstrate that this latter number is not statistically significant.

Figure 3.30


% RLU
0
20
40
60
80
100
120
140
+ Sox9
+ MitF
+ 92KR
+ M2KR
Xenopus Embryos
- Grg4
+ Grg4

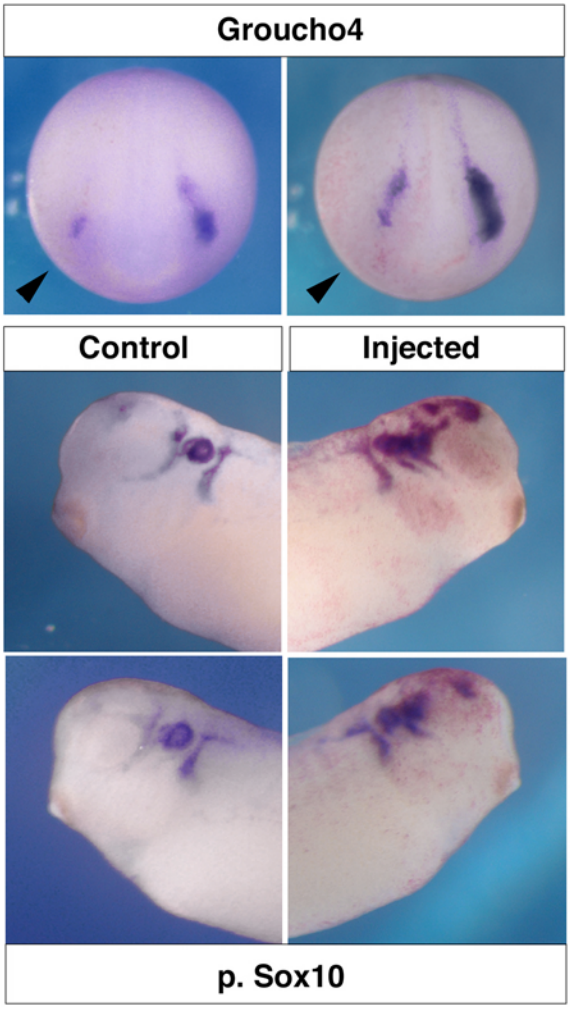
interference as possible in the embryo. Clearly, as Groucho4 is a co-repressor in many different systems, misexpressing it in the embryo could lead to potential complications. It would have been beneficial to perform luciferase assays in the presence of CBP/p300 as well, however, I did not have access to this DNA construct. I would have expected that co-injection of CBP/p300 with the Sox9 and MitF double lysine mutants would have produced even greater synergy than seen without the addition of CBP/p300.

If the SUMOylation of Sox9 is a mechanism used to recruit the co-repressor, Groucho4 to the Dct promoter and inhibit transcriptional synergy of this promoter, it was possible that SoxE factors use this as a general mechanism to control other promoters/cell types. The Groucho4 data I obtained was particularly interesting given that a previous paper had shown that the misexpression of Groucho4 gave rise to enlarged or ectopic otocysts in zebrafish (Bajoghli et al., 2005). Interestingly, when I misexpress constitutively SUMOylated Sox9 in the embryo, I also see enlarged or ectopic ears forming (Figure 2.4). Due to this overlap in function, I wanted to investigate the effects of misexpressing Groucho4 in *Xenopus* embryos. The misexpression of Groucho4 led to two very interesting phenotypes. First, I saw a clear inhibition of neural crest precursors at neurula stages, and second, I saw the formation of ectopic otocysts at tailbud stages (Figure 3.31). This data clearly mimicked the phenotypes seen with misexpression of constitutively SUMOylated Sox9 (Figure 2.10 and Figure 2.14). Also important to note is that there are no ectopic melanocytes forming on the flank of these embryos, which also correlates with the biochemical and luciferase assay data. Given the data demonstrating that Sox9 is predominantly SUMOylated when Sox9 is bound to DNA, Groucho4 interacts only with constitutively SUMOylated Sox9, and that misexpression of Groucho4 leads to the same phenotype as constitutively SUMOylated Sox9, I hypothesize that the embryo uses the

Figure 3.31: Misexpression of Groucho4 leads to an inhibition of neural crest precursors at neurula stages and ectopic otocysts at tailbud stages in *Xenopus* embryos.

Misexpression of Groucho4 in *Xenopus* embryos inhibits neural crest precursor formation, as depicted by the loss of the neural crest precursor marker, Sox10, in the *in situ* hybridizations shown in the top panel. Later, at tailbud stages, the bottom four pictures demonstrate that the misexpression of Groucho4 leads to ectopic ear formation, as depicted in whole mount *in situ* hybridizations performed with the glial, melanocyte, and otocyst marker, Sox10.

Figure 3.31



SUMOylation of Sox9 as a mechanism to recruit the co-repressor Groucho4 and any number of other factors to a particular promoter when the embryo does not want to produce any more transcript from that gene. I also hypothesize that the effects seen on the ear by these two constructs is secondary to the primary inhibition of the neural crest. Perhaps when these two factors work together to inhibit transcription both at early and late neural crest stages, the competency zone for ear formation is enlarged, leading then to the ectopic and enlarged ear formation seen in the *Xenopus* embryos. These data clearly demonstrate a mechanism by which SUMOylation of Sox9 dynamically regulates cell type formation in the developing embryo.

Discussion

The work presented in this chapter demonstrates several new important findings with respect to how the Dct promoter is regulated by SoxE factors and MitF. Previous data presented by other researchers showed that MitF and Sox10 could synergistically activate the Dct promoter and a form of MitF that could not be SUMOylated led to an increase in this synergy (Jiao et al., 2004; Ludwig et al., 2004; Murakami and Arnheiter, 2005). I was interested in looking at the transcriptional control of the Dct promoter for several reasons. The first being that I had shown SoxE factors to also be SUMOylated and thought perhaps the SUMOylation of these two factors would have an impact on melanocyte development. I had previously seen an increase in melanocyte formation with misexpression of a Sox9 or Sox10 construct that could not be SUMOylated and lack of ectopic melanocyte formation with misexpression of a Sox9 or Sox10 construct that was constitutively SUMOylated. The second reason being that I wanted to provide further evidence demonstrating that Sox9 could perform tasks normally reserved for Sox10 in the developing embryo.

In the first portion of this chapter, I have shown that MitF SUMOylation mutants have similar effects on melanocyte formation as do their counterpart SoxE SUMOylation mutants (as was demonstrated in Chapter 2). While misexpression of MitF and MitF^{K182,316R} can give rise to ectopic Dct expression, indicating an abundance of melanocyte precursors, misexpression of MitF^{K182,316R}/SUMO-1 cannot perform this task in the embryo. This data told me that perhaps the presence or absence of SUMOylated proteins on the Dct promoter was a mechanism used by this promoter to control melanocyte development.

I then went on to demonstrate that misexpression of either SoxE double lysine mutant in combination with misexpression of the MitF double lysine mutant gave rise to ectopic Dct expression on the flank of the embryo in a synergistic manner. This was the first piece of data in this chapter that again verified Sox9 could perform equivalently to Sox10 with respect to melanocyte development. Had Sox9 not been able to perform equivalently, I would have seen a difference in the ectopic Dct phenotype in these embryos in comparison to their Sox10 counterparts, which I did not.

I then further demonstrated that the MitF double lysine mutant was sufficient to induce the melanocyte differentiation program by turning on Dct expression in animal cap explants. This data had never been shown before and truly confirms MitF as a master regulator of initiating melanocyte formation. Interestingly, however, while this construct alone is sufficient to turn on Dct expression, it does not appear sufficient to form actual melanocytes in these animal caps. This data indicates that at least one further downstream component is missing to complete the melanocyte differentiation program.

Furthermore, dual misexpression of Sox9 and MitF double lysine mutants in animal cap explants also showed synergistic ectopic Dct expression. However, the constitutively

SUMOylated form of Sox9 could block ectopic Dct expression when misexpressed in combination with MitF^{K182,316R}. This data demonstrated that the presence of a SUMO-1 moiety could at least partially inhibit the function of MitF in these animal caps, suggesting that SUMOylation of these factors could indeed be a mechanism used by the embryo to control for an improper number of melanocytes from forming.

The data summarized above was also recapitulated in luciferase assays and further confirmed that the SUMOylation status of Sox9 and MitF was extremely important to the regulation of this promoter. Misexpression of the Sox9 and MitF double lysine mutants led to synergistic activation of this promoter and the presence of one constitutively SUMOylated mutant inhibited this synergy. This luciferase data also led to the second piece of evidence suggesting that Sox9 could perform equivalently to Sox10 in melanocyte development, as it can promote synergistic activation on the Dct promoter in combination with MitF.

Until this point, all of the data I had obtained told me that SUMOylation was important to Dct promoter regulation, but it did not tell me why it was important. I wanted to understand the exact mechanism used by SUMOylation to control this synergy. SUMOylation has been found to regulate transcriptional activation in many ways. It can prevent proper DNA binding of specific transcription factors necessary to the activation of a promoter, it can recruit HDAC activity to make the DNA so tightly wound that no factors can bind properly, it can alter subcellular localization of those proteins important for activation, or it can alter the binding of different co-factors that might be necessary to or inhibit the activation of a specific promoter (reviewed in (Hay, 2005)). In this chapter, I have ruled out the first two options. SUMOylation of MitF and Sox9 is not promoting HDAC recruitment or sterically interfering with binding to the Dct promoter.

However, I found some interesting data with respect to the other two options mentioned above. I found that the Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1 mutants had different, but complex cellular localization patterns. Both factors appeared to be shuttling back and forth from the nucleus to the cytoplasm. This data makes sense, as the Sox9 protein is encoded with both nuclear import and nuclear export signals.

It was the subnuclear data that was perplexing. Sox9^{K61,365R} appeared to have a punctate staining pattern, located within very small subnuclear bodies. While punctate staining patterns exist, such a staining pattern has been better demonstrated for SUMOylated proteins. These SUMOylated factors can become trapped in either small PML bodies or larger SNBs. Sox9^{K61,365R}/SUMO-1 appeared to have both a small punctate pattern, as well as larger punctate pattern. This was perhaps indicative of both PML body and SNB inhabitance. Interestingly, PML bodies act as repressive bodies, but given the data described in this chapter, I do not feel that this is a likely spot for my SUMOylated Sox9 protein to be located. It is also possible that the SUMOylated Sox9 species could be dually located in the SNBs. A function of the SNB is to act as a site for active SUMOylation. However, the Sox9^{K61,365R}/SUMO-1 protein has both of its internal SUMOylation sites mutated and has a SUMO-1 moiety attached to the end of the protein. This would suggest to the cell that this protein is already SUMOylated and would not need to be recruited to the SNBs. There is always the possibility that perhaps the SUMO-1 moiety itself could act as an attractant for other SUMO-1 moieties. While, the formation of SUMO chains has only been found to occur with SUMO-2 and SUMO-3, and not with SUMO-1, that does not mean it is not possible (Tatham et al., 2001). The subnuclear differences between the locations of the double lysine mutant and the constitutively SUMOylated mutant are extremely interesting and warrant further investigation.

After sifting through the above data, I felt that the most likely way in which SUMOylation was regulating Sox9 was by inhibiting the recruitment of a co-activator or promoting the binding of a co-repressor. The three factors I chose to look at were Pax3, Groucho4, and CBP/p300. I found that only $Sox9^{K61,365R}$ but not $Sox9^{K61,365R}$/SUMO-1 could interact with the co-activator, CBP/p300. This and other work suggests that Sox9 recruits CBP/p300 to the Dct promoter through its C-terminal activation domain (Tsuda et al., 2003), and works together with MitF to synergistically activate Dct. When Sox9 is SUMOylated, however, the site of CBP/p300 interaction on Sox9 may become covered up. The most plausible explanation for how this occurs is that the SUMOylation of Sox9 is causing a conformational change in this interaction region that will not allow proper co-activator binding.

I also found that Pax3 and the co-repressor Groucho4 could only interact with $Sox9^{K61,365R}$/SUMO-1 and not $Sox9^{K61,365R}$. This data, together with the data showing that Sox9 needs to be able to bind to DNA in order to be efficiently SUMOylated, suggested that when a SUMO-1 moiety was present, Groucho4 and Pax3 are recruited to the Dct promoter and do not allow synergistic activation of the Dct promoter, possibly by displacing the proper binding location of MitF. I then went on to show that misexpression of Groucho4 in the embryo leads to identical phenotypes as the misexpression of constitutively SUMOylated Sox9. They can both lead to the inhibition of neural crest precursors and to an enlargement of or ectopic ear formation. This suggests a more broad mechanism of transcriptional control by the SUMOylation of Sox9. My hypothesis is that, with respect to the neural crest and the derivatives formed by the neural crest, SoxE factors become SUMOylated on SoxE-specific promoters and recruit Groucho4 to these promoters in order to turn down transcription when necessary. This would allow for the proper defining of the neural crest-related competency zones. However,

when constitutively SUMOylated Sox9 is misexpressed, the neural crest forming region is further reduced, allowing for other cell types, such as the ear, to invade and expand into this territory.

In the data presented thus far in this thesis, I have shown that SUMOylation of SoxE factors impacts several different aspects of development. However, SUMOylation is not the only way in which these factors are managed and in Chapter 4, I will discuss various other ways that SoxE factors may be regulated throughout development.

CHAPTER 4

OTHER FORMS OF SOXE REGULATION

Introduction

The previous two data chapters are based on results that are or will be in published journal format. This chapter includes additional data I have gathered over the course of my thesis work that has not been formally published. In the first section of this chapter, I will describe work I have done that shows Sox10 to be post-translationally modified via phosphorylation. Thus far, Sox10 phosphorylation has not been published, nor has Sox8. However, Sox9 has been shown to be phosphorylated by Protein Kinase A (PKA) at two sites (Huang et al., 2000; Malki et al., 2005). Neither of these two sites overlaps with my hypothesized sites described below. Huang et al found that the phosphorylation of Sox9 by PKA led to an increase in DNA binding as well as transcriptional activity (Huang et al., 2000). Malki et al found that phosphorylation at these two sites in Sox9 enhances nuclear localization of the protein (Malki et al., 2005). Interestingly, the novel, putative phosphorylation area that I have found in Sox10 is conserved in Sox8 and Sox9. This conservation indicates a potentially important site for protein regulation.

SoxE factors are also known to use partner proteins to enhance and regulate their transcriptional responses in different cell types. I began to investigate potential novel partner proteins by performing a yeast two-hybrid screen, using Sox10 as the bait. As previously mentioned in Chapter 2, I pulled both UBC9 and SUMO-1 out of this screen. I also pulled out three other potentially interesting factors that I will describe in the second section of this chapter.

The different conserved domains of SoxE proteins were also of great interest to me. Interestingly, Aoki and colleagues showed that a construct consisting only of the E2 domain and activation domain of Sox10 could perform equivalently to full-length Sox10 with respect to early neural crest precursor formation (Aoki et al., 2003). I wanted to investigate this claim, as well as

identify what other domains were imperative for SoxE function throughout the development of both the neural crest and the ear. To do this, I created a number of deletion constructs, whose functional data I will describe in the third section of this chapter.

Methods

DNA Constructs and PCR-directed mutagenesis

The Sox10$_{T221,224,225A(or\ D)}$ mutant was generated using overlapping, mutation-containing PCR primers. From the full-length, wildtype *XSox10* cDNA, primer set one amplified Sox10 amino acids 1-225 and primer set two amplified amino acids 221-438. The products of these two PCRs were then mixed together and then the entire length of the Sox10 DNA was amplified in order to include the mutations created. All PCRs performed used low copy number PCR and a high-fidelity polymerase (Tgo: Roche, Indianapolis, IN). These cDNAs were then cloned into a pCS2 variant that adds five C-terminal myc tags (gift of R. Davis) and confirmed by sequencing.

Embryo Methods

All injection, collection, and *in situ* hybridization of *Xenopus* embryos were performed as stated in Chapter 2 methodology. The constructs for making the *X*CG-1 probe and Pax8 probes were provided by Hazel Sive and A. Brandli, respectively.

Western blot Assays

Wildtype or mutant Sox10 proteins were expressed in *Xenopus* embryos and collected in sets of five at late blastula stages. Embryos were lysed in Lysis Buffer (1xPBS, 1%NP40),

supplemented with phenylmethylsulfonyl fluoride and an inhibitor tablet (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets; Roche; Indianapolis, IN). Samples were resolved on SDS-PAGE and proteins were detected using an antibody against the myc epitope tag at 1:3000 (Myc: 9E10; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody (at 1:5000) was horseradish peroxidase coupled and detected by chemiluminescence (Pierce, Rockford, IL).

In vitro *translation*

Sox10 mRNA (Message Machine kit; Ambion, Austin, TX) was heat-inactivated for 3 minutes at 70˚C, then mixed with amino acid mix –methionine, RNAsin, Rabbit Reticulocyte Lysates and ^{35}S-Methionine. After a 90-minute incubation at 30˚C, the samples were resolved on SDS-PAGE. The gel was then fixed, rinsed in Sodium Salicylate and dried. Proteins were then detected by autoradiography.

Lambda Phosphatase assay

Wildtype or mutant Sox10 proteins were expressed in *Xenopus* embryos and collected in sets of five at late blastula stages. Embryos were lysed in 100 ul of Lysis Buffer (1xPBS, 1% NP40) supplemented with phenylmethylsulfonyl fluoride, an inhibitor tablet (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets; Roche; Indianapolis, IN), aprotinin and leupeptin. To a fresh tube, 12.5 ul of this lysate was then added to a volume of 26.5 ul of Lysis Buffer (with fresh inhibitors), 5 ul of lambda phosphatase buffer, 5 ul of $MnCl_2$, and 1 ul of lambda phosphatase (New England BioLabs, Ipswich, MA) (in the case of the control, 27.5 ul of Lysis Buffer is added due to no lambda phosphatase being added). The samples were incubated at

30˚C for thirty minutes. The samples were then resolved on SDS-PAGE and proteins were detected as stated above in the Western blot protocol.

Kinase assay

Wildtype or mutant Sox10 was expressed in *Xenopus* embryos and collected in sets of five at late blastula stages. The embryos were then lysed in Lysis Buffer (1xPBS, 1% NP40) supplemented with phenylmethylsulfonyl fluoride and an inhibitor tablet (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets; Roche; Indianapolis, IN). Then, IP Buffer was added to the cleared lysates (supplemented with 5mM EDTA, 10mM beta-glycerol phosphate, 2 mM sodium orthovanadate, 20 mM NaF, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). The pull antibody was then added (Myc: 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:500 and incubated at 4˚C for two hours. After this time, 50 ul of protein A-sepharose beads (Sigma-Aldrich) were added to each sample and rocked at 4˚C for two hours. The samples were then washed into Kinase buffer (50mM HEPES pH 7.2, 5mM EGTA, 20mM MgCl2, 1mM DTT, 10mM beta-glycerol phosphate, 2mM sodium orthovanadate, 20mM NaF, 50μM ATP (non-radioactive), 0.5 μM gamma ATP per sample, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). The samples were then incubated for 10 minutes at room temperature. The reaction was quenched with SDS sample buffer. The samples were then resolved by SDS-PAGE and visualized by autoradiography.

Yeast Two-Hybrid screen

The protocol used for this screen was adapted from Current Protocols in Molecular Biology Unit 20.1. The constructs and strains used for this screen can be found in Chapter 2 methodology, as

well as in the results section below. All incubations were done at 30˚C unless stated otherwise. Briefly, the bait, Sox10ΔAD, was subcloned into the pEG202 plasmid and verified through sequencing. The bait was then transformed into the mating strain, EGY48 and plated onto Glucose/Complete Media (Glu/CM) –Ura, -His plates. Glycerol stocks were then made from overnight liquid cultures (Glu/CM -Ura, -His) of the colonies that grew on the drop out plates. Next, a 20 ml liquid culture of bait+mating strain was grown overnight in Glu/CM –Ura, -His. The next morning, the culture was diluted into 300 ml of Glu/CM –Ura-His media to an OD_{600}=0.10 ($2x10^6$ cells/ml). The culture was then incubated until it reached an OD_{600}=0.60 ($1x10^7$ cells/ml). The cells were then spun down at 1500 g for 5 minutes at room temperature. They were then resuspended in 30 ml of sterile water and transferred to a 50 ml conical. The cells were then spun down again at 1500 g for 5 minutes at room temperature. The supernatant was decanted and the cells were resuspended in 1.5 ml of TE buffer/0.1 M Lithium Acetate. Then, 1 ug of library DNA (in pJG4-5 vector) and 50 ug of sheared salmon sperm DNA (Eppendorf, Westbury, New York) was added to each of 30 eppendorf tubes. Then, 50 ul of resuspended yeast solution and 300 ul of sterile 40% PEG4000 was added to each tube and inverted to mix. The tubes were then incubated for 30 minutes. Then, 40 ul of Dimethyl Sulfoxide (DMSO) was added to each tube, mixed, and the samples were then heat shocked for 15 minutes at 42˚C. Each transformation (400 ul) was then plated on a large (4 inch diameter) Glu/CM –Ura-His-Trp dish (30 plates total). The plates were then incubated for several days until growth occurred. Also, on smaller plates, 1:10, 1:100, and 1:1000 dilutions were plated in order to determine transformation efficiency.

Once the plates had fully grown, all 30 plates were put into the 4˚C to harden for four hours. The growth was then scraped down with a cell scraper (with the aid of adding 4 ml of 1xTE to the plate). The plates were then washed again with 2 ml 1xTE. The conicals were then spun down for five minutes at 1500 g, room temperature. The cells were then combined into one conical by being resuspended/rinsed with 25 ml of 1xTE. The final volume of cells was 15 ml. The suspension was then spun down again for five minutes at 1500g, room temperature. The cells were then resuspended in 15 ml of 50% glycerol (want a final of 25% glycerol). The suspension was mixed well, aliquoted into 1 ml samples, and frozen at –80˚C.

Next, the interactor hunt was performed by taking a 1 ml aliquot of library + bait glycerol stock, adding 9 ml Gal/Raff –Ura-His-Trp, and incubating for four hours. On the experimental plates (Gal/Raff –Ura-His-Trp-Leu), the culture was diluted to OD_{600}=0.5 with Gal/Raff –Ura-His-Trp-Leu (giving $1x10^7$ cells/ml). Then, 2x100 ul of this culture was plated onto two plates and incubated for several days. After the plates grew, individual colonies were restreaked on new Gal/Raff –Ura-His-Trp-Leu plates. Glycerol stocks were then made of each individual colony that regrew. Then, rapid isolation of plasmid DNA from yeast was performed (E. Weiss lab protocol-use the high-powered vortexer, do not do this by hand). PCR was performed directly on the plasmid DNA preps to obtain a PCR product of the interactor to send for sequencing. The PCR was performed with the following conditions. The DNA was diluted 1:10, then 0.5 ul of pJG4-5 UP primer (the prey plasmid primer), 0.5 ul of DN primer, 1 ul of dNTPs, 1 ul of diluted DNA, 2.5 ul 10x Taq buffer, 0.5 ul Taq polymerase, 19 ul of sterile water was added. The PCR parameters were as follows: 94˚C for 4’, 94˚C for 30”, 53˚C for 30”, 72˚C for 1’, 72˚C for 5’ and 4˚C forever, for 30 cycles. Then, the PCR product was gel purified and sent for sequencing. The

fragments were then dropped into the pGEM®-T vector (Promega, Madison, WI) so as to be easily used for *in situ* hybridization probes.

If an interactor of further interest appeared, it was retested by shuttling the yeast plasmid isolation product through KC8 cells (*E. coli*) using an electroporation-based transformation, which were plated on LB+ Ampicilin plates. Then, the plasmid of interest was obtained by picking colonies and testing them with the pJG4-5 primers, miniprepping them, and then doing a yeast transformation. Finally, the interaction was retested in the yeast mating strain EGY48 with Sox10ΔADpEG202. If growth appeared on a Gal/Raff –Ura-His-Trp-Leu plate, then the interaction was confirmed as real. If the plates did not grow, then the interaction was not real.

SoxE Deletion Constructs

The SoxE deletion constructs were all created using PCR-based methods. Wildtype *XSox9* or *XSox10* was used as the template for the PCRs. All PCRs performed used low copy number PCR and a high-fidelity polymerase (Tgo: Roche, Indianapolis, IN). All constructs were made in several different steps. For example, the first step to making Sox10ΔHMG was by using a primer set that amplified amino acids 1-86 and a primer set that amplified amino acids 166-438. The primers that sandwiched the HMG area had the BglII restriction site built in (same for all constructs), which adds an arginine and a serine between the fragments. Once these two fragments were digested with BglII and gel purified, they were ligated together in an overnight reaction. The ligation product was then PCR-amplified by using the two outer primers, thereby creating a construct consisting of amino acids 1-86 and 166-438. The primers used for each construct are as follows:

- Sox9ΔE1 (no K61): 100-477 (construct does not contain the first SUMOylation site)
- Sox9ΔE1 (yes K61): 1-65; 100-477 (construct does contain the first SUMOylation site)
- Sox9ΔHMG: 1-101; 181-477
- Sox9ΔE2: 1-225; 307-477
- Sox9ΔAD: 1-306
- Sox9ΔE1, HMG: 184-477
- Sox10ΔE1 (no K44): 85-438 (construct does not contain the first SUMOylation site)
- Sox10ΔE1 (yes K44): 1-50; 85-477 (construct does contain the first SUMOylation site)
- Sox10ΔHMG: 1-86; 166-438
- Sox10ΔE2: 1-209; 291-438
- Sox10ΔAD: 1-333
- Sox10ΔE1, HMG: 169-438

All of these cDNAs were then cloned into a pCS2 variant that adds either five N- or C-terminal myc tags (gift of R. Davis and D. Turner, respectively) or a pCS2 variant that adds three N-terminal Flag tags. All constructs were confirmed by sequencing.

Results

Phosphorylation of Sox10

Upon examining the amino acid sequence of Sox10, I identified a conserved group of threonines within the E2 domain of all SoxE members; Sox10^{T221,224,225}, Sox9^{T236,239,240} and Sox8^{T224,227,228} (Figure 4.1). Being that threonines are the second-most phosphorylated amino acid, I wanted to investigate this conserved area further. These threonines are also surrounded by prolines, which is a common amino acid scenario used by the Ras-MAPK(inase) pathway.

The way in which the Ras-MAPK pathway works is that once a signal binds to a receptor, the receptor becomes phosphorylated leading to the activation of Ras, a small G protein. Ras then binds to and activates the kinase Raf. This then sets off a cascade of phosphorylation, first through the phosphorylation of MAPK Kinase (MAPKK), which in turn phosphorylates and activates MAPK. At this point, MAPK can either phosphorylate/activate other kinases, or travel into the nucleus where it can phosphorylate and activate specific target transcription factors.

Focusing on Sox10, I began to investigate this area by making several point mutations. I made threonine to alanine mutations at these sites in order to silence phosphorylation, Sox10^{T221,224,225A}; and I also made threonine to aspartate mutations at these sites in order to mimic phosphorylation, Sox10^{T221,224,225D}. I began to look at these Sox10 variants by first injecting these myc-epitope tagged constructs in *Xenopus* embryos. I found that the alanine mutant migrated more rapidly on an SDS-PAGE gel than the wildtype, suggesting that this could be a site of post-translational modification, as modifications such as phosphorylation add an additional charge to a protein, thereby making the protein migrate at a slower pace through an SDS-PAGE gel (Figure 4.2A). Upon translating these proteins in an *in vitro* system, the migratory abilities of these factors no longer differed (Figure 4.2B). To examine if this shift was due to phosphorylation, I performed a λ-phosphatase assay on both wildtype- and alanine mutant-injected embryo lysates. Phosphatases are enzymes used to dephosphorylate target substrates, and treatment with this enzyme would dephosphorylate wildtype Sox10. I found that treatment with λ-phosphatase caused the wildtype Sox10 band to collapse to the size of the alanine mutant, suggesting that this area was a modified area of the protein (Figure 4.3). To

Figure 4.1

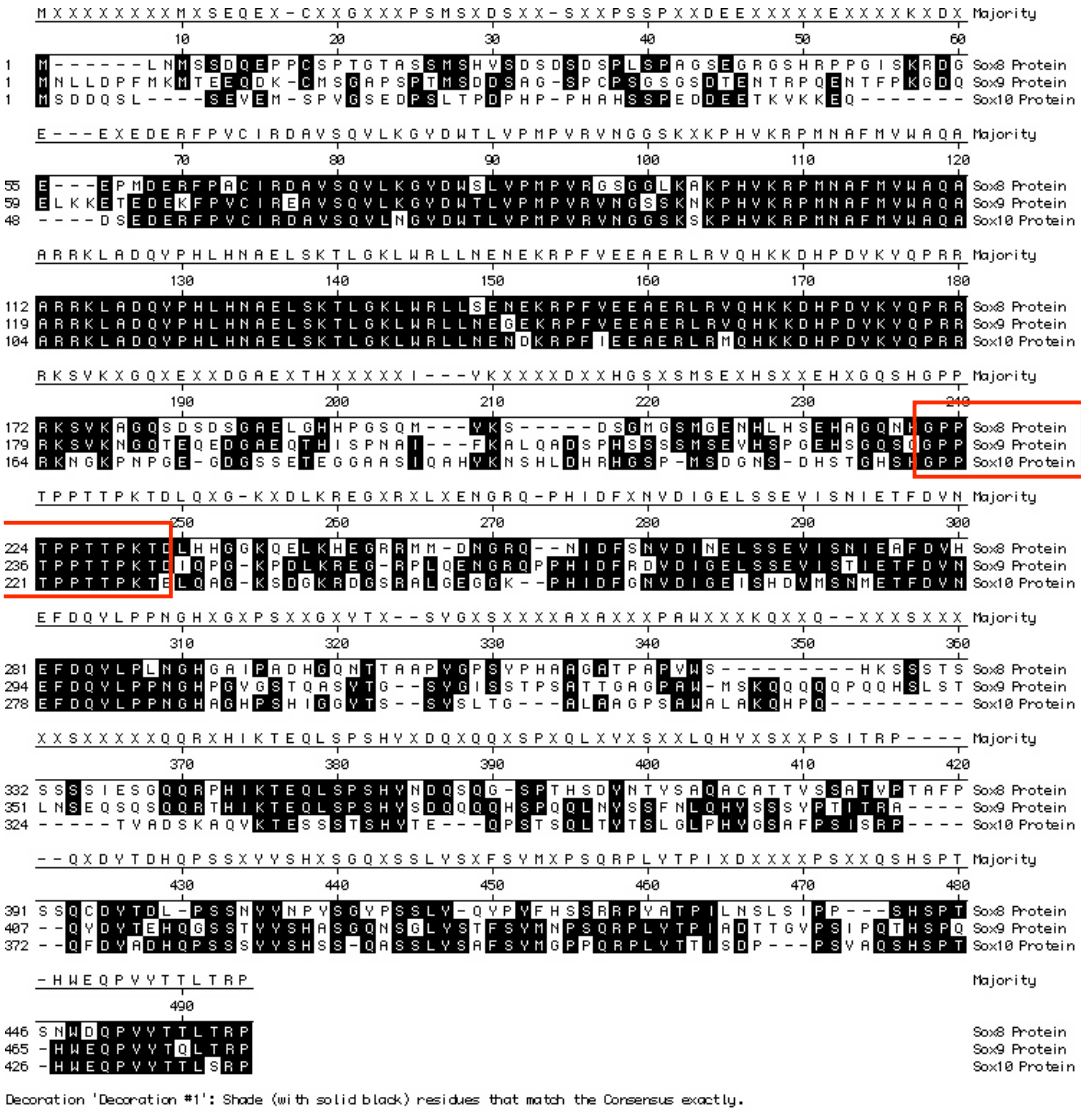


Figure 4.1: SoxE Protein Schematic

The proline-directed threonines highlighted in the red box are a possible area of MAP kinase phosphorylation.

Figure 4.2

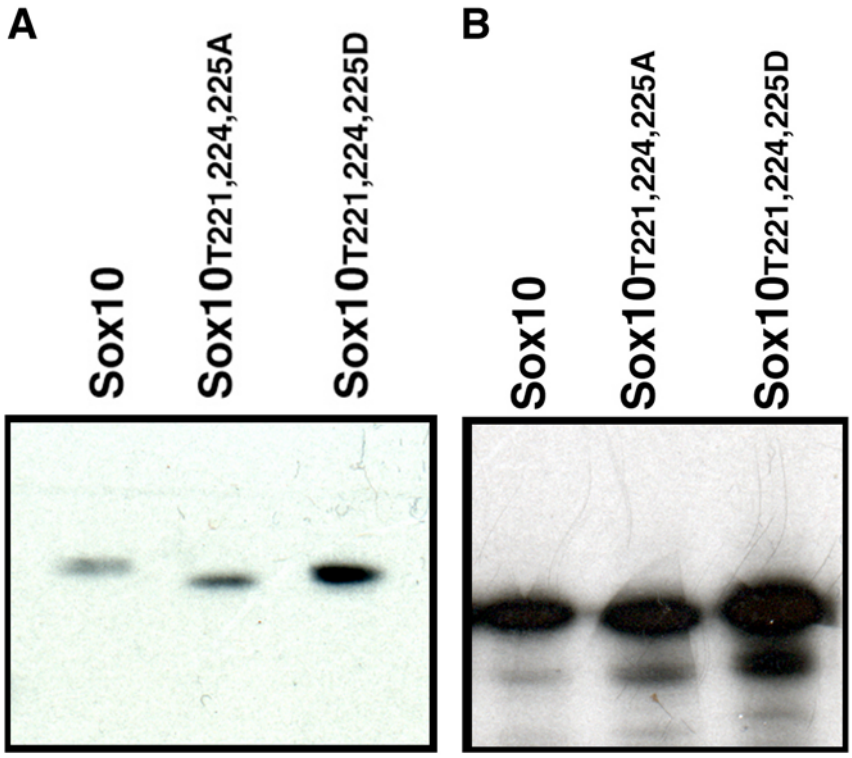


Figure 4.2: *In vivo* translation versus *in vitro* translation of Sox10 and mutants.

A. Microinjection of wildtype Sox10, the alanine mutant or the aspartate mutant into *Xenopus* embryos to allow *in vivo* translation shows varying migration patterns on SDS-PAGE. The wildtype and aspartate mutant migrate more slowly than the alanine mutant, suggesting the presence of a modification. B. Translating the wildtype Sox10, alanine, and aspartate mutants *in vitro* no longer shows a size difference with SDS-PAGE analysis.

Figure 4.3

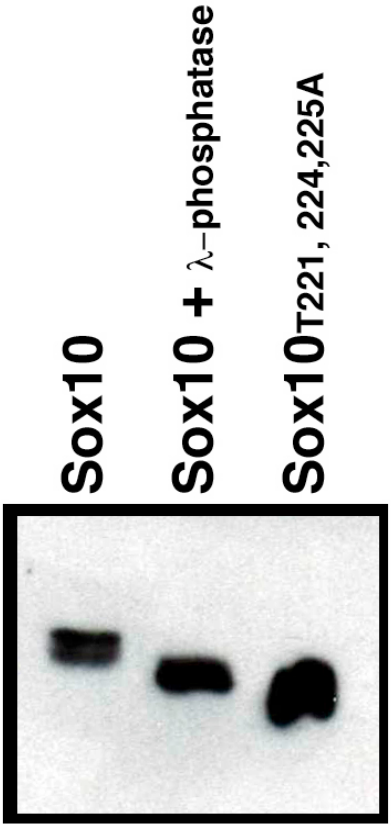


Figure 4.3: λ-phosphatase assay collapses the size difference between wildtype Sox10 and the alanine mutant.

Wildtype Sox10 runs at a molecular weight of approximately 48 kDa on SDS-PAGE (lane 1). The alanine mutant runs at a slightly lower molecular weight (lane 3). Treatment of Sox10 with λ-phosphatase collapses the size of Sox10 to that parallel with the alanine mutant.

ensure that this was in fact a site of phosphorylation, I performed a crushed kinase assay on Sox10 and found that it was phosphorylated, while the alanine mutant could not be phosphorylated (Figure 4.4).

I then moved into embryological studies to observe the phosphorylation-mutant forms of Sox10 that I had created. I first began by analyzing the effects these mutants had on early and late neural crest development. I microinjected 5-50pg of either construct into one cell of the *Xenopus* embryo at the two- or four-cell stage. All constructs were C-terminally myc epitope-tagged and protein levels were monitored by SDS-PAGE to ensure that I was examining equivalent amounts of proteins across the mutant forms. After injection, the embryos were cultured until late neurula stages or neural crest differentiation stages, fixed, and then analyzed by whole mount *in situ* hybridization. To examine the effects of these mutants on neural crest formation, I used a variety of neural crest markers, such as Slug, Sox9 and Sox10. The 3' untranslated regions of Sox9 and Sox10 were used as probes, so as to not cross-react with the coding region that was injected into the embryo.

I have previously demonstrated the varying affects of wildtype Sox10 in Chapter 2, with the most typical effect being an expansion of neural crest precursors and a small fraction showing a decrease in neural crest precursors. Here, I again show varying effects of both the alanine and aspartate mutants with respect to neural crest formation. Each construct varies slightly in their response to different *in situ* hybridization probes. Misexpression of the alanine mutant leads to a slight shift of Sox9-expressing cells more distal than normal from the neural plate and neural crest precursor regions (Figure 4.5A). While these cells do appear to be shifted in comparison to the uninjected side, the neural crest and otic placode are both present,

Figure 4.4

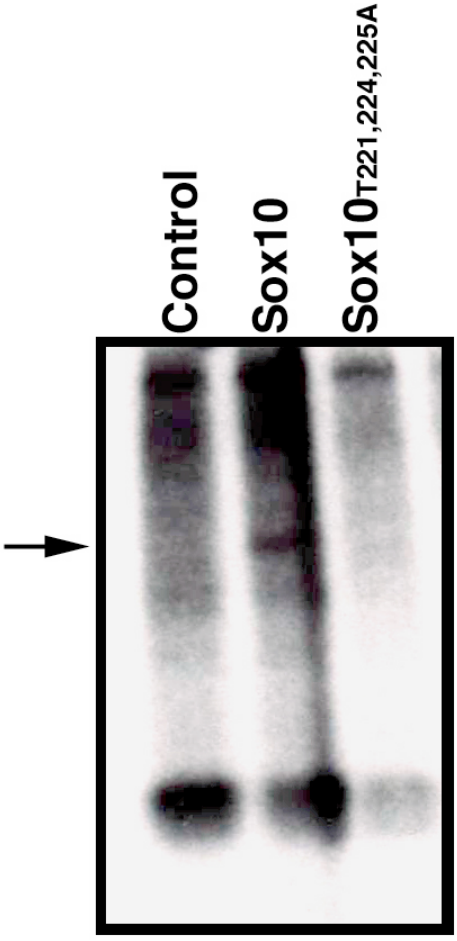


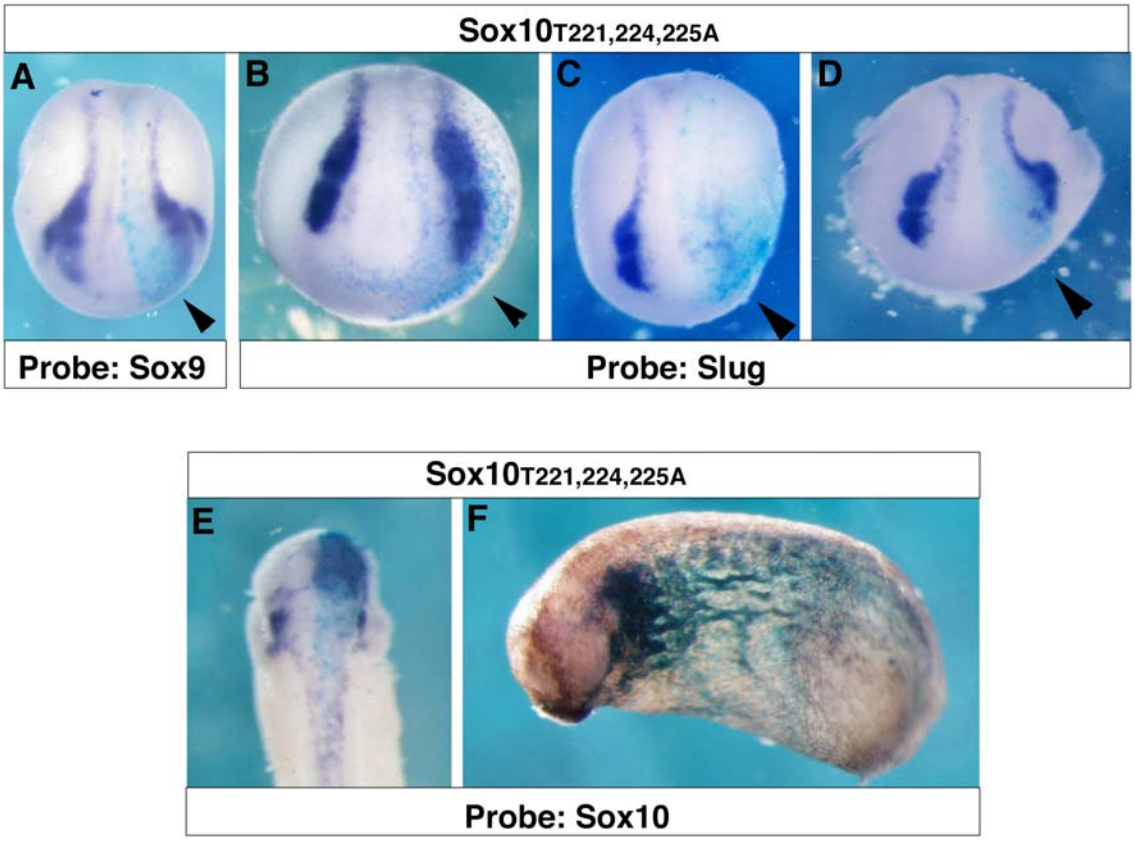
Figure 4.4: Sox10 is phosphorylated, while the alanine mutant is not.

Kinase assay performed on embryo lysates. The control lane shows no phosphorylation, while the Sox10 lane shows a phosphorylated species. However, the alanine mutant does not appear to be phosphorylated, confirming that this site is phosphorylated.

Figure 4.5: Effects of the Sox10 alanine mutant in *Xenopus*.

Misexpression of Sox10^{T221,224,225A} on early neural crest precursor formation (A-D) has variable effects, from expansion to inhibition of the neural crest precursors. Misexpression of this construct expands the field of Sox10-expressing cells at later neural crest differentiation stages (E-F).

Figure 4.5


Sox10T221,224,225A
A
B
C
D
Probe: Sox9
Probe: Slug
Sox10T221,224,225A
E
F
Probe: Sox10

suggesting perhaps an effect on a different cell population, such as an expansion of the neural plate.

However, when looking at those cells expressing the neural crest precursor marker, Slug, I observe different effects that the alanine mutant has on the neural crest. The phenotypes depicted here are reminiscent of how misexpression of wildtype Sox10 affects the neural crest. Misexpression of the alanine mutant leads to both an expansion and inhibition of Slug (Figure 4.5B,C). Interestingly, I also observed a similar phenotype with the Slug marker as I do with the Sox9 marker, a slight shift of the neural crest precursors (Figure 4.5D).

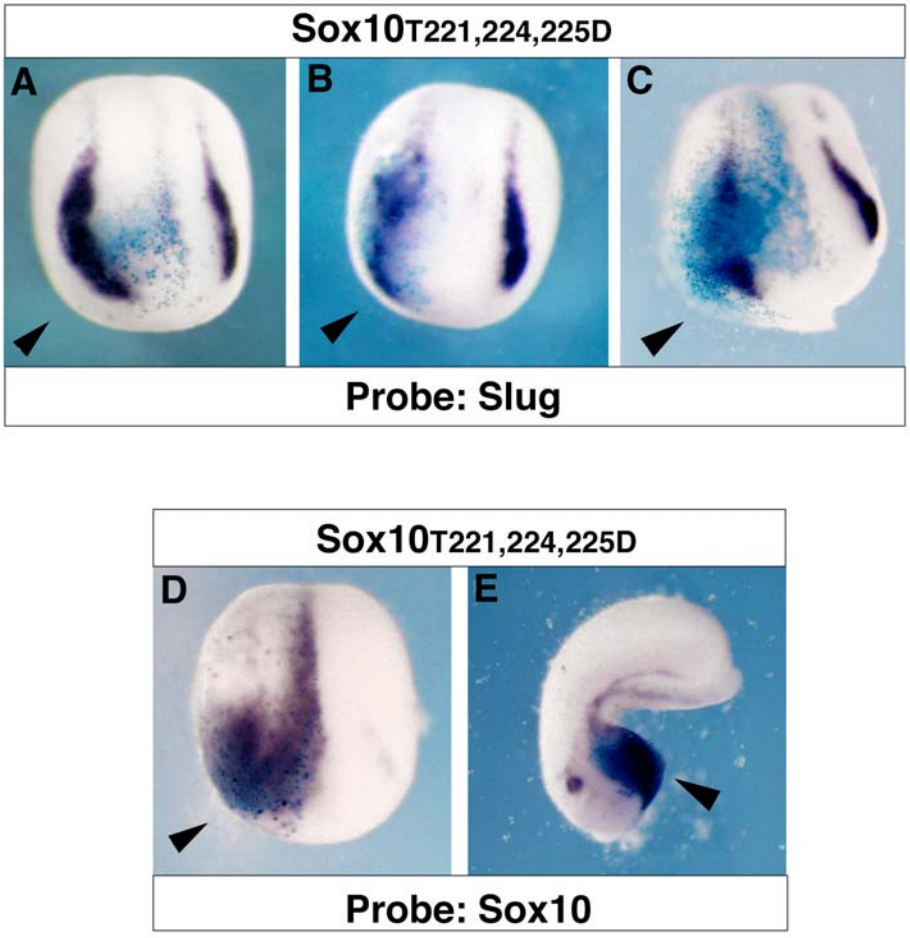
Wildtype Sox10 also plays a large role at neural crest differentiation stages, affecting melanocytes and cranial ganglia. In examining the effects of the alanine mutant on this later stage of development, I see a vast expansion of the Sox10-expressing cells, which is similar to what I observe with wildtype Sox10 misexpression (Figure 4.5 EF). This most likely points to an expansion of the cranial ganglia, melanocytes, and/or ear. Interestingly, I also observe "crenulations", or wrinkles, on the injected side of the embryo (Figure 4.5F). While I am unsure of what these wrinkles are, they are a prevalent phenotype and would be interesting to pursue further. One thought is that they are melanocyte-derived outgrowths in the skin, as some of the crenulations appear to stain with markers for melanocytes, such as Sox10.

Examination of the aspartate mutant in *Xenopus* embryos showed different, interesting affects on the neural crest. While it too can expand the neural crest precursor population, as shown by the expansion of Slug (Figure 4.6 A), misexpression of this mutant also leads to diffused expression of Slug or even places where Slug expressing cells are no longer there, forming a hole in the neural crest precursor domain (Figure 4.6 B,C). Misexpression of the aspartate mutant also leads to a vast expansion of the Sox10 probe at early stages, which looks

Figure 4.6: Effects of the Sox10 aspartate mutant in *Xenopus*.

Misexpression Sox10^{T221,224,225D} on early neural crest precursor development (A-C) leads to expansion, diffusion, and spotted inhibition of the neural crest precursor marker Slug. Misexpression of this construct leads to expansion of Sox10-expressing cells both early and in later neural crest development (D, E).

Figure 4.6


Sox10T221,224,225D
A
B
C
Probe: Slug
Sox10T221,224,225D
D
E
Probe: Sox10

similar to the phenotype seen in misexpression of nonSUMOylated Sox10 (Figure 4.6 D and refer to Figure 2.10B). At later neural crest differentiation stages, Sox10 expression is expanded, as it is with misexpression of the alanine mutant and wildtype Sox10 (Figure 4.6 E).

To review this data, the alanine mutant appears to function similarly to wildtype Sox10 with respect to neural crest formation, suggesting that perhaps the normal setting of Sox10 at these stages is to be unphosphorylated. However, the aspartate mutant, or phosphomimetic version of Sox10, leads to complex phenotypes in comparison to wildtype. In looking at the patchy appearance of the neural crest precursors that express Slug, the highest level of beta-galactosidase corresponds to "missing" neural crest cells. Perhaps a phosphorylated version of Sox10 acts to turn off Slug expression, acting in a negative feedback. With respect to the expression pattern of Sox10, the aspartate mutant appears to have no different effect on Sox10-expressing neural crest cells in comparison to the effects seen in wildtype misexpression. Both of these mutants function in a manner similar to that of wildtype Sox10 during neural crest differentiation stages, suggesting that phosphorylation of this particular area is not a mechanism used by Sox10 to control proper neural crest derivative formation. Interestingly, SUMOylation is sometimes controlled by phosphorylation or vise versa. I wanted to investigate whether my Sox10 phosphorylation site had any impact on the SUMOylation of Sox10. During SDS-PAGE analysis of embryos co-injected with both SUMO and the alanine or aspartate mutant, I found that neither of these mutations had any impact on the ability of Sox10 to be SUMOylated at both positions (Figure 4.7).

While the effects of these mutants are interesting with respect to neural crest formation, they may also disturb other regions of the embryo. Because of this, I looked at early ear

Figure 4.7

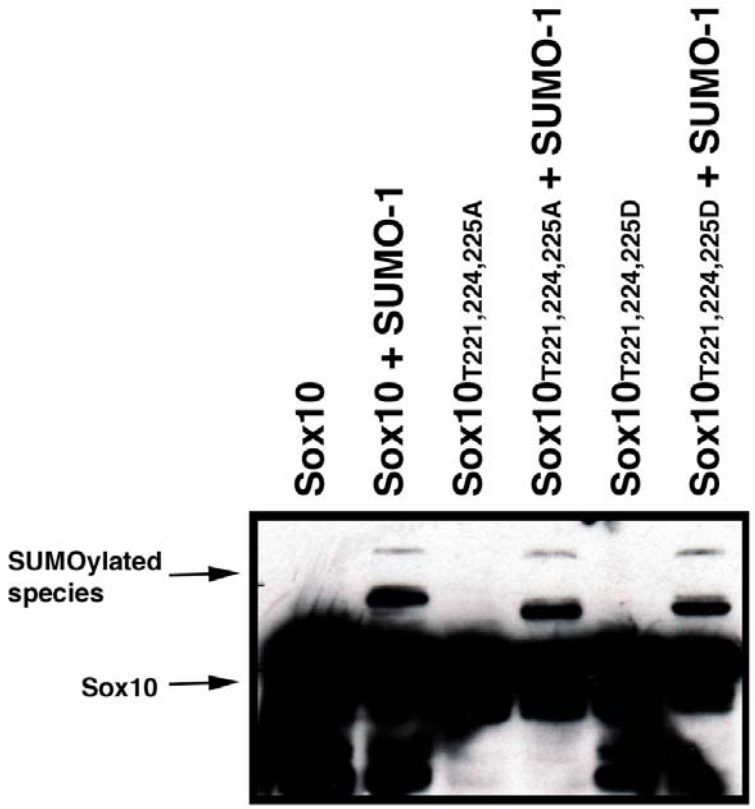


Figure 4.7: Sox10 phosphorylation mutants do not impact the ability of the protein to be SUMOylated at either SUMOylation site.

Co-injection of wildtype Sox10, Sox10^{T221,224,225A}, or Sox10^{T221,224,225D} with SUMO-1 shows no impact on the ability of Sox10 to be SUMOylated at its two SUMOylation sites via SDS-PAGE analysis. The SUMOylated species are located above the wildtype protein because SUMOylation is a modification on the protein that causes it to migrate more slowly.

formation and a marker for the cement gland. The cement gland is an organ found in the developing tadpole that secretes a glue-like substance to help the tadpole stick to rocks. The reason why I chose to look at this marker is not to look at the effect of these mutants on the formation of the cement gland, but instead to look and see if other cell types formed in the same area as the early neural crest were disturbed.

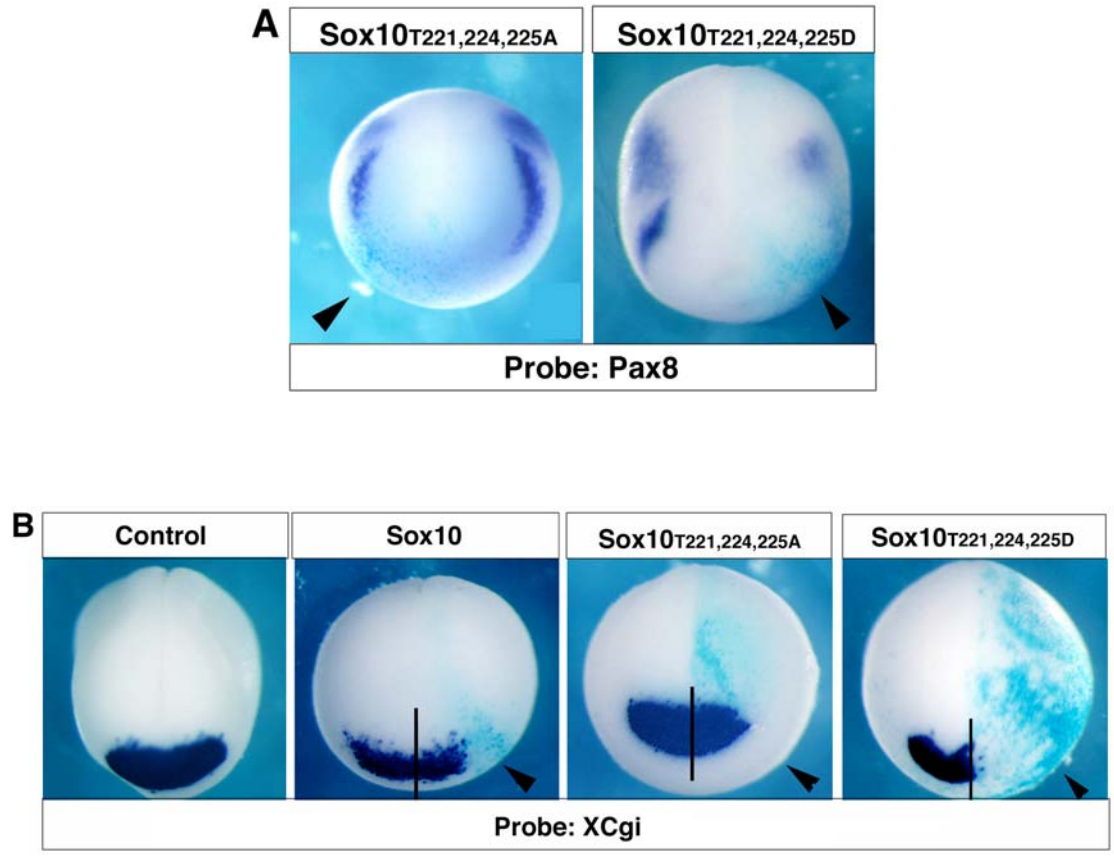
I have previously shown that SoxE factors are involved in ear formation as well as the neural crest. I therefore wanted to examine the effects of my mutants on the ear by using the otic placode- and kidney-specific marker, Pax8. While I have shown that wildtype Sox10 leads to an expansion of the otic placode at early neurula stages in Chapter 2, the alanine and aspartate mutants appear to have different effects on the ear. Misexpression of the alanine mutant leads to a partial inhibition of the otic placode, while the aspartate mutant leads to almost complete inhibition of the otic placode (Figure 4.8 A). Interestingly, the aspartate mutant phenotype here looks similar to the ear phenotype seen with the nonSUMOylated form of Sox10 shown in Chapter 2. This result suggests that phosphorylation of Sox10 at these sites is detrimental to early ear formation. It would be of great benefit to visit this effect at later stages of ear development. It is also possible that the ear cannot properly form due to the expansion of another cell population, which will be discussed at a later point.

Because misexpression of these mutants at times appears to alter the location of the neural crest cell population, by a slight shift away from the neural plate, and inhibit otic placode formation, I next wanted to examine other cell populations in this area of the developing embryo. The cement gland, as mentioned above, is an organ found in the developing embryo. As shown by this whole mount *in situ* hybridization performed on a control embryo, the cement gland, marked by the probe XCg1, is located at the anterior most portion of the embryo at the transverse

Figure 4.8: Comparing the effects of the alanine versus aspartate mutant on the otic placode and cement gland.

Misexpression of the alanine mutant leads to a slight inhibition of the otic placode, whereas misexpression of the aspartate mutant leads to a more severe inhibition of the otic placode (A). The misexpression of both wildtype and alanine mutant Sox10 leads to a slight inhibition of the cement gland, while misexpression of the aspartate mutant leads to a dramatic loss of the cement gland (B).

Figure 4.8


A
Sox10T221,224,225A
Sox10T221,224,225D
Probe: Pax8
B
Control
Sox10
Sox10T221,224,225A
Sox10T221,224,225D
Probe: XCgi

neural fold (Figure 4.8 B). I looked to see if misexpression of my phosphorylation mutants affected the formation of this cell population. In Figure 4.6 B, I show that misexpression of wildtype Sox10 leads to a slight inhibition of XCg1, which corresponds nicely to there being a slight expansion of the neural crest precursors. Misexpression of the alanine mutant also leads to a slight inhibition of the cement gland cell population (Figure 4.8 B). This too corresponds to the alanine misexpression data showing a slight expansion of the neural crest region, which again, may alter the formation of the cement gland cells. Misexpression of the aspartate mutant, however, leads to an almost complete inhibition of XCg1-expressing cells. Given the effects this mutant has on Sox10-expressing neural crest precursors (Figure 4.8 B), perhaps the vast expansion of the neural crest is altering the ability of the cement glad to form. It would be informative to look at other cell populations that form in this area, such as the neural plate and placodes. It is possible that the misexpression of these mutants has an effect on the neural plate, given the information that the neural crest precursors are more distal than normal. Piecing apart the regulatory functions of this phosphorylation site could provide researchers with a mechanism that SoxE proteins use to control the development of different cell types, just as SUMOylation did in the data presented in Chapter 2.

Yeast Two Hybrid Screen

SoxE factors are known to use partner proteins to carry out their transcriptional events (reviewed in (Kamachi et al., 2000; Wilson and Koopman, 2002)). As previously described, *in vivo* these factors bind to DNA at the minor groove with a low affinity. The recruitment of a partner helps to stabilize this interaction and promote transcriptional activity. As demonstrated

in Chapter 1, there are several published examples of such interactions, whether they are physical interactions or within very close, spatial proximity to one another.

I was interested in looking for novel SoxE partner proteins. To do this, I performed a yeast two-hybrid screen. All of the plasmids and the library that I used in my screen were a generous gift of S. Sokol. The library used as the "prey" is a gastrula stage cDNA library isolated from 300 *Xenopus* embryos and was amplified once. I constructed Sox10 without the activation domain as my "bait" (Sox10ΔAD; amino acids 1-333). I used the LexA based yeast two-hybrid reporter system for this screen, which is also essentially described in (Itoh et al., 2000) and in the beginning of this section. In brief, I inserted Sox10ΔAD into the yeast plasmid pEG202, fused just downstream of the heterologous DNA-binding protein LexA. I then transformed this plasmid into the mating strain, EGY48, which contains the reporter plasmid p8op-lacZ. It should be noted that p8op-lacZ was originally published under the name of pSHI8-34 (Estojak et al., 1995; Golemis and Brent, 1992). This mating strain is used because it is a dual reporter system responding to activation through the LexA operator. The strain EGY48 itself has six LexA binding sites (operators) located upstream and in transcriptional control of the LEU2 gene reporter. This gene will only be activated then when there is a transcriptional response from the bait/LexA fusion gene. Therefore, this allows for selection when transcriptional activation is achieved by growing in medium without leucine.

A second round of selection can be achieved through the reporter plasmid, which contains *lacZ* under the control of eight *LexA* operators and the minimal TATA region from the GAL1 promoter. By using this reporter, I was able to undergo a second round of discrimination based on my selecting for those colonies achieving differences in transcriptional activation strength by a change in color. By plating these cells on plates containing Xgal, the colonies will

turn light blue if there low levels of activation and bright blue with the highest levels of activation present. To actually identify interactors with the bait, the library plasmid, pJG4-5 is co-transformed into the mating strain with the Sox10ΔADpEG202. The library uses the inducible yeast *GAL1* promoter. Proteins within the library are fused to an acidic motif, which is used as the activation domain. To then identify which proteins will interact with the bait, the co-transformed yeast must be grown in galactose-containing media, as opposed to just glucose-containing media. This will activate expression of the library proteins, which can then interact with the bait. As previously mentioned then, to eliminate all library proteins but those positively interacting with the bait, the co-transformed strains are also grown in galactose-containing, leucine-minus media. This will ensure that those proteins not interacting with the bait and not activating transcription of the *LexA* operators will not be able to grow then without the presence of leucine.

Also, as mentioned above, the strength of these interactions can be measured by looking at the differences in blue color on Xgal plates. It was my experience, however, that the Xgal assay does not work as well as proposed. In the end, it did not correlate well with the interactions I achieved and did not discriminate against false-positives. I would not recommend using this assay.

After the initial screen is performed, the positive interactors must be isolated via PCR-based methods described above. These cleaned PCR fragments are then sequenced and submitted to nBLAST through the NCBI website to reveal their identities. However, when an interactor looks as though it is of interest, further testing must be done. Unfortunately, the yeast two-hybrid system produces many false-positives, or factors that interact together in yeast, but cannot be repeated outside of this system by methods such as co-immunoprecipitation. The way

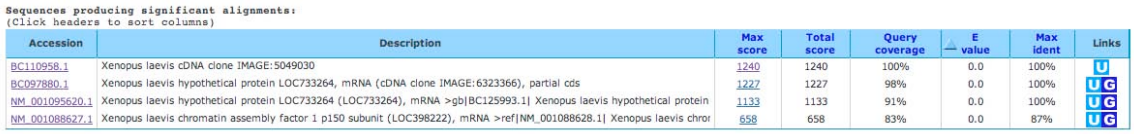
in which to eliminate false positives is by shuttling the interacting protein through *E. coli* and then putting them back through the yeast two-hybrid process, in which the protein of interest is inserted into the "prey" pJG4-5 strain. Those interactions that are not real will fail to grow. Those that passed this retest are ultimately found to be true positive interacting factors. One caveat to this screen was the removal of the activation domain from Sox10 when introduced as the bait. This must be done so that enhanced, false activation will not interfere with the yeast system, which uses the activation domain during the interaction process. This is a problem with the SoxE factors because several of the previously identified SoxE partners, such as ß-catenin and CBP/p300, interact through the SoxE activation domain (Akiyama et al., 2004; Furumatsu et al., 2005b; Tsuda et al., 2003).

During this screen, I successfully pulled out SUMO-1 and UBC9 as Sox9/10 interaction partners, as previously described in Chapter 2. However, I also pulled out a number of other interesting factors that were not false positives. From the many interesting factors that I retested, only three remained real interactors.

The first of these three factors is the clone with Genbank accession number BP700053. This is an EST (expressed sequence tag) that was identified in a screen of the anterior neuroectoderm, and according to this screen, it is expressed during late gastrula stages. Interestingly, upon blasting this EST, a number of hypothetical proteins come up, as well as *X. laevis* chromatin assembly factor 1 p150 subunit (Figure 4.9). No conserved protein domains were detected, however. As detected by *in situ* hybridization, the location of this clone's transcripts corresponds with a time and place consistent with Sox10 expression (Figure 4.10).

The second Sox10-interacting factor I identified during my screen is the Genbank clone BC073360. This factor is listed under a Core Nucleotide sequence and is defined as *Xenopus*

Figure 4.9



Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
BC110958.1	Xenopus laevis cDNA clone IMAGE:5049030	1240	1240	100%	0.0	100%	U
BC097880.1	Xenopus laevis hypothetical protein LOC733264, mRNA (cDNA clone IMAGE:6323366), partial cds	1227	1227	98%	0.0	100%	U G
NM_001095620.1	Xenopus laevis hypothetical protein LOC733264 (LOC733264), mRNA >gb|BC125993.1| Xenopus laevis hypothetical protein	1133	1133	91%	0.0	100%	U G
NM_001088627.1	Xenopus laevis chromatin assembly factor 1 p150 subunit (LOC398222), mRNA >ref|NM_001088628.1| Xenopus laevis chror	658	658	83%	0.0	87%	U G

Figure 4.9: Sox10-Interacting Factor BP700053

The first Sox10-interacting clone I pulled out of the yeast two-hybrid hit as Genbank accession number BP700053. This is an EST (expressed sequence tag) identified in a screen of genes found in the anterior neuroectoderm, and it is expressed during late gastrula stages. In the alignment performed, this clone was closely related to the chromatin assembly factor, p150.

Figure 4.10

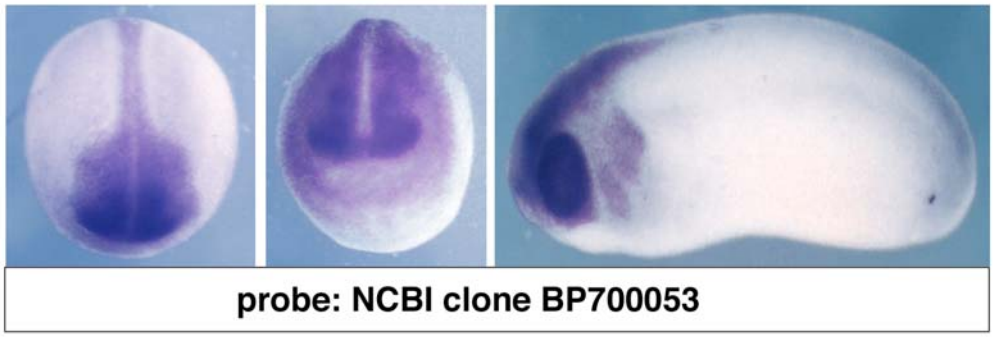


Figure 4.10: *In situ* hybridizations on Sox10-interactor BP700053

The first Sox10-interacting clone I pulled out of the yeast two-hybrid hit as Genbank accession number BP700053. The transcripts of this clone are located in a time and place consistent with Sox10 expression. Both in the early neural crest precursor population as well as the later migratory neural crest cell population.

laevis MGC80778 protein, which was originally found in the adult spleen. The protein that this factor is identified as has two putative conserved domains within it (Figure 4.11). This protein may be homologous to p23, a protein that binds to hsp90. It also hits to a conserved CS domain, which is also involved in heat shock proteins. It is potentially part of a scaffolding complex that may bind to Sox10. The results from *in situ* hybridization performed with the antisense form of this factor, reveal that it's transcripts are located in a time and place consistent with Sox10 expression (Figure 4.12).

The third positive Sox10 interacting factor I pulled out of my screen is the hypothetical protein BC055984. This factor was found during a screen of stage 31/32 *Xenopus* embryos. Interestingly, this stage corresponds to neural crest differentiation stages. A possible identification for this factors is ARKadia-like 1 isoform a, which is a novel E3 ubiquitin ligase. At this point in time, no *in situ* hybridizations were performed with this factor and therefore, I do not know what the expression pattern looks like in *Xenopus* embryos. However, this protein is very well conserved through several different species and may prove to be extremely interesting.

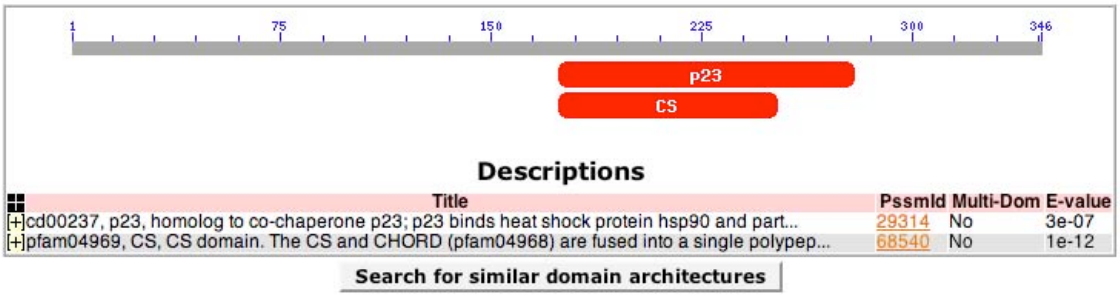
SoxE Deletion Constructs

To further examine the functional aspects of the SoxE proteins, I created a number of deletion constructs, each taking out a different conserved domain. As shown in Chapter 2, SoxE factors have a central HMG, or DNA binding domain, a C-terminal activation domain and two other conserved domains specific to SoxE factors, termed E1 and E2. Currently, there is little information known about the functions of the E1 and E2 domains. I wanted to examine whether one particular domain was required for a specific aspect of SoxE activity. The constructs are

Figure 4.11

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NM_001092330.1	Xenopus laevis MGC80778 protein (MGC80778), mRNA >gb|BC073360.1| Xenopus laevis MGC80778 protein, mRNA (cDN	4961	4961	100%	0.0	100%	U G
NM_001097659.1	Xenopus laevis hypothetical protein LOC100036878 (LOC100036878), mRNA >gb|BC129581.1| Xenopus laevis hypotheti	1735	1882	53%	0.0	90%	U G
NM_001017320.2	Xenopus tropicalis NudC domain containing 3 (nudcd3), mRNA >emb|CR848659.2| Xenopus tropicalis finished cDNA, clo	1596	1596	44%	0.0	90%	U G
NM_001015778.1	Xenopus tropicalis MGC108341 protein (MGC108341), mRNA >gb|BC089719.1| Xenopus tropicalis MGC108341 protein, r	1596	1596	44%	0.0	90%	U G
BC070646.1	Xenopus laevis hypothetical protein LOC431986, mRNA (cDNA clone IMAGE:3396590), partial cds	161	161	12%	1e-35	77%	U G



http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=9GJ6RXPR015&mode=all

Figure 4.11: Sox10-Interacting Factor BC073360

The second Sox10-interacting protein I pulled out of my screen is Genbank clone BC073360. This factor is listed as a Core Nucleotide sequence and is defined as Xenopus laevis MGC80778 protein found in the adult spleen. The protein that this factor is identified as has two putative conserved domains within it, shown above, a p23 domain and a CS domain. This clone hits to nothing other then hypothetical proteins.

Figure 4.12

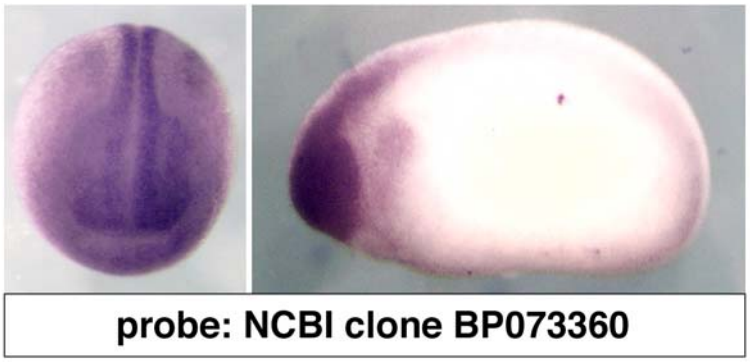


Figure 4.12: *In situ* hybridizations done on Sox10-interacting factor BP073360

The second Sox10-interacting protein I pulled out of my screen is Genbank clone BC073360. This factor is listed as a Core Nucleotide sequence and is defined as Xenopus laevis MGC80778 protein found in the adult spleen. The *in situ* hybridizations performed with this clone show that it is expressed in a time and place consistent with the Sox10 expression pattern.

shown in Figure 4.13.

My interest was first peaked in this area of research by the results obtained in a study by Aoki and colleagues (Aoki et al., 2003). These researchers found that misexpression of a construct including only the E2 domain and activation domain of Sox10 in *Xenopus* embryos could evoke a similar phenotype with respect to early neural crest precursor formation as wildtype Sox10 misexpression. The result they saw was an expansion of this precursor population. I wanted to create this construct (Sox10ΔE1, HMG) to see if I could recapitulate these results. Interestingly, upon misexpressing Sox10ΔE1, HMG mRNA into 2-celled *Xenopus* embryos, I found variable results. I found both a slight expansion of the precursor population, which mimicked their results, and I also saw inhibition and spotted expression of the neural crest precursor population (Figure 4.14). These results are very similar to those I have shown in Chapter 2 with misexpression of wildtype Sox9 and 10. Perhaps then this construct can perform equivalently as the wildtype protein with respect to neural crest precursor formation and only the E2 and activation domain are necessary for the formation of this cell type. However, this may not be the case for all cell populations that Sox10 is involved in forming.

I next looked at early ear formation using the *in situ* hybridization probe, *Pax8*. *Pax8* marks both the otic placode and the embryonic kidney, which is located more posterior than the ear. Misexpression of Sox10ΔE1, HMG in *Xenopus* embryos led to an inhibition of early ear formation, as can be seen by the absence of *Pax8* staining in the otic placode region (Figure 4.15). This differs from the otic placode expansion seen with wildtype Sox10 misexpression (Figure 2.4). This result indicates that the E2 domain and activation domain are not sufficient to produce the same phenotype that wildtype Sox10 misexpression gives in this particular

Figure 4.13

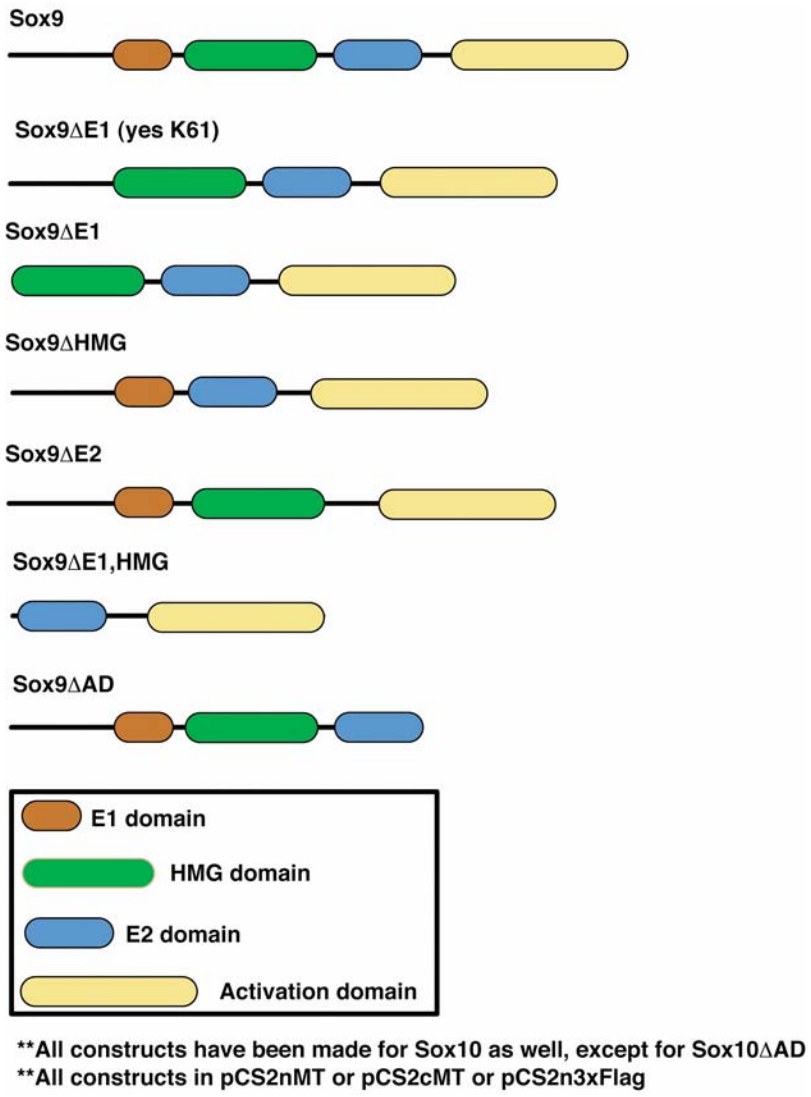


Figure 4.13: Schematic of Sox9 and Sox10 conserved domain deletion constructs.

Figure 4.14

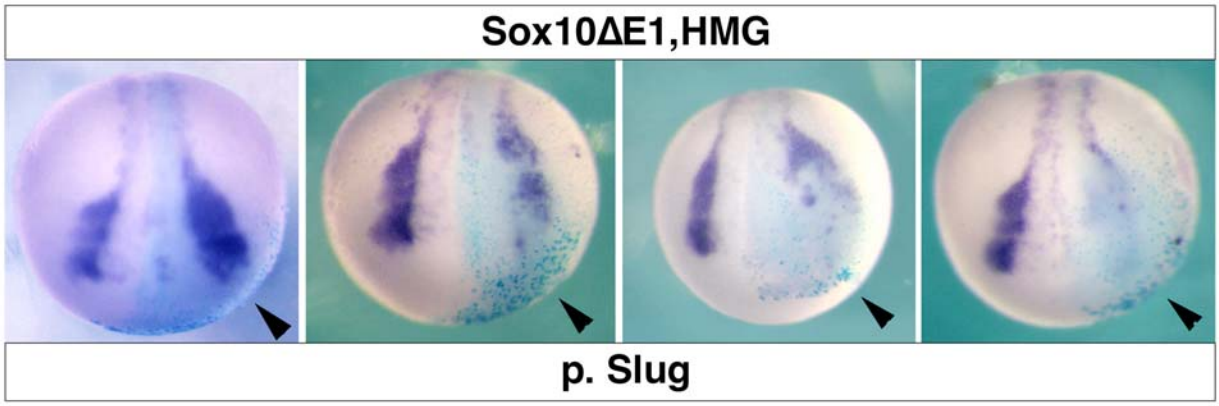


Figure 4.14: The effects of Sox10ΔE1, HMG on neural crest precursor formation. Misexpression of Sox10ΔE1, HMG leads to variable phenotypes with respect to early neural crest precursor formation. These include expansion and inhibition of this cell type. These phenotypes mirror what is seen with misexpression of wildtype Sox10, as seen in Chapter 2.

Figure 4.15

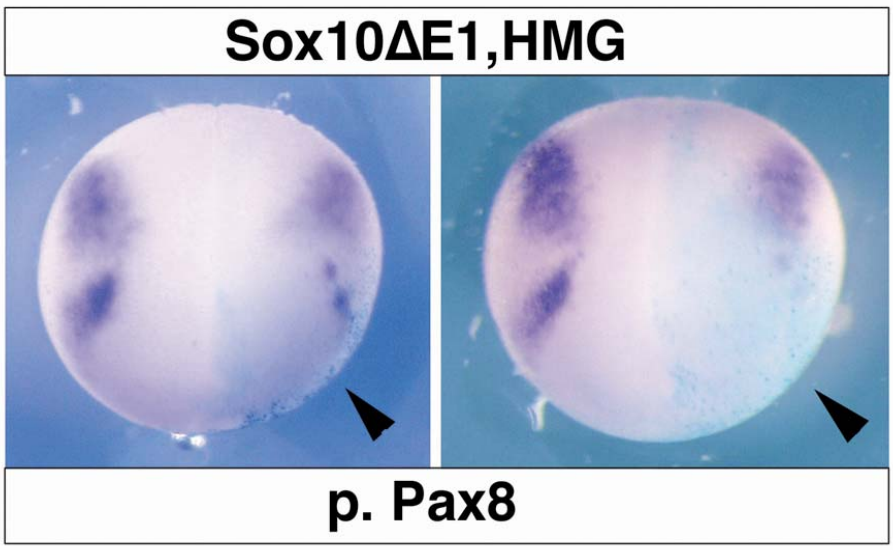


Figure 4.15: The effects of misexpressing Sox10ΔE1, HMG on the otic placode.

Misexpression of Sox10ΔE1,HMG results in inhibition of the otic placode at early neurula stages. Misexpression of wildtype Sox10 results in the expansion of the otic placode at this stage (as seen in Figure 2.4).

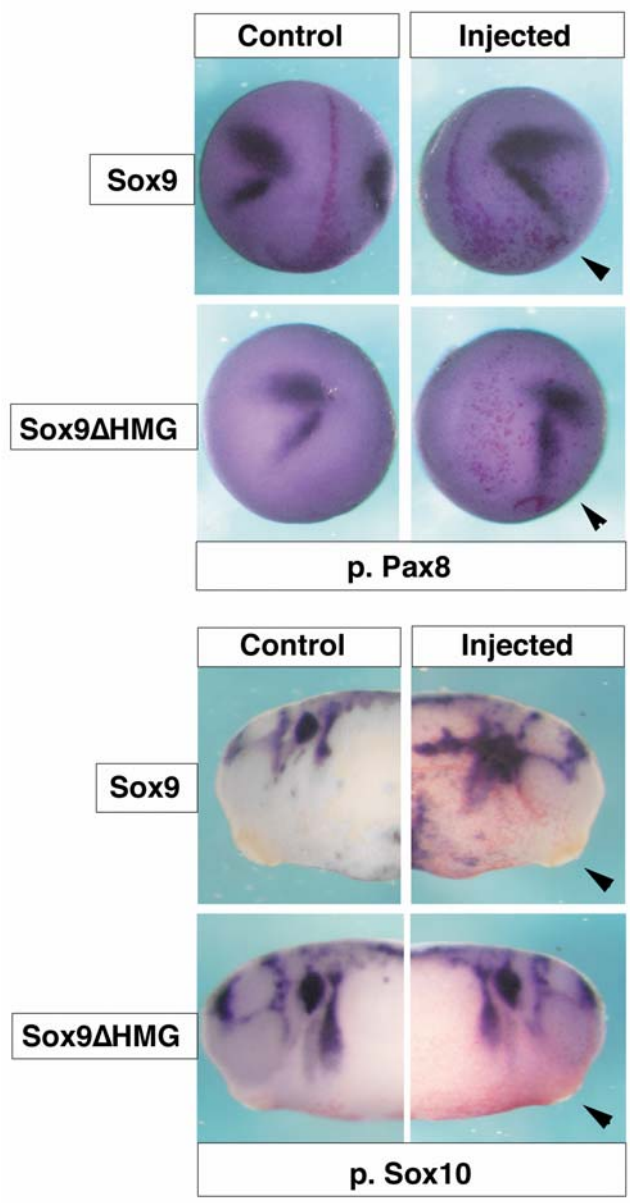
cell population. It also demonstrates that there is perhaps something inhibitory about this construct with respect to early ear development. The way in which to fully understand this would be to perform rescue experiments. By co-expressing the Sox10 morpholino with this deletion construct, I would be able to determine whether the deletion construct is sufficient to perform the functions of wildtype Sox10. For example, the Sox10 morpholino alone results in inhibition of the early neural crest precursors, as shown in Chapter 2. However, if I misexpressed Sox10ΔE1,HMG with the Sox10 morpholino and found a complete restoration of the early neural crest precursors, then I would be able to conclude that the E2 domain and activation domain were sufficient in the formation of this cell type. The same experiments would need to be performed when looking at otic placode formation as well.

I next wanted to look at a different deletion construct, Sox9ΔHMG, and wanted to focus on ear formation. The preliminary results that I have obtained show that the HMG domain of Sox9 may not be necessary for the formation of the otic placode, but is necessary for the formation of neural crest derivatives. In Figure 4.16, I show that misexpression of wildtype Sox9 leads to the expansion of the otic placode region. I also see that the misexpression of equivalent levels of Sox9ΔHMG protein also leads to the same phenotype. This suggests that the HMG domain is not necessary for the effects this protein has on this cell type. Indeed, there is precedence in the literature indicating that the ability of SoxE factors to bind to DNA is not necessary for the formation of certain cell types. Instead, the SoxE factor binds to other cofactors and exhibits a transcriptional response through the cofactor. Lang and Epstein demonstrated that Sox10 physically interacts with Pax3. This interaction is necessary for the activation of the c-RET enhancer and these factors can still activate this enhancer when the

Figure 4.16: The effects of misexpressing Sox9ΔHMG on the neural crest.

Misexpression of wildtype Sox9 and Sox9ΔHMG leads to the expansion of the otic placode region during early neurula stages. Misexpression of wildtype Sox9 leads to the expansion of melanocytes, cranial ganglia and the otocyst during neural crest differentiation stages. However, misexpression of Sox9ΔHMG does not lead to any phenotypes at this later stage.

Figure 4.16


Control
Injected
Sox9
Sox9ΔHMG
p. Pax8
Control
Injected
Sox9
Sox9ΔHMG
p. Sox10

Sox10 HMG domain is mutated, thus providing evidence that Sox10 does not need to bind DNA to carry out its response in this particular setting (Lang and Epstein, 2003).

However, at later developmental stages when I look at the otocyst and neural crest derivatives, such as melanocytes and cranial ganglia, I find that misexpression of wildtype Sox9 expands all of these cell types, but misexpression of Sox9ΔHMG does none of the above (Figure 4.16). These cell types all have a normal phenotype on the injected side. This would suggest that the HMG domain does play an important role in mediating the formation of these cell types. Again, this would need to be verified with rescue experiments, as suggested above. It is interesting that the HMG domain would not be necessary during early ear formation, but is needed during otocyst formation.

Discussion

This chapter provides data from many of the experiments that I have started doing throughout my thesis research, but have not had the opportunity to finish. They remain as excellent starting points for any future lab member that would want to continue them.

The phosphorylation data indicates a potential new mechanism by which SoxE factors may be controlled throughout development. It will be of great interest to investigate whether all three of these factors are phosphorylated at this point, as well as confirming which kinase is responsible for the phosphorylation. Preliminary data indicates that the phosphorylation of Sox10 has no impact on the protein's ability to be SUMOylated; however, that does not rule out the possibility that the SUMOylation state of the protein could influence it's ability to be phosphorylated. Also, the phenotypes caused by the mutant Sox10 proteins that either eliminate or mimic the phosphorylated state need much further evaluation through *in situ* hybridization. It

would also be interesting to see if the phosphorylation state of these proteins is altered throughout the course of development. One way in which this could be achieved is by creating a phospho-specific antibody to endogenous Sox10. This way, only the phosphorylated protein would be detected via SDS-PAGE. This project has the potential to lead to some very interesting findings.

The yeast two-hybrid screen was also very beneficial to the laboratory. Not only did it bring a new technique to the lab, but also provided the lab with several new constructs that may have an impact on neural crest development. To begin, a much more thorough panel of *in situ* hybridization experiments will need to be done on the novel clones isolated in order to determine their staining patterns throughout development. It would also be very beneficial to obtain the full-length clones of these interactors so that both confirmation of the interaction through co-immunoprecipitations and misexpression studies in the *Xenopus* embryo could be carried out. It should also be noted that I have twenty-nine other glycerol stocks of the Sox10-library screen in the –80˚C. These could be plated and more novel interactors could be identified. Other potential ways in which the yeast two-hybrid could be used is by performing the screen with the Sox10 alanine mutant or a Sox10 protein that cannot be SUMOylated. This could identify partner proteins that have interactions under very specific conditions. One caveat to this screen, as previously mentioned, is that a growing number of SoxE partner proteins that appear to interact with the SoxE activation domain, which has been partially removed in this screen.

The deletion construct project is particularly interesting and will be of great use to the laboratory. The greatest tool that this project will take advantage of is the Sox10 morpholino. To really get a good idea of what domains function in what cell type, the endogenous protein will first need to be eliminated. Only then will the question at hand truly be able to be answered.

These constructs will also be beneficial to the novel partner proteins identified in the yeast two-hybrid screen. Once the full-length clones of these potential partners are obtained, co-immunoprecipitations could be done with the different deletions constructs in order to identify what area of the protein is important for the interaction.

To conclude, the experiments presented here make excellent starting off points for any researcher in the laboratory that would like to focus on SoxE factors. While the data is all extremely preliminary, it supports new and interesting ideas surrounding neural crest development.

CHAPTER 5

GENERAL DISCUSSION

The neural crest is an intriguing cell type to study because these cells are unique in their multipotency until various stages of development. They also have a complex regulatory network of signaling pathways and transcription factors. By understanding how certain neural crest-specific pathways and factors are mechanistically regulated, the scientific community can gain important insight into how this and other cell populations are formed and proliferated. As these cells form all of the derivatives that create the difference between invertebrates and vertebrates, they are clearly essential to the proper formation and development of the embryo. When defects occur in these cells, a wide number of birth defects and childhood cancers can develop. Clearly, understanding the way in which these cells form and are maintained within the developing organism will greatly benefit mankind.

The steps of neural crest development are extremely complex. While many advances have been made in understanding the different signaling pathways and transcription factors involved in the propagation of these cells, there is a vast expanse of knowledge that still needs to be gained. While understanding what factors and pathways are involved in the neural crest and at what time they are involved is very important, going more in-depth to obtain more specific mechanistic data is imperative. Much of the research in this field has been focused on signaling pathways; however, as the way in which these pathways transduce signals is becoming more clear, the downstream targets of these pathways and how they are then mechanistically regulated is less clear. Interestingly, a common theme throughout development is the use of the same downstream factors simultaneously and consecutively throughout development to promote very different outcomes. This thesis has demonstrated one way in which the homologous factors, Sox9 and Sox10, are used to in this manner to promote different outcomes during development. This thesis then shows the mechanism by which this cell type distinction may be regulated.

I began studying the role of these factors during neural crest precursor stages. SoxE factors are necessary to the proper formation of the neural crest precursors and overexpression of these factors leads to an increase in neural crest precursor formation (Aoki et al., 2003; Honore et al., 2003; Spokony et al., 2002; Taylor and Labonne, 2005). At this neurula stage of development, the expression patterns of these two factors are completely overlapping with respect to the neural crest precursors. This data led me to believe that Sox9 and Sox10 are functionally redundant. However, once the neural crest cells have begun to differentiate, at tailbud stages, the expression patterns of Sox9 and Sox10 are completely non-overlapping with respect to neural crest derivatives. This is interesting, given that during neurula stages these factors completely overlap and they are so highly homologous. While the early expression pattern data suggests that these factors could act redundantly for one another, the later expression pattern data suggests otherwise. In fact, until the data contained in Chapter 2 of this thesis was published, it was suggested that Sox9 and Sox10 did have divergent activity (Aoki et al., 2003). Aoki and colleagues found that the misexpression of only Sox10, and not Sox9, could lead to ectopic Dct expression in *Xenopus* animal cap explants (Aoki et al., 2003). This thesis presents evidence that this result was not correct and goes on to further investigate the overlapping activities of Sox9 and Sox10.

To briefly summarize my results in the first portion of Chapter 2, I found that Sox9 could in fact perform equivalent activities in the developing embryo as Sox10. I find clear evidence demonstrating that the misexpression of Sox9 can form ectopic melanocytes in *Xenopus* embryos, parallel to the activity of Sox10. Sox9 can also inhibit neuronal differentiation, as can Sox10. These factors are also both involved in ear formation and the misexpression of both Sox9 and Sox10 can form ectopic and enlarged ear-like structures on the injected side of the

embryo. These two factors can also perform equivalent activities during neurula stages, with respect to both neural crest and the otic placode. This result was more expected, however, given that their expression patterns overlap at this stage. These data greatly benefit the scientific community by providing the knowledge that while expression patterns may diverge throughout the course of evolution, the function of highly related proteins may not diverge. Why then would the embryo need to retain more than one copy of this gene? This phenomenon can be explained in evolutionary terms.

Sox9 and Sox10 most likely arose via duplication of a single ancestral SoxE factor, and the most commonly employed explanations for the retention of such duplicate genes during vertebrate evolution are neofunctionalisation and subfunctionalisation. Neofunctionalisation assumes that the duplication event frees one copy of the gene from selective pressure to maintain essential functions, allowing this copy to evolve new functions. While examples of neofunctionalisation have been reported (McClintock et al., 2001), subfunctionalisation may be a more common explanation for the high retention rate of duplicate genes. Under the duplication-degeneration-complementation (DDC) model of subfunctionalisation, the reciprocal loss of aspects of the ancestral expression pattern in each of the duplicates could account for the selective pressure to maintain both copies (Lynch and Force, 2000). Although *Sox9* and *Sox10* are initially expressed in all neural crest precursors in *Xenopus*, their later expression is restricted to distinct subsets of neural crest derivatives in all model organisms examined, consistent with a role for subfunctionalisation in retaining these paralogs subsequent to their duplication. While neofuntionalisation suggests some degree of functional divergence, duplicate genes maintained as a result of subfunctionalisation are likely to retain similar activities.

This portion of my project has potential for further investigation. I would suggest determining whether Sox10 can function equivalently to Sox9. I expect that this be true, but I feel it would be important to investigate this matter. Both data analysis and expression pattern studies show a clear role for Sox9 in cartilage formation (Bi et al., 1999). The future of this project would be to test what the effects of misexpressing Sox9 and Sox10 had on the neural crest-derived facial cartilage. This would answer the first question of whether both Sox9 and Sox10 can lead to ectopic cartilage. This would be examined by looking at injected embryos stained with cartilage-specific probes or by performing Alcian blue staining, which stains cartilage at late stages of development. However, I feel that the most impressive data in Chapter 2 is when Sox10 is specifically depleted through the use of a morpholino and Sox9 rescues neural crest precursor formation and later Sox10-specific neural crest derivatives, such as melanocytes. Clearly, this would be the most informative experiment to perform in asking whether Sox10 can compensate for a loss of Sox9. However, the current Sox9 morpholino is extremely toxic to embryos and they cannot survive past gastrulation. I would suggest designing different forms of this morpholino to see if they are also toxic to the embryo.

While the data discussed thus far provides insight into evolutionary biology, the bigger question that this thesis addresses is how these factors are able to perform many divergent activities within the developing embryo. Sox proteins are well known for their use of partner proteins to carry out their transcriptional events, and partnering with different factors in different cell types could be one way in which these factors are able to diversify function (Bondurand et al., 2000; Jiao et al., 2004; Lang and Epstein, 2003; Ludwig et al., 2004; Yasumoto et al., 2002). Also, while at the start of my research, only Sox9 had been found to be a post-translationally modified protein through phosphorylation, it was possible that the presence or absence of novel

post-translational modifications could influence cell fate decisions in the embryo. I addressed these hypotheses by performing a yeast two-hybrid screen with Sox10 as my bait, out of which I pulled SUMO-1 and UBC9. I then found that both Sox9 and Sox10 were post-translationally modified by SUMO on two sites within each protein.

Although SUMOylation was first described as a post-translational modification almost ten years ago, the cellular consequences of SUMO modification remain poorly understood at the molecular level, and appear to differ significantly from substrate to substrate. Moreover, SUMO modification of different sites within the same protein can have different consequences for that protein's activity (Gill, 2004; Hay, 2005; Poukka et al., 2000). Among the reported effects of SUMOylation on transcription factors are the modulation of protein-protein interactions, protein-DNA interactions, and protein localization; as well as the regulation of protein stability via antagonization of ubiquitination (Gill, 2004; Hay, 2005). The consequences of SUMOylation for transcriptional activity are also diverse. In a number of cases, SUMO modification of transcriptional activators inhibits their potency as activators (Gill, 2004; Girdwood et al., 2004); however, SUMO-modification of other proteins leads to an increased ability to activate transcription (Goodson et al., 2001; Gostissa et al., 1999; Hong et al., 2001; Rodriguez et al., 1999).

The consequences of SUMO-modification can also be highly context dependant. For example, on some promoters SUMOylation of Smad4 results in transcriptional repression, while on other promoters this modification has been found to enhance Smad4-dependant transcriptional activation (Long et al., 2004). Once the effects of SUMOylation are better understood, it may prove to be the case that, like other aspects of transcriptional regulation, such promoter context dependent-effects are the rule rather than the exception. Indeed, given my

findings that SoxE isoforms that cannot be SUMOylated or that mimic a constitutively SUMOylated state have distinct effects on neural crest and otic placode formation, cellular context appears likely to play an important role in determining SoxE function in these two cell types. The way in which SUMOylation altered these fate decisions, however, was not clear. Possibilities include that the SUMOylation status alters partner protein preference or that when the neural crest cannot properly form due to SUMOylation of SoxE, the ear-competent zone is expanded.

This newly identified knowledge allows a glimpse into how the same transcription factor can be used several times over throughout development as well as in close proximity to one another to promote different events. This knowledge contributed to the scientific community's understanding of the true impact that a post-translational modification can make on a protein. This discovery will likely become a common mechanism used throughout development with a number of transcription factors.

There are several future direction pathways to be taken from this portion of my thesis other than the path I took, which is summarized below. While this thesis focuses on the ear, neural crest precursors, and melanoblasts, there are many other Sox9 and Sox10-derived structures within the embryo. Perhaps the SUMOylation state of these proteins is important for glia or cartilage formation. In fact, upon misexpression of the constitutively SUMOylated SoxE proteins, there is ectopic head staining, some of which is the ear, but may also be glial fates. Looking at glial- and cartilage-specific probes would provide additional useful information about the impact that SUMOylation has on SoxE factors. The answer to this question could also be addressed by obtaining cartilage- or glial-specific promoter constructs upstream of luciferase. Then, transcriptional activation assays could be performed using the SUMOylation mutant

constructs to assay the effects of SUMOylation on these cell-type specific promoters. Examples of promoters that would be beneficial to study are the glial-specific promoters, Po and MBP (Lefebvre et al., 1997; Peirano et al., 2000; Stolt, 2004; Stolt et al., 2002), and the chondrocyte-specific promoter, Col2a1, which is bound and regulated by Sox9 (Lefebvre et al., 1997; Tsuda et al., 2003). Unfortunately, no direct targets of SoxE transcriptional regulation have been identified at early stages of neural crest development. It would have also been beneficial to have had an ear specific promoter available to test if the constitutively SUMOylated mutant activated this promoter to a greater extent than the unSUMOylated construct, as indicated by my *in situ* results. However, ear-specific promoters are not available at this time, and I will come back to the role of these constructs in the ear later in this chapter. One additional direction to be taken could be to perform the yeast two-hybrid screen again with the different SUMOylation constructs to see if any novel partners exist with one form over the other.

Insight into a potential mechanism by which SUMO modification might regulate SoxE activity came about from work on MitF. In that study, SUMO modification of MitF was found to have no effect on the regulation of promoters containing a single MitF binding site but suppressed synergistic activation of promoters with multiple binding sites (Murakami and Arnheiter, 2005). If SUMOylation regulates SoxE-mediated transcription by selectively modulating cooperative interaction among factors constituting transcriptional complexes, then my findings suggest that distinct types of complexes may be deployed in tissues such as the neural crest and the inner ear. From this point, my thesis then went on to elucidate the precise mechanism through which SUMOylation modifies SoxE activity. There are broad implications for understanding how developmental regulatory factors with conserved activity can mediate very different functional outcomes when expressed in different tissues.

Interestingly, Murakami and Arnheiter used the melanocyte-specific promoter, Dct, to study the role of SUMOylation in MitF regulation. In Chapter 2, I saw a strong phenotype in melanocyte formation with my SoxE SUMOylation mutants, so I investigated this phenomenon more in depth. Briefly, misexpression of Sox9 or Sox10 that cannot be SUMOylated gives rise to ectopic melanocytes, while the constitutively SUMOylated form of these proteins does not allow ectopic melanocytes to form. As mentioned previously, MitF, the proposed master regulator of melanocyte formation, has been shown to work in synergy with Sox10 to promote melanocyte formation (Jiao et al., 2004; Ludwig et al., 2004; Yasumoto et al., 2002). Of course, this synergy had not been demonstrated for Sox9, as the scientific community previously felt that Sox9 did not perform a role in melanocyte formation. Now, we of course know this is not the case, as several reports, including the data presented here has clearly shown a role for Sox9 in melanocyte formation (Cook et al., 2005; Passeron et al., 2007; Taylor and Labonne, 2005). As demonstrated, SUMO also modifies MitF and both SoxE and MitF have binding sites adjacent to one another on the melanocyte-specific Dct promoter.

Upon investigating the SUMOylation of these two factors in relation to melanocyte formation, I found some very interesting and novel discoveries. I first looked at the effects of SUMOylation of MitF in *Xenopus* embryos. Similar to the SoxE data presented in Chapter 2, I found that analogous SUMOylation mutant constructs in a MitF background had similar effects on melanocyte formation as the SoxE SUMOylation mutants. Misexpression of MitF that could not be SUMOylated led to ectopic *Dct* expression on the flank of the embryo, whereas misexpression of constitutively SUMOylated MitF could not led to ectopic melanocyte formation. In fact, I found that MitF alone was sufficient to induce ectopic *Dct* expression in animal cap explants, and unSUMOylated MitF led to an even greater enhancement of *Dct*

expression in the animal caps. However, the constitutively SUMOylated form of MitF could not perform these activities.

Interestingly, however, MitF alone, in any form, was not sufficient to give rise to fully differentiated ectopic melanocytes. Clearly, part of the melanocyte-determining pathway is not present in this system. In the future, it will be of great interest to investigate what specific factors and combination of factors are necessary to give rise to fully differentiated melanocytes.

I also demonstrated that when expressed in combination, MitF^{K182,316R} and Sox9^{K61,365R}/SUMO-1 could not give rise to ectopic *Dct* expression in animal caps or whole embryos. This indicated that the presence of SUMO blocked the function of the unSUMOylated construct. When misexpressing both the Sox9 and MitF double lysine mutants in the embryo, I found that these factors worked synergistically to regulate *Dct* expression. Clearly the SUMOylation state of these factors is important for the proper regulation of the Dct promoter. Because of this phenomenon, I chose to investigate the actual mechanism by how SUMOylation functioned at the level of transcription by doing luciferase assays using the Dct promoter. While I ruled out more clear cut mechanisms that SUMO was using to inhibit synergy on the Dct promoter, such as HDAC recruitment and steric interference, I found some interesting data with respect to subcellular localization and cofactor recruitment.

When I looked for differences between the subcellular localization of Sox9^{K61,365R} and Sox9^{K61,365R}/SUMO-1, I found the differences to be complex, but interesting. I found that the Sox9^{K61,365R} construct had very punctate staining within the nucleus. The pattern appeared as tiny dots, which could be indicative of some type of nuclear sequestration or compartmentalization. When I looked at the localization pattern of Sox9^{K61,365R}/SUMO-1, I found a very similar punctate staining pattern to Sox9^{K61,365R}, but also found some larger spots of staining. While these patterns

were extremely interesting, I did not have the tools to properly investigate what nuclear compartments these factors were being sent into. This would be an excellent future project for someone in the lab, as I feel that there may be exciting data present. As described in Chapter 3, a few subnuclear compartments do sequester SUMOylated factors, such as the PML bodies and SNBs (reviewed in (Hay, 2005; Navascues et al., 2007)). While the PMLs are indicative of the smaller punctate pattern, the SNBs are indicative of the larger punctate staining pattern. PML bodies are used to sequester SUMOylated proteins and repress their activity. SNBs are thought to be sites of SUMOylation. While I do not think that my SUMOylated SoxE factors are being sequestered in these environments in order to repress their activity, given that I demonstrated the majority of Sox9 SUMOylation occurred under DNA binding conditions, it is still a possibility that they may shuttle into these compartments after they have been SUMOylated and are no longer necessary to the cell at the level of the DNA. There are markers available to multiple subnuclear compartments and co-localization experiments would need to be performed to fully understand the staining pattern of these factors.

The most solid evidence I obtained leading to the actual mechanism by which SUMOylation regulates SoxE factors was from the discovery that the SUMOylation state of SoxE factors led to the recruitment of different co-factors. Briefly, I found that $Sox9^{K61,365R}$ could interact with the co-activator CBP/p300, while $Sox9^{K61,365R}$/SUMO-1 could not. I then discovered that $Sox9^{K61,365R}$/SUMO-1 could interact with Pax3 and the co-repressor Groucho4, while $Sox9^{K61,365R}$ could not. Below is an in depth analysis of these interactions, as well as other important data supporting these interactions.

Before I examined the interactions with these extraneous factors, I first looked at the interactions between MitF and Sox9. What I found was at first exciting, but then became

somewhat perplexing. I found that MitF bound with the greatest affinity to $Sox9^{K61,365R}$/SUMO-1. However, previous investigators had found that MitF was no longer associated with the Dct promoter when Pax3 was present (Lang et al., 2005). However, this result is still very interesting to a development standpoint. As I mentioned, the chromatin immunoprecipitation data suggesting that Pax3 displaced MitF on the Dct promoter may not have been properly carried out (Lang et al., 2005). It is possible that MitF is displaced by Pax3, but still maintains an interaction off or on a different location of the DNA with $Sox9^{K61,365R}$/SUMO-1. Interestingly, I also found that Pax3 binds to $Sox9^{K61,365R}$/SUMO-1, but not to $Sox9^{K61,365R}$. One would expect then that MitF would have to interact with Pax3 given that they may be in a transcriptional complex with $Sox9^{K61,365R}$/SUMO-1. However, when I performed co-immunoprecipitations with MitF and Pax3, I did not visualize a solid interaction. Therefore, the question to answer is how is it that MitF can bind to $Sox9^{K61,365R}$/SUMO-1 and $Sox9^{K61,365R}$/SUMO-1 can bind to Pax3, but Pax3 does not appear to bind to MitF. One answer is that perhaps the co-immunoprecipitation washing conditions are too stringent to see a bridged interaction. Another thought is that the interaction seen with Pax3 and $Sox9^{K61,365R}$/SUMO-1 or MitF and $Sox9^{K61,365R}$/SUMO-1 is important for another aspect of development and not an interaction that is used during Dct regulation. To gain some insight into the second possibility, in the future, one could perform the same co-immunoprecipitations in a melanoma cell line using endogenous antibodies so as to isolate the interaction to a specific cell type, as SoxE factors and Pax3 are associated with one another in multiple cell types. A more clear answer would be to perform ChIP assays to investigate interactions of these two factors on several different promoters/enhancers, such as Dct, MitF, and c-RET. As this data complicated matters slightly,

and I could not get the ChIP assays to work during the time of this thesis, I chose to investigate the other interactions I found with the co-factors mentioned above.

As demonstrated, I found that $Sox9^{K61,365R}$ and not $Sox9^{K61,365R}$/SUMO-1 could interact with CBP/p300. Given that both Sox9 and MitF have previously been shown to interact with this co-activator complex in independent circumstances, this result is not surprising (Sato et al., 1997; Tsuda et al., 2003). Also, Tsuda and colleagues found that Sox9 interacted with CBP/p300 through its activation domain (Tsuda et al., 2003). This data suggests that when SUMO is not present, Sox9 is able to interact with this co-activator and this may enhance the synergy seen on the Dct promoter, in collaboration with MitF. However, when SUMO is present, this interaction is no longer possible. This is most likely due to the fact that CBP/p300 has been shown to bind to Sox9 around the same area that the SUMOylation site is located in the activation domain. The presence of this moiety could potentially be covering up the binding site or altering the conformation in a way that prevents CBP/p300 from interacting with Sox9. This in itself is an important discovery into one way in which SoxE factors are transcriptionally controlled.

However, while the CBP/p300 data clearly indicates a potential mechanism used by SUMO to alter transcriptional activity on the Dct promoter and a general mechanism by which SoxE factors function, I cannot be certain that this interaction is actually occurring on the Dct promoter. One experiment that would help solidify this hypothesis is to again perform co-immunoprecipitation assays in the melanoma cell line using all endogenous antibodies. This would at least indicate that this interaction is important in the melanocyte cell type. Furthermore, it will be necessary to perform ChIP assays with the Dct promoter to first investigate whether CBP/p300 is recruited to a complex on the Dct promoter, and second, to

investigate whether the presence of SUMO-1 inhibits this recruitment. Obtaining a CBP/p300 *Xenopus* construct would be of benefit as well. Expression pattern studies, misexpression studies and luciferase assays could then be performed with the addition of this construct. I would expect that the additional misexpression of CBP/p300 with $Sox9^{K61,365R}$ and $MitF^{K182,316R}$ would lead to an even greater synergy on the Dct promoter. This interaction may also be important for other cell types in which SoxE factors function, so in the future, when more SoxE cell type-specific promoters become available, it will be imperative to test the effects of this complex on those promoters as well.

However, CBP/p300 is a co-activator complex, and I did not feel that the mere elimination of CBP/p300 binding could account for such an inhibition in synergy on the Dct promoter because SoxE factors have only been seen to function as activators and not repressors. I felt that there must be additional factors present that were recruited to the promoter in order to inhibit the synergy seen on the Dct promoter. Although there are many instances where the activity of SoxE factors can be repressed, the factors themselves have not been shown to exhibit the repressive activity. Stolt and colleagues found that SoxD proteins interfere with the activity of SoxE proteins in oligodendrocyte development (Stolt et al., 2006). SUMOylation of SoxE factors has been shown to negatively regulate wildtype SoxE activity as well (Girard and Goossens, 2006; Komatsu et al., 2004; Oh et al., 2007). However, as mentioned, these data do not pinpoint a precise mechanism by which this inhibition of activity occurs.

The data I have presented at the end of Chapter 3 greatly enhances knowledge surrounding the mechanistic impact that SUMOylation of SoxE factors has on a cell because it explains how these factors are able to have a negative impact on transcription and cell type formation. Along with the co-activator interactions I described earlier, I also found that only

Sox9^{K61,365R}/SUMO-1 and not Sox9^{K61,365R} interacted with the co-repressor Groucho4 by recruiting Groucho4 to the DNA. I have demonstrated that the majority of Sox9 SUMOylation occurs on the DNA, as a construct missing the Sox9 DNA binding domain was not efficiently SUMOylated. This was an important finding because it demonstrated that the inhibitory effects I had seen on the Dct promoter were most likely occurring on the DNA and also told me that my SUMOylated construct was not being sequestered elsewhere in the nucleus. The recruitment of Groucho4 to the Dct promoter by Sox9^{K61,365R}/SUMO-1 explains the inhibition of synergy I see on the Dct promoter. Important to note is that SUMO-1 itself does not bind to Groucho4. I feel that the SUMOylation of Sox9 is imperative to the formation of a transcriptional regulatory complex, which includes Pax3 and Groucho4, on the Dct promoter. The SUMOylation of Sox9 appears to strength protein-protein interactions, and this will most likely become a common theme of regulation used by SoxE proteins throughout development in multiple cell types.

Interestingly, Groucho4 has a role in ear development. Bajoghli and colleagues found that in zebrafish, overexpression of Groucho4 led to the enlargement or ectopic formation of the embryonic ear (Bajoghli et al., 2005). They found that this was in part due to an enhancement of Eya1 expression, which plays a positive role in ear development (Bajoghli et al., 2005). This is interesting, given that my constitutively SUMOylated Sox9 interacts with Groucho4. In novel data presented in this thesis, I found that misexpression of Groucho4 in *Xenopus* also leads to the formation of ectopic/enlarged otocysts. In Chapter 2, I demonstrated that the misexpression of constitutively SUMOylated Sox9 also leads to an enlarged ear phenotype, while Sox9 that cannot be SUMOylated leads to partial inhibition of the ear. Interestingly, I also found that the misexpression of Groucho4 in *Xenopus* led to an inhibition of the neural crest precursors, just as misexpression of my constitutively SUMOylated SoxE factors does. The neural crest precursors

and otic placode develop very closely to one another. Specific neural crest derivatives and the otocyst also form in close proximity to one another. Cells in these fields have a binary choice in what cell type they participate in forming. I feel that the misexpression of constitutively SUMOylated Sox9 is recruiting Groucho4 to neural crest-specific promoters at multiple stages of development. This, in turn, is allowing these factors to partially shut down transcription in the neural crest domains, which may then allow the competency zone that forms the ear to expand. In the wildtype developing embryo, this may be used as a mechanism to clearly define the neural crest precursor zone. One could imagine that SoxE factors are SUMOylated in areas around the neural crest precursor zone so that there is a proper amount of neural crest precursors forming.

It is interesting that misexpression of Groucho4 alone can give rise to ectopic ears. While I cannot prove that the effects of misexpressing Groucho4 are not directly effecting ectopic otocyst formation or the inhibition of the neural crest precursors, it appears to be at least related to the SUMOylation of Sox9. Perhaps the presence of ectopic Groucho4 increases the amount of SUMOylation machinery around Sox9 and this, in turn, leads to a decrease in transcription of certain factors. The closest experiment I can propose to answer this question is to create a hormone-inducible SoxE dominant negative. By misexpressing a hormone-inducible SoxE dominant negative alongside of Groucho4, and not allowing the dominant negative to be active until just prior to neural crest differentiation stages, one could imagine that if Groucho4 acted through the SUMOylation of SoxE factors, then the addition of the dominant negative would block ectopic ear formation. However, this approach is scattered with difficultly, as the addition of a SoxE dominant negative affects many areas of development and may skew result interpretation. A tool that would be extremely useful would be a SoxE targeted ear-specific

promoter. No such promoter has been identified, but it would be helpful to examine the direct effects of SoxE SUMOylation and Groucho4 interaction in this cell type.

SUMOylation is not the only way in which SoxE factors are regulated, and Chapter 4 examines some of these regulatory events. For example, I found a novel site for SoxE factor phosphorylation. These proline-directed threonines are located in the E2 domain of Sox9, Sox10, and the additional SoxE factor, Sox8. To verify this was a real phosphorylation site, I performed several experiments. First, I found that wildtype Sox10 migrated at a slower rate than a mutant form Sox10 that had these threonines mutated to alanines to silence any phosphorylation. This suggested the presence of a modification, as phosphorylation causes proteins to migrate more slowly due to an increased charge on the protein. Then, I was also able to collapse this size difference when treating the wildtype protein with a phosphatase. Lastly and most convincing, upon performing a kinase assay on Sox10, I found that it was in fact a phosphorylated protein and that mutation of these specific threonines silenced this phosphorylation.

Upon looking at the developmental effects this phosphorylation site has on the embryo, I found some interesting data. The alanine mutant, which silences any phosphorylation at this site, behaves similar to misexpression of wild type Sox10 at neural crest precursor stages. It can both expand the precursor population, while also inhibiting this population of cells. Also, with respect to neural crest differentiation stages, the alanine mutant acts analogous to wild type Sox10 misexpression. This data suggests that perhaps the normal setting of Sox10 when it plays a role in neural crest development is to be unphosphorylated. However, the aspartate mutant, which acts as a phosphoprotein, has more exaggerated phenotypes. The expansion of neural crest precursors is much more pronounced. The phenotype this mutation presents looks

strikingly like the phenotype associated with misexpression of the unSUMOylated SoxE protein. I investigated whether the phosphorylation status of Sox10 could influence its ability to be SUMOylated and it did not. However, I did not check to see whether the SUMOylation status of Sox10 could influence the ability of the protein to be phosphorylated. Perhaps SUMOylation of SoxE inhibits phosphorylation at this site and is used as a mechanism to define the neural crest precursor domain during neurula stages. This could tie into the data I obtained with respect to otic placode formation upon misexpression of these mutants. Misexpression of the aspartate mutant leads to a complete loss of ear formation and I know from previous data that SUMOylation of Sox10 enhances ear formation, so perhaps achieving the correct balance of phosphorylated to unphosphorylated Sox10 helps to properly form the ear and neural crest. Interestingly, the phosphorylation of ELK-1 results in the removal of SUMO-1 (reviewed in (Girdwood et al., 2004). However, this mechanism would not quite fit in my scenario, given that I have shown that the aspartate mutant can be SUMOylated. Perhaps though, this should be tried in the kinase assay, as the SoxE factor may need to be truly phosphorylated in order to remove the SUMO moiety.

I feel that some level of phosphorylation may be necessary given that misexpression of the alanine mutant leads to slight inhibition of the otic placode. It could be that the SUMOylation and phosphorylation status of SoxE proteins work together to achieve the proper balance of these factors in the developing embryo. Interestingly, data has recently emerged that defines a phosphorylation-dependent SUMOylation motif (PDSM). This PDSM contains the original SUMOylation consensus site, but also extends to include a serine and proline (ψKXExxSP) (Hietakangas et al., 2006). In some factors that contain this consensus site, phosphorylation of the serine located within this site enhances SUMOylation of the factor

(Gregoire et al., 2006; Hietakangas et al., 2006; Jambunathan and Fontes, 2007; Kang et al., 2006). Jambunathan and Fontes found that in the zinc finger X-linked duplicated family member C (ZXDC), disruption of the PDSM site, by mutating either the lysine or the serine, decreases transcriptional activation of this factor (Jambunathan and Fontes, 2007). Interestingly, SoxE factors contain a PDSM in the activation domain SUMOylation site. In the future, I would suggest testing whether mutation of this serine to alanine disrupts SUMOylation of SoxE factors and if this site has any impact of the function of SoxE factors with respect to either neural crest or ear development.

There are several future directions that could be taken from this project. It would extremely useful to repeat the kinase assays, as the data included in Chapter 4 was performed only once. Clearly defining which kinase is responsible for phosphorylation at these sites would also be beneficial. Numerous *in situ* hybridizations using different neural crest and ear markers would need to be done on phospho-mutant-injected embryos in order to obtain a clear picture of how these sites are affecting development. As mentioned earlier, I did not examine if the SUMOylation state of the SoxE protein affects phosphorylation. I suggest performing the kinase assay on both the double lysine mutant and the constitutively SUMOylated form of the SoxE proteins to address this thought. I would also perform the kinase assay on embryos which were co-injected with both wild type Sox10 and SUMO alone, as having the SUMO moiety attached to the C-terminus of the protein could potentially alter the results of this assay.

Another way in which SoxE factors are most likely dynamically regulated is through the use of different partner proteins. I performed a yeast two-hybrid screen to look for novel Sox10 interactors and subsequently pulled out many other proteins besides SUMO and UBC9. It is important to note for future lab members that I only screened a small pool of the interactors that I

obtained. I have twenty-nine other glycerol stocks from this screen in the –80˚C freezer. In the future, these could easily be plated and screened to hunt for other potentially interesting interactors. However, in the glycerol stock that I did screen, I found three novel interactors that had expression patterns in the correct time and place consistent with a role as a Sox10-interacting protein. While I performed some preliminary *in situ* hybridizations with these factors, this would need to be done again in much more detail to grasp a full understanding of the expression patterns of these factors. Obtaining the full-length versions of these factors would also be highly beneficial in order to fully characterize the effects of these factors in the developing embryo. It is my feeling that at least one of these three novel factors will have an important impact on neural crest development and I highly suggest carrying experiments through with these factors.

Clearly, as it is a common theme throughout this thesis, SoxE factors are regulated throughout development by the use of different partner proteins. The SoxE proteins have four potential protein-protein interaction areas, the E1 domain, E2 domain, HMG domain, and activation domain. One way to begin piecing together what domains are important for the formation of different cell types is to try deleting the different SoxE domains and see what effect this has on the embryo. The reason I wanted to look at different deletion constructs is because of the data that came out of the Saint-Jeanett lab. Aoki and colleagues found that misexpression of a form of Sox10 with only the E2 domain and activation domain could expand the neural crest precursor marker Slug in a similar manner as the misexpression of full-length Sox10 (Aoki et al., 2003). This was interesting, as it suggested that perhaps neither the E1 domain nor the DNA binding domain were necessary to elicit the proper developmental response with respect to neural crest precursor formation. In my hands, I found similar, but slightly altered, results. First of all, as presented in Chapter 2, I find variable phenotypes, both expansion and inhibition of

neural crest precursors with misexpression of full-length Sox10, which I feel is due to the SUMOylation state of the factor. With the same deletion constructs used by Aoki and colleagues, I find both expansion and inhibition of the neural crest marker Slug, mirroring my full-length results. Interesting is that the C-terminal SUMOylation site is still present in this deletion construct. In the future, it would beneficial to create my SUMOylation mutants in the background of this deletion construct, as misexpression of the full-length SUMO mutants always produced very consistent phenotypes. By doing this, a better grasp of whether this construct acts similarly to the full-length construct will be obtained, as the results will be more consistent and directly comparable to the full-length results. Interestingly, though, is that in my hands, the misexpression of this construct led to an inhibition of the otic placode. There are several possible conclusions to be drawn from this result. One conclusion that can be drawn from this result is that in the embryos examined, the neural crest precursor domain had expanded and the ear was no longer able to properly form. Again, looking at several more *in situ* hybridization probes both early and late would further the understanding of what domains are necessary for what cell types. Also, an important additional future direction would be to perform rescue experiments with this construct to see whether depletion of endogenous Sox10 can be rescued by misexpression of this deletion construct. This would provide the most clear and direct evidence to support the claim that this deletion construct can functionally replace the full-length protein.

I also investigated the effects of a Sox9 construct that had the HMG, or DNA binding domain, taken out (Sox9ΔHMG). I looked specifically at the effects this construct had on otic placode development, as well as later neural crest derivative and otocyst formation. Interestingly, I found that misexpression of wildtype Sox9 and misexpression of Sox9ΔHMG gave similar phenotypes with respect to early ear formation. Both of these factors led to ectopic

Pax8 staining, which marks the otic placode. This data suggests that the HMG domain is not necessary for early ear formation. If it were necessary, one would expect no effect on the ear when misexpressing this construct. It is possible that the HMG domain not be necessary, as on the cRET enhancer, Pax3 binds the DNA and Sox10 binds to Pax3 and not the DNA (Lang and Epstein, 2003). However, in this case, Pax3 still binds to Sox10 in the HMG domain, but not in the area required for the physical association with DNA, so this phenomenon would not be possible with the loss of the entire HMG domain (Lang and Epstein, 2003). However, there are several known SoxE partners that bind outside of the HMG domain, such as ß-catenin and CBP (Akiyama et al., 2004; Tsuda et al., 2003). This suggests it would still be possible for the HMG domain to not be necessary.

However, with respect to later neural crest and otocyst markers, I saw no effect with misexpression of Sox9ΔHMG, while the misexpression of wildtype Sox9 gave a clear expansion of the ear and melanoblasts. This data suggests that the HMG domain is necessary for the formation of the neural crest derivatives and later ear specification. If it were not necessary, I would expect to see the same, or at least some of the same, ectopic expansion as in wildtype. Clearly, misexpression studies do not lead to strong developmental answers when questioning the necessity of a factor or a particular domain. As previously suggested, first knocking down protein levels of endogenous SoxE and rescuing with the deletion construct would be ideal to answer these questions. In fact, I have created a full panel of Sox9 and Sox10 deletion constructs as shown in Figure 4.13. This is an excellent project for a future lab member who is interested in piecing together the complex puzzle of domain necessity. I would suggest beginning with morpholino studies and proceeding with the deletion construct rescues. Using different *in situ* hybridization probes specific to either the ear or SoxE neural crest derivatives,

such as melanocytes, would be useful to understand what domains are necessary to form what cell types. Then, a yeast two-hybrid screen could be performed to look for alternate partner proteins specific to different domains, using the deletion constructs as bait.

By investigating which deletion constructs are necessary for the cell types SoxE factors form, scientists can begin to understand how the developing embryo can offer a multitude of uses for the same transcription factor. Also, by studying the necessity or lack there of the DNA binding domain in SoxE factors, we can embrace the possibility that transcription factors may not need to bind DNA to produce results in the embryo.

Significance of the thesis

In summary, this thesis has taken two transcriptional activators thought to have different functions in the developing embryo and proved that they are functionally equivalent. It has also demonstrated a novel way in which SUMOylation of a protein can function by dynamically regulating cell fate decisions. The SUMOylation of Sox9 and Sox10 can alter between neural crest and inner ear formation. This provided one answer to the development question of how one individual transcription factor can function very differently to produce diverse results.

This thesis then went on to demonstrate the mechanism by which SUMOylation regulates SoxE factors on the promoter of the melanocyte-specific gene, Dct. First, I found evidence suggesting that the majority of SoxE factor SUMOylation occurs on the DNA. When the HMG domain is deleted, the amount of SUMOylated SoxE species heavily decreases. I also found that an alternating network of co-factor recruitment occurs during specific SUMOylation states. When SoxE factors are not SUMOylated, the co-activator, CBP, is recruited to potentially synergistically enhance Dct transcription (along with the help of MitF). When SoxE factors are

constitutively SUMOylated, a complex mechanism involving recruitment of the co-repressor, Groucho4 ensues. This recruitment inhibits synergistic activation of the Dct promoter. Intriguingly, misexpression of Groucho4 leads to the formation of ectopic ears in the developing embryo, just as SUMOylation of SoxE factors can. As constitutively SUMOylated Sox9 interacts with Groucho4, this suggests that it is the SUMOylation of Sox9 and henceforth interaction with Groucho4 that is giving rise to ectopic ears. These ectopic ears may form because the competency zone of neural crest formation is inhibited and the ear zone may be expanded.

This thesis also presents several new lines of SoxE factor regulation in Chapter 4. While the data is very preliminary, there is strong evidence suggesting that SoxE proteins contain a novel phosphorylation site within them and this site has a clear effect on neural crest and ear formation. Several novel SoxE partner proteins were also identified and may lead to a greater understanding of how these factors function in different cell types. And finally, by deleting different conserved SoxE domains, preliminary evidence suggests that the HMG, or DNA binding domain of Sox9, may not be necessary for early otic placode development.

Taken together, the data presented in this thesis demonstrates several clear and novel lines of SoxE factor regulation in the developing embryo. One of the main themes of this thesis is that SUMO is not an independent moiety, but is a tool the cell uses to dynamically regulate developmental events by building and regulating the formation of transcriptional complexes. This data has greatly contributed to the overall knowledge of how transcription factors are dynamically regulated throughout the time course of development.

REFERENCES

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J Neurosci* **14**, 3475-86.

Akitaya, T. and Bronner-Fraser, M. (1992). Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration. *Dev Dyn* **194**, 12-20.

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J. M., Taketo, M. M., Nakamura, T., Behringer, R. R. et al. (2004). Interactions between Sox9 and ß-catenin control chondrocyte differentiation. *Genes Dev* **18**, 1072-1087.

Amiel, J., Watkin, P. M., Tassabehji, M., Read, A. P. and Winter, R. M. (1998). Mutation of the MITF gene in albinism-deafness syndrome (Tietz syndrome). *Clin Dysmorphol* **7**, 17-20.

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y. H., Credidio, C. and Saint-Jeannet, J. P. (2003). Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. *Dev Biol* **259**, 19-33.

Bajoghli, B., Aghaallaei, N. and Czerny, T. (2005). Groucho corepressor proteins regulate otic vesicle outgrowth. *Dev Dyn* **233**, 760-71.

Barald, K. F. and Kelley, M. W. (2004). From placode to polarization: new tunes in inner ear development. *Development* **131**, 4119-4130.

Barembaum, M. and Bronner-Fraser, M. (2005). Early steps in neural crest specification. *Semin Cell Dev Biol* **16**, 642-6.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2**, 84-9.

Bellmeyer, A., Krase, J., Lindgren, J. and LaBonne, C. (2003). The Protooncogene c-Myc Is an Essential Regulator of Neural Crest Formation in Xenopus. *Developmental Cell* **4**, 827-839.

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nat Genet* **22**, 85-9.

Bi, W., Huang, W., Whitworth, D. J., Zhang, Z., Deng, J. M., Behringer, R. R. and de Crombrugghe, B. (2001). Haploinsufficiency of Sox9 results in defective precartilaginous mesenchymal condensations and premature skeletal mineralization. *Proc Natl Acad Sci U S A* **98**, 6698-6703.

Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O. and Goossens, M. (2001). Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. *Hum Mol Genet* **10**, 2783-95.

Bondurand, N., Kuhlbrodt, K., Pingault, V., Enderich, J., Sajus, M., Tommerup, N., Warburg, M., Hennekam, R. C., Read, A. P., Wegner, M. et al. (1999). A molecular analysis of the yemenite deaf-blind hypopigmentation syndrome: SOX10 dysfunction causes different neurocristopathies. *Hum Mol Genet* **8**, 1785-9.

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Le Caignec, C., Wegner, M. and Goossens, M. (2000). Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Hum Mol Genet* **9**, 1907-17.

Bonstein, L., Elias, S. and Frank, D. (1998). Paraxial-fated mesoderm is required for neural crest induction in Xenopus embryos. *Dev Biol* **193**, 156-68.

Bowles, J., Schepers, G. and Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* **227**, 239-55.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev* **15**, 66-78.

Bronner-Fraser, M. and Fraser, S. (1989). Developmental potential of avian trunk neural crest cells in situ. *Neuron* **3**, 755-66.

Bronner-Fraser, M. and Fraser, S. E. (1988). Application of new technologies to studies of neural crest migration and differentiation. *Am J Med Genet Suppl* **4**, 23-39.

Budd, P. S. and Jackson, I. J. (1995). Structure of the mouse tyrosinase-related protein-2/dopachrome tautomerase (Tyrp2/Dct) gene and sequence of two novel slaty alleles. *Genomics* **29**, 35-43.

Cai, Y., Brophy, P. D., Levitan, I., Stifani, S. and Dressler, G. R. (2003). Groucho suppresses Pax2 transactivation by inhibition of JNK-mediated phosphorylation. *Embo J* **22**, 5522-9.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F. and Nieto, M. A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2**, 76-83.

Chan, K. K., Wong, C. K. Y., Lui, V. C. H., Tam, P. K. H. and Sham, M. H. (2003). Analysis of SOX10 Mutations Identified in Waardenburg-Hirschsprung Patients: Differential Effects on Target Gene Regulation. *J Cell Biol* **90**, 573-585.

Chang, C. and Hemmati-Brivanlou, A. (1998). Neural crest induction by Xwnt7B in Xenopus. *Dev Biol* **194**, 129-34.

Chang, W., Nunes, F. D., De Jesus-Escobar, J. M., Harland, R. and Wu, D. K. (1999). Ectopic noggin blocks sensory and nonsensory organ morphogenesis in the chicken inner ear. *Dev Biol* **216**, 369-81.

Chang, W., ten Dijke, P. and Wu, D. K. (2002). BMP pathways are involved in otic capsule formation and epithelial-mesenchymal signaling in the developing chicken inner ear. *Dev Biol* **251**, 380-94.

Cheung, M. and Briscoe, J. (2003). Neural crest development is regulated by the transcription factor Sox9. *Development* **130**, 5681-5693.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt-8, a Xenopus Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-55.

Cook, A. L., Smith, A. G., Smit, D. J., Leonard, J. H. and Sturm, R. A. (2005). Co-expression of SOX9 and SOX10 during melanocytic differentiation in vitro. *Exp Cell Res* **308**, 222-35.

Deardorff, M. A., Tan, C., Saint-Jeannet, J. P. and Klein, P. S. (2001). A role for frizzled 3 in neural crest development. *Development* **128**, 3655-63.

Eberhard, D., Jimenez, G., Heavey, B. and Busslinger, M. (2000). Transcriptional repression by Pax5 (BSAP) through interaction with corepressors of the Groucho family. *Embo J* **19**, 2292-303.

Ekker, M., Akimenko, M. A., Bremiller, R. and Westerfield, M. (1992). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* **9**, 27-35.

Ellies, D. L., Stock, D. W., Hatch, G., Giroux, G., Weiss, K. M. and Ekker, M. (1997). Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish dlx genes. *Genomics* **45**, 580-90.

Elworthy, S., Lister, J. A., Carney, T. J., Raible, D. W. and Kelsh, R. N. (2003). Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development. *Development* **130**, 2809-18.

Estojak, J., Brent, R. and Golemis, E. A. (1995). Correlation of two-hybrid affinity data with in vitro measurements. *Mol Cell Biol* **15**, 5820-9.

Ferguson, C. A. and Kidson, S. H. (1997). The regulation of tyrosinase gene transcription. *Pigment Cell Res* **10**, 127-38.

Furumatsu, T., Tsuda, M., Taniguchi, N., Tajima, Y. and Asahara, H. (2005a). Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. *J Biol Chem* **280**, 8343-50.

Furumatsu, T., Tsuda, M., Yoshida, K., Taniguchi, N., Ito, T., Hashimoto, M. and Asahara, H. (2005b). Sox9 and p300 cooperatively regulate chromatin-mediated transcription. *J Biol Chem*.

Fuse, N., Yasumoto, K., Suzuki, H., Takahashi, K. and Shibahara, S. (1996). Identification of a melanocyte-type promoter of the microphthalmia-associated transcription factor gene. *Biochem Biophys Res Commun* **219**, 702-7.

Gerlach, L. M., Hutson, M. R., Germiller, J. A., Nguyen-Luu, D., Victor, J. C. and Barald, K. F. (2000). Addition of the BMP4 antagonist, noggin, disrupts avian inner ear development. *Development* **127**, 45-54.

Gill, G. (2003). Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. *Curr Opin Genet Dev* **13**, 108-13.
Gill, G. (2004). SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* **18**, 2046-2059.

Gilmour, D. T., Maischein, H. and Nüsslein-Volhard, C. (2002). Migration and Function of a Glial Subtype in the Vertebrate Peripheral Nervous System. *Neuron* **34**, 577-588.

Girard, M. and Goossens, M. (2006). Sumoylation of the SOX10 transcription factor regulates its transcriptional activity. *FEBS Lett* **580**, 1635-41.

Girdwood, D. W. H., Tatham, M. H. and Hay, R. T. (2004). SUMO and transcriptional regulation. *Semin Cell Dev Biol* **15**, 201-210.

Golemis, E. A. and Brent, R. (1992). Fused protein domains inhibit DNA binding by LexA. *Mol Cell Biol* **12**, 3006-14.

Goodson, M. L., Hong, Y., Rogers, R., Matunis, M. J., Park-Sarge, O. K. and Sarge, K. D. (2001). Sumo-1 modification regulates the DNA binding activity of heat shock transcription factor 2, a promyelocytic leukemia nuclear body associated transcription factor. *J Biol Chem* **276**, 18513-8.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M. and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. *Embo J* **18**, 6462-71.

Gregoire, S., Tremblay, A. M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z., Wu, Z., Giguere, V. and Yang, X. J. (2006). Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J Biol Chem* **281**, 4423-33.

Hay, R. (2005). SUMO: a history of modification. *Mol Cell* **18**, 1-12.

Hearing, V. J. and Tsukamoto, K. (1991). Enzymatic control of pigmentation in mammals. *Faseb J* **5**, 2902-9.

Hecker, C. M., Rabiller, M., Haglund, K., Bayer, P. and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* **281**, 16117-27.

Heller, N. and Brandli, A. W. (1999). Xenopus Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. *Dev Genet* **24**, 208-19.

Hemesath, T. J., Steingrimsson, E., McGill, G., Hansen, M. J., Vaught, J., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., Jenkins, N. A. and Fisher, D. E. (1994). microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. *Genes Dev* **8**, 2770-80.

Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M. (1998). Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. *Proc Natl Acad Sci U S A* **95**, 5161-5.

Herbrand, H., Guthrie, S., Hadrys, T., Hoffmann, S., Arnold, H. H., Rinkwitz-Brandt, S. and Bober, E. (1998). Two regulatory genes, cNkx5-1 and cPax2, show different responses to local signals during otic placode and vesicle formation in the chick embryo. *Development* **125**, 645-54.

Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A. and Sistonen, L. (2006). PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* **103**, 45-50.

Holmstrom, S., Van Antwerp, M. E. and Iniguez-Lluhi, J. A. (2003). Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. *Proc Natl Acad Sci U S A* **100**, 15758-63.

Hong, Y., Rogers, R., Matunis, M. J., Mayhew, C. N., Goodson, M. L., Park-Sarge, O. K. and Sarge, K. D. (2001). Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J Biol Chem* **276**, 40263-7.

Honore, S. M., Aybar, M. J. and Mayor, R. (2003). Sox10 is required for the early development of the prospective neural crest in Xenopus embryos. *Dev Biol* **260**, 79-96.

Huang, W., Zhou, X., Lefebvre, V. and de Crombrugghe, B. (2000). Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Mol Cell Biol* **20**, 4149-58.

Hutson, M. R., Lewis, J. E., Nguyen-Luu, D., Lindberg, K. H. and Barald, K. F. (1999). Expression of Pax2 and patterning of the chick inner ear. *J Neurocytol* **28**, 795-807.

Iniguez-Lluhi, J. A. and Pearce, D. (2000). A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. *Mol Cell Biol* **20**, 6040-50.

Isaacs, H. V., Tannahill, D. and Slack, J. M. (1992). Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-20.

Itoh, K., Antipova, A., Ratcliffe, M. J. and Sokol, S. (2000). Interaction of dishevelled and Xenopus axin-related protein is required for wnt signal transduction. *Mol Cell Biol* **20**, 2228-38.

Jambunathan, S. and Fontes, J. D. (2007). Sumoylation of the zinc finger protein ZXDC enhances the function of its transcriptional activation domain. *Biol Chem* **388**, 965-72.

Jiao, Z., Mollaaghababa, R., Pavan, W. J., Antonellis, A., Green, E. D. and Hornyak, T. J. (2004). Direct interaction of Sox10 with the promoter of murine Dopachrome Tautomerase (Dct) and synergistic activation of Dct expression with Mitf. *Pigment Cell Res* **17**, 352-62.

Kamachi, Y., Uchikawa, M. and Kondoh, H. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* **16**, 182-7.

Kang, J., Gocke, C. B. and Yu, H. (2006). Phosphorylation-facilitated sumoylation of MEF2C negatively regulates its transcriptional activity. *BMC Biochem* **7**, 5.

Kelsh, R. N., Dutton, K., Medlin, J. and Eisen, J. S. (2000). Expression of zebrafish *fkd6* in neural crest-derived glia. *Mech Dev* **93**, 161-64.

Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron* **38**, 17-31.

Kimura, Y., Matsunami, H., Inoue, T., Shimamura, K., Uchida, N., Ueno, T., Miyazaki, T. and Takeichi, M. (1995). Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos. *Dev Biol* **169**, 347-58.

Komatsu, T., Mizuaski, H., Mukai, T., Ogawa, H., Baba, D., Shirakawa, M., Hatakeyama, S., Nakayama, K.I., Yamamoto, H., Kikuchi, A. and Morohashi, K. (2004). SUMO-1 Modification of the Syngery Control Motif of Ad4BP/SF-1 Regulates Synergistic Transcription between Ad4BP/SF-1 and Sox9. *Mol Endocrinol* **18**, 2451-2462.

Komatsu, T., Mizusaki, H., Mukai, T., Ogawa, H., Baba, D., Shirakawa, M., Hatakeyama, S., Nakayama, K. I., Yamamoto, H., Kikuchi, A. et al. (2004). Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. *Mol Endocrinol* **18**, 2451-62.

Koopman, P., Schepers, G., Brenner, S. and Venkatesh, B. (2004). Origin and diversity of the SOX transcription factor gene family: genome-wide analysis in Fugu rubripes. *Gene* **328**, 177-86.

Kumasaka, M., Sato, S., Yajima, I., Goding, C. R. and Yamamoto, H. (2005). Regulation of melanoblast and retinal pigment epithelium development by Xenopus laevis Mitf. *Dev Dyn* **234**, 523-34.

LaBonne, C. and Bronner-Fraser, M. (1998a). Induction and patterning of the neural crest, a stem cell-like precursor population. *J Neurobiol* **36**, 175-89.

LaBonne, C. and Bronner-Fraser, M. (1998b). Neural crest induction in Xenopus: evidence for a two-signal model. *Development* **125**, 2403-14.

LaBonne, C. and Bronner-Fraser, M. (1999). Molecular mechanisms of neural crest formation. *Annu Rev Cell Dev Biol* **15**, 81-112.

LaBonne, C. and Bronner-Fraser, M. (2000). Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev Biol* **221**, 195-205.

Lang, D., Chen, F., Milewski, R., Li, J., Lu, M. M. and Epstein, J. A. (2000). Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. *J Clin Invest* **106**, 963-71.

Lang, D. and Epstein, J. A. (2003). Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. *Hum Mol Genet* **12**, 937-45.

Lang, D., Lu, M. M., Huang, L., Engleka, K. A., Zhang, M., Chu, E. Y., Lipner, S., Skoultchi, A., Millar, S. E. and Epstein, J. A. (2005). Pax3 functions at a nodal point in melanocyte stem cell differentiation. *Nature* **433**, 884-7.

Lawoko-Kerali, G., Rivolta, M. N. and Holley, M. (2002). Expression of the transcription factors GATA3 and Pax2 during development of the mammalian inner ear. *J Comp Neurol* **442**, 378-91.

Lee, M., Goodall, J., Verastegui, C., Ballotti, R. and Goding, C. R. (2000). Direct Regulation of the Microphthalmia Promoter by Sox10 Links Waardenburg-Shah Syndrome (WS4)-associated Hypopigmentation and Deafness to WS2. *J Biol Chem* **275**, 37978-37983.

Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N. and de Crombrugghe, B. (1997). SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol* **17**, 2336-46.

Levy, C., Khaled, M. and Fisher, D. E. (2006). MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med* **12**, 406-14.

Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. and Raible, D. W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-67.

Liu, D., Chu, H., Maves, L., Yan, Y., Morcos, P. A., Postlethwait, J. H. and Westerfield, M. (2003). Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. *Development* **130**, 2213-2224.

Long, J., Wang, G., Dongming, H. E. and Lui, F. (2004). Repression of Smad4 transcriptional activity by SUMO modification. *Biochem. J.* **379**, 23-29.

Ludwig, A., Rehberg, S. and Wegner, M. (2004). Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. *FEBS Lett* **556**, 236-44.

Lynch, M. and Force, A. (2000). The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154**, 459-73.

Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F. and Boizet-Bonhoure, B. (2005). Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation. *Embo J* **24**, 1798-809.

Marchant, L., Linker, C., Ruiz, P., Guerrero, N. and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev Biol* **198**, 319-29.

Mayor, R., Guerrero, N. and Martinez, C. (1997). Role of FGF and noggin in neural crest induction. *Dev Biol* **189**, 1-12.

McClintock, J. M., Carlson, R., Mann, D. M. and Prince, V. E. (2001). Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes. *Development* **128**, 2471-84.

Miller, A. J., Levy, C., Davis, I. J., Razin, E. and Fisher, D. E. (2005). Sumoylation of MITF and its related family members TFE3 and TFEB. *J Biol Chem* **280**, 146-55.

Moore, K. J. (1995). Insight into the microphthalmia gene. *Trends Genet* **11**, 442-8.

Moury, J. D. and Jacobson, A. G. (1989). Neural fold formation at newly created boundaries between neural plate and epidermis in the axolotl. *Dev Biol* **133**, 44-57.

Moury, J. D. and Jacobson, A. G. (1990). The origins of neural crest cells in the axolotl. *Dev Biol* **141**, 243-53.

Murakami, H. and Arnheiter, H. (2005). Sumoylation modulates transcriptional activity of MITF in a promoter-specific manner. *Pigment Cell Res* **18**, 265-277.

Nakagawa, S. and Takeichi, M. (1995). Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **121**, 1321-32.

Navascues, J., Bengoechea, R., Tapia, O., Vaque, J. P., Lafarga, M. and Berciano, M. T. (2007). Characterization of a new SUMO-1 nuclear body (SNB) enriched in pCREB, CBP, c-Jun in neuron-like UR61 cells. *Chromosoma* **116**, 441-51.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. *Dev Biol* **199**, 93-110.

Nieto, M. A. (2001). The early steps of neural crest development. *Mech Dev* **105**, 27-35.

Nissen, R. M., Yan, J., Amsterdam, A., Hopkins, N. and Burgess, S. M. (2003). Zebrafish foxi one modulates cellular responses to Fgf signaling required for the integrity of ear and jaw patterning. *Development* **130**, 2543-54.

Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P. (1990). Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development* **109**, 797-809.

Oh, H. J., Kido, T. and Lau, Y. F. (2007). PIAS1 interacts with and represses SOX9 transactivation activity. *Mol Reprod Dev* **74**, 1446-55.

Oliver, G., Wehr, R., Jenkins, N. A., Copeland, N. G., Cheyette, B. N., Hartenstein, V., Zipursky, S. L. and Gruss, P. (1995). Homeobox genes and connective tissue patterning. *Development* **121**, 693-705.

Ozaki, H., Nakamura, K., Funahashi, J., Ikeda, K., Yamada, G., Tokano, H., Okamura, H. O., Kitamura, K., Muto, S., Kotaki, H. et al. (2004). Six1 controls patterning of the mouse otic vesicle. *Development* **131**, 551-62.

Paratore, C., Eichenberger, C., Suter, U. and Sommer, L. (2002). Sox10 haploinsufficiencty affects maintenance of progenitor cells in a mouse model of Hirschsprung disease. *Hum Mol Genet* **11**, 3075-85.

Passeron, T., Valencia, J. C., Bertolotto, C., Hoashi, T., Le Pape, E., Takahashi, K., Ballotti, R. and Hearing, V. J. (2007). SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. *Proc Natl Acad Sci U S A* **104**, 13984-9.

Peirano, R. I., Goerich, D. E., Riethmacher, D. and Wegner, M. (2000). Protein zero gene expression is regulated by the glial transcription factor Sox10. *Mol Cell Biol* **20**, 3198-209.

Pfeffer, P. L., Gerster, T., Lun, K., Brand, M. and Busslinger, M. (1998). Characterization of three novel members of the zebrafish Pax2/5/8 family: dependency of Pax5 and Pax8 expression on the Pax2.1 (noi) function. *Development* **125**, 3063-74.

Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H. and Pavan, W. J. (2000). Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. *Hum Genet* **107**, 1-6.

Potterf, S. B., Mollaaghababa, R., Hou, L., Southard-Smith, E. M., Hornyak, T. J., Arnheiter, H. and Pavan, W. J. (2001). Analysis of SOX10 function in neural crest-derived melanocyte development: SOX10-dependent transcriptional control of dopachrome tautomerase. *Dev Biol* **237**, 245-57.

Poukka, H., Karvonen, U., Janne, O. A. and Palvimo, J. J. (2000). Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). *Proc Natl Acad Sci U S A* **97**, 14145-50.

Raven, C. P. and Kloos, J. (1945). Induction by medial and lateral pieces of the archenteron roof with special reference to the determination of the neural crest. *Acta Neerl Morphol* **5**, 348-362.

Read, A. P. and Newton, V. E. (1997). Waardenburg syndrome. *J Med Genet* **34**, 656-65.

Riley, B. B., Chiang, M., Farmer, L. and Heck, R. (1999). The deltaA gene of zebrafish mediates lateral inhibition of hair cells in the inner ear and is regulated by pax2.1. *Development* **126**, 5669-78.

Riley, B. B. and Phillips, B. T. (2003). Ringing in the new ear: resolution of cell interactions in otic development. *Dev Biol* **261**, 289-312.

Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P. and Hay, R. T. (1999). SUMO-1 modification activates the transcriptional response of p53. *Embo J* **18**, 6455-61.

Ross, S., Best, J. L., Zon, L. I. and Gill, G. (2002). SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-842.

Saint-Germain, N., Lee, Y., Zhang, Y., Sargent, T. D. and Saint-Jeannet, J. (2004). Specification of the otic placode depends on Sox9 function in Xenopus. *Development* **131**, 1755-1763.

Saint-Jeannet, J. P., He, X., Varmus, H. E. and Dawid, I. B. (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc Natl Acad Sci U S A* **94**, 13713-8.

Sakai, D., Suzuki, T., Osumi, N. and Wakamatsu, Y. (2006). Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development. *Development* **133**, 1323-33.

Sato, S., Roberts, K., Gambino, G., Cook, A., Kouzarides, T. and Goding, C. R. (1997). CBP/p300 as a co-factor for the Microphthalmia transcription factor. *Oncogene* **14**, 3083-92.

Savarirayan, R., Robertson, S. P., Bankier, A. and Rogers, J. G. (2003). Variable expression of campomelic dysplasia in a father and his 46, XY daughter. *Pediatr Pathol Mol Med* **22**, 37-46.

Selleck, M. A. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-38.

Selleck, M. A. and Bronner-Fraser, M. (1996). The genesis of avian neural crest cells: a classic embryonic induction. *Proc Natl Acad Sci U S A* **93**, 9352-7.

Selzer, E., Wacheck, V., Lucas, T., Heere-Ress, E., Wu, M., Weilbaecher, K. N., Schlegel, W., Valent, P., Wrba, F., Pehamberger, H. et al. (2002). The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. *Cancer Res* **62**, 2098-103.

Shibahara, S., Yasumoto, K., Amae, S., Udono, T., Watanabe, K., Saito, H. and Takeda, K. (2000). Regulation of pigment cell-specific gene expression by MITF. *Pigment Cell Res* **13 Suppl 8**, 98-102.

Smith, S. D., Kelley, P. M., Kenyon, J. B. and Hoover, D. (2000). Tietz syndrome (hypopigmentation/deafness) caused by mutation of MITF. *J Med Genet* **37**, 446-8.

Solomon, K. S., Kudoh, T., Dawid, I. B. and Fritz, A. (2003). Zebrafish foxi1 mediates otic placode formation and jaw development. *Development* **130**, 929-40.

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat Genet* **18**, 60-4.

Spokony, R. F., Aoki, Y., Saint-Germain, N., Magner-Fink, E. and Saint-Jeannet, J. P. (2002). The transcription factor Sox9 is required for cranial neural crest development in Xenopus. *Development* **129**, 421-32.

Steel, K. P., Davidson, D. R. and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-9.

Steingrimsson, E., Copeland, N. G. and Jenkins, N. A. (2004). Melanocytes and the microphthalmia transcription factor network. *Annu Rev Genet* **38**, 365-411.

Stolt, C. C., Lommes, P., Friedrich, R.P., and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. *Development* **131**, 2349-2358.

Stolt, C. C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., Bartsch, U. and Wegner, M. (2002). Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev* **16**, 165-70.

Stolt, C. C., Schlierf, A., Lommes, P., Hillgartner, S., Werner, T., Kosian, T., Sock, E., Kessaris, N., Richardson, W. D., Lefebvre, V. et al. (2006). SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. *Dev Cell* **11**, 697-709.

Streit, A. (2002). Extensive cell movements accompany formation of the otic placode. *Dev Biol* **249**, 237-54.

Sulaimon, S. S. and Kitchell, B. E. (2003). The biology of melanocytes. *Vet Dermatol* **14**, 57-65.

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P. and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-5.

Tan, C., Deardorff, M. A., Saint-Jeannet, J. P., Yang, J., Arzoumanian, A. and Klein, P. S. (2001). Kermit, a frizzled interacting protein, regulates frizzled 3 signaling in neural crest development. *Development* **128**, 3665-74.

Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H. and Hay, R. T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* **276**, 35368-74.

Taylor, K. M. and Labonne, C. (2005). SoxE factors function equivalently during neural crest and inner ear development and their activity is regulated by SUMOylation. *Dev Cell* **9**, 593-603.

Torres, M. and Giraldez, F. (1998). The development of the vertebrate inner ear. *Mech Dev* **71**, 5-21.

Torres, M., Gomez-Pardo, E. and Gruss, P. (1996). Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-91.

Tsuda, M., Takahashi, S., Takahashi, Y. and Asahara, H. (2003). Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. *J Biol Chem* **278**, 27224-9.

Tsukamoto, K., Palumbo, A., D'Ischia, M., Hearing, V. J. and Prota, G. (1992). 5,6-Dihydroxyindole-2-carboxylic acid is incorporated in mammalian melanin. *Biochem J* **286 (Pt 2)**, 491-5.

Vachtenheim, J. and Novotna, H. (1999). Expression of genes for microphthalmia isoforms, Pax3 and MSG1, in human melanomas. *Cell Mol Biol (Noisy-le-grand)* **45**, 1075-82.

Vachtenheim, J., Novotna, H. and Ghanem, G. (2001). Transcriptional repression of the microphthalmia gene in melanoma cells correlates with the unresponsiveness of target genes to ectopic microphthalmia-associated transcription factor. *J Invest Dermatol* **117**, 1505-11.

Vachtenheim, J., Sestakova, B. and Tuhackova, Z. (2007). Inhibition of MITF transcriptional activity independent of targeting p300/CBP coactivators. *Pigment Cell Res* **20**, 41-51.

Valentine, S. A., Chen, G., Shandala, T., Fernandez, J., Mische, S., Saint, R. and Courey, A. J. (1998). Dorsal-mediated repression requires the formation of a multiprotein repression complex at the ventral silencer. *Mol Cell Biol* **18**, 6584-94.

Vallin, J., Thuret, R., Giacomello, E., Faraldo, M. M., Thiery, J. P. and Broders, F. (2001). Cloning and characterization of three Xenopus slug promoters reveal direct regulation by Lef/beta-catenin signaling. *J Biol Chem* **276**, 30350-8.

Verastegui, C., Bille, K., Ortonne, J. P. and Ballotti, R. (2000). Regulation of the Microphthalmia-associated Transcription Factor Gene by the Waardenburg Syndrome Type 4 Gene, SOX10. *J Biol Chem* **275**, 30757-30760.

Watanabe, K., Takeda, K., Katori, Y., Ikeda, K., Oshima, T., Yasumoto, K., Saito, H., Takasaka, T. and Shibahara, S. (2000). Expression of the Sox10 gene during mouse inner ear development. *Brain Res Mol Brain Res* **84**, 141-5.

Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* **27**, 1409-20.

Wilson, M. and Koopman, P. (2002). Matching SOX: partner proteins and co-factors of the SOX family of transcriptional regulators. *Curr Opin Genet Dev* **12**, 441-6.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-3.

Wilson, P. A. and Hemmati-Brivanlou, A. (1997). Vertebrate neural induction: inducers, inhibitors, and a new synthesis. *Neuron* **18**, 699-710.

Wilson, P. A., Lagna, G., Suzuki, A. and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. *Development* **124**, 3177-84.

Wu, J., Yang, J. and Klein, P. S. (2005). Neural crest induction by the canonical Wnt pathway can be dissociated from anterior-posterior neural patterning in Xenopus. *Dev Biol* **279**, 220-32.

Yasumoto, K., Takeda, K., Saito, H., Watanabe, K., Takahashi, K. and Shibahara, S. (2002). Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. *Embo J* **21**, 2703-14.

Yasumoto, K., Yokoyama, K., Shibata, K., Tomita, Y. and Shibahara, S. (1994). Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol Cell Biol* **14**, 8058-70.

Yasumoto, K., Yokoyama, K., Takahashi, K., Tomita, Y. and Shibahara, S. (1997). Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J Biol Chem* **272**, 503-9.

Zheng, W., Huang, L., Wei, Z. B., Silvius, D., Tang, B. and Xu, P. X. (2003). The role of Six1 in mammalian auditory system development. *Development* **130**, 3989-4000.

APPENDIX I

MODULATING THE ACTIVITY OF NEURAL CREST REGULATORY FACTORS

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Modulating the activity of neural crest regulatory factors

Kimberly M Taylor[1] and Carole LaBonne[1,2]

Substantial progress has been made in defining the regulatory factors involved in generating multipotent neural crest cells at the neural plate border of vertebrate embryos, controlling the onset of their migratory behavior, and directing their differentiation into one of a diverse array of derivatives. Growing evidence suggests that these factors function as a complex network, in some cases displaying overlapping functions and cross-regulatory interactions. Mechanisms are emerging for how some of these regulatory components are controlled post-translationally and the extent to which their activities are conserved across species.

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Introduction

Patterning the vertebrate body plan depends upon the neural crest, a transient population of stem cell-like progenitors that distinguishes the craniates/vertebrates from other metazoans. Neural crest cells arise at the neural plate border in response to signals from the adjacent non-neural ectoderm and/or the underlying mesoderm. After undergoing an Epithelial Mesenchymal and Transition (EMT), these cells migrate to sites throughout the embryo where they give rise to a diverse array of derivatives that include melanocytes, the neurons and glia of the peripheral nervous system (with the exception of several placode-derived cranial sensory nerves), and mesectodermal cells that form much of the craniofacial skeleton as well as the fin mesenchyme of anamniote vertebrates [1] (Figure 1).

Many aspects of neural crest ontogeny have been studied intensely, including the formation and maintenance of the premigratory stem cell population, the process by which these cells acquire migratory abilities, and the signals that direct individual multipotent cells to give rise to a single type of derivative. Moreover, as the neural crest is such a lineage rich tissue, it is an excellent system for investigating the post-transcriptional mechanisms by which the activity of widely deployed signaling pathways and regulatory proteins can be modulated in order to elicit appropriate cell type specific responses. Such studies are of great importance to understand the neural crest; not only are the extracellular signals that direct specific aspects of neural crest development utilized reiteratively for distinct events [2], but in addition, the nuclear factors that function downstream of these signals are not dedicated regulators of neural crest cell fate, and mostly appear to play multiple distinct roles in the development of neural crest cells. Accordingly, this review will examine recent advances in our understanding of the patterning events underlying neural crest cell formation, migration and lineage diversification, with particular emphasis on insights into the regulatory mechanisms that control the activity of key factors such as Snail, SoxE proteins, and *N*-cadherin.

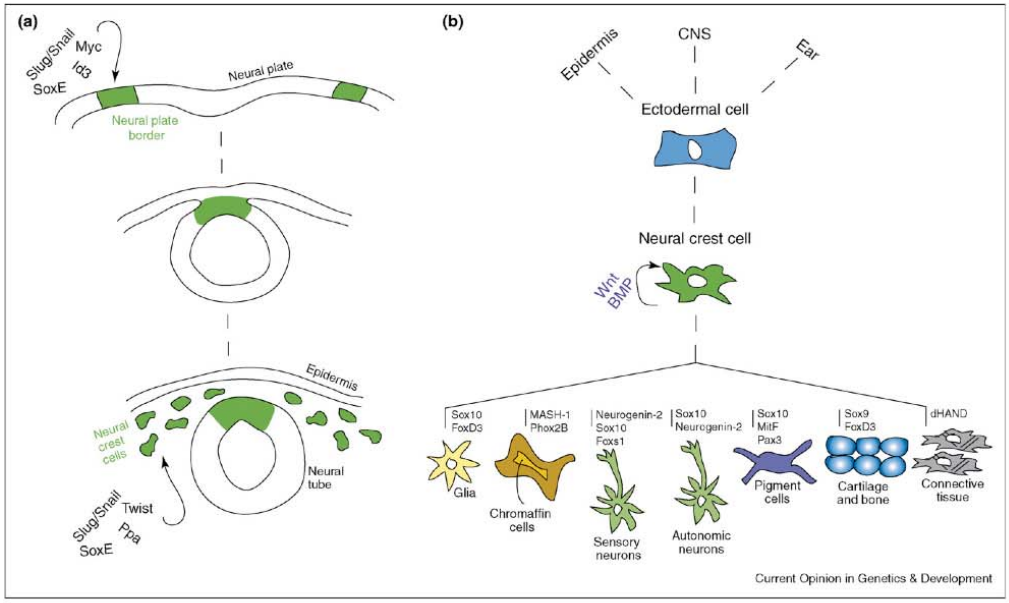
Snail family factors

Zinc finger transcriptional repressors of the Snail superfamily, most commonly Snail and Slug (also known as Snail2), mark the neural crest forming regions of all vertebrate embryos as well as the presumptive ancestors of these cells at the neural plate border of non-vertebrate chordates [3]. Although it has been proposed that these factors function primarily as regulators of cell movement and survival [4], there are also numerous examples of roles for these proteins that do not fit readily into either of these categories, including left–right asymmetry [5,6,7••], endoreduplication [8] and in the formation of neural crest precursor population at the neural plate border [9,10].

Snail and cell survival

In at least some cellular contexts, Snail and Slug can function as anti-apoptotic factors, both in the neural crest [11–13,14] as well as in other cell types [4]. Consistent with a role for these factors in promoting of cell survival, a recent study in *Xenopus* suggested that Caspase9 may be a direct target of Slug–Snail mediated repression [14]. However, there are also examples of a clear disconnect between Snail–Slug function and cell survival. For instance, a recent study demonstrated that Snail-family transcription factors actually promote cell death in the *Drosophila* eye periphery in a non-cell autonomous manner, the first example of such a role [15••]. Moreover, the Snail expressing cells, themselves, undergo apoptosis in this system, which is inconsistent with a strict correlation between *Snail* function and cell survival. Similarly, *Xenopus* neural crest cells expressing high levels of a stabilized Slug isoform have also been found to undergo apoptosis [16]. It is clear from these studies that much remains to be

Figure 1



(a) Schematic summarizing the stages of neural crest precursor formation, and regulatory factors involved. The neural crest arises as two populations of cells (green) on either side of the neural plate (CNS precursors). Following neural tube closure, neural crest cell populate the dorsal-most aspect of the neural tube. Around the time of neural tube closure these cells will undergo EMT and commence migration. Factors such as Slug/Snail and SoXE proteins are implicated in both the initial formation of these cells and their migratory behavior. (b) Neural crest lineage diversification. Many of the factors that play essential roles at earlier stages of neural crest development, such as SoxE factors, are also involved in directing multipotent neural crest progenitors to give rise to a specific neural crest derivative cell type.

learned about the cellular contexts in which Snail family factors do and do not promote cell survival, and this is essential in understanding the patterning events that these factors participate in.

Snail and EMT

The ability of Snail family proteins to promote Epithelial-Mesenchymal transitions (EMTs) is also context dependant. During EMTs, cells undergo transient structural changes resulting in a loss of polarity and contact with neighboring cells and the acquisition of motility [17]. The expression of Snail-related factors frequently correlates with these changes during embryonic development [4]. However, Slug plays an essential role in the formation of neural crest precursor cells many hours before they will undergo EMT and commence migration [9]. Moreover, in Amphioxus, a non-vertebrate chordate, cells at the neural plate border express *Snail* even though this organism lacks definitive neural crest and these *Snail* expressing cells will not become migratory [18]. Together these findings suggest that there is a dynamic context-dependent regulation of Slug and Snail that allows them to regulate essential targets, such as those involved in neural crest precursor formation, without always inducing an EMT.

Post-translational modification of Snail

How then is the activity of Snail factors directed to one or another of these activities? Recent work in *Xenopus* has shown that the stability of Slug and Snail is dynamically regulated during neural crest development via the ubiquitin-proteasome system, and that the F-box protein Ppa is essential for directing their degradation [16]. Point mutations that abrogate Ppa targeting were found to stabilize the Slug protein and allow it to elicit premature migration. This work suggests that Snail family proteins may carry out different functions at distinct threshold concentrations, and that accumulation of high levels of these factors is necessary for them to promote EMTs. Work in mammalian cell culture has suggested that GSK-mediated phosphorlyation directs β-Trcp mediated degradation of Snail [19–21]; however, this mechanism does not appear to function in the neural crest, at least in *Xenopus* [16]. Indeed, Slug and many other Snail factors

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lack the β-Trcp destruction box present in mammalian Snail, suggesting that this is unlikely to be an evolutionarily conserved means of targeting Snail proteins for degradation. Snail is also regulated at the level of nuclear translocation [21,22], and it will be important to determine the role that this mechanism plays in neural crest development.

Conservation of Snail function and targets

Slug and Snail arose via duplication and are believed to be functionally equivalent factors [10] with their relative participation in specific developmental events being due to changes in tissue specific enhancer-driven expression [23]. However, a recent microarray study of Slug and Snail transfected MDCK cell lines found that the gene sets that were altered in response to these factors were only partially overlapping. This finding led the authors to suggest that Slug and Snail might, in fact, control distinct aspects of EMT and other cellular processes [24]. It is possible that the expression levels of the two proteins in the cell lines utilized were not equivalent, however, allowing the possibility that the observed differences in activity were quantitative rather than qualitative.

Interestingly, whereas Slug and/or Snail clearly play essential roles in neural crest formation in both *Xenopus* and avian embryos, a recent double knockout of their orthologs in the mouse epiblast displays no defects in neural crest development, at least in cranial regions before E9.5 [7••]. These mutant mice do have defects in left–right asymmetry, however, further highlighting the essential role of Snail family proteins in patterning and regulatory events beyond cell survival and EMT. Neural crest specific Snail1 deletion on a Snail2 $(slug)^{-/-}$ background does lead to later craniofacial and palate defects [7••]; [25]. Nevertheless, the surprising finding that neural crest cells form and migrate normally in the double mutant mice suggests that murine neural crest cells express one or more factors that can carry out the essential functions that Snail family proteins mediate in frog and chick. With respect to regulation of EMT and cell behavior, Twist and Sip1 are attractive candidates for such a factor given that they, like Snail and Slug, are also linked to these processes during tumor progression [26]. To better understand the apparent dichotomy in the requirement for these factors, it will be important to identify the essential targets of Slug–Snail regulation in *Xenopus* and avian neural crest and then determine how these targets are regulated in murine embryos. The best-characterized target of Snail mediated repression in tumor cells is E-cadherin [27], but this factor does not appear to be a Slug–Snail target in avian neural crest cells. Importantly, recent work from the Bronner-Fraser lab found that cadherin6B is a direct target of Slug in avian neural crest cells, and this study set the groundwork for the identification of additional essential targets [28]. Members of the Claudin and Occludin families, important components of tight junctions, might prove worthwhile targets to investigate, as both Claudin-1 and Occludin have recently been shown to be a direct target of Snail–Slug-mediated repression in cultured epithelial cells [29,30].

SoxE proteins

Like Snail-family proteins, the HMG transcription factors of the SoxE family play multiple distinct roles in neural crest formation and also in other cell types, such as the ear. Sox10, for example, is required not only for survival and maintenance of pluripotency in neural crest stem cells but also for the specification and differentiation of melanocytes and peripheral glia [31]. SoxE factors thus provide another model for understanding how the activity of widely deployed regulatory factors is controlled in order to direct the appropriate developmental outcome.

Sox factors are weak transcriptional activators and are thought to regulate target gene expression in conjunction with partner proteins. Sox Group E, comprised Sox8, 9, and 10, are all initially expressed in all multipotent neural crest progenitors at the neural plate border and at least in *Xenopus* are required for the formation of these cells. Later expression of the different SoxE factors becomes restricted to specific subsets of neural crest derivatives. Most work on the role of these factors in the neural crest has focused on Sox9 and 10; however, two recent studies have more closely examined the role of Sox8 in mouse and *Xenopus* [32••,33•].

Sox 8 function and functional redundancy

In *Xenopus*, unlike in mouse and chick, expression of Sox8 in the neural crest precedes expression of Sox9 and Sox10. Morpholino-mediated knockdown of Sox8 in *Xenopus* delays the expression of a number of early neural crest markers, but expression of these genes eventually recovers, presumably because of the compensatory activities of Sox9 and Sox10 [33•]. This recovery supports previous findings that SoxE factors can substitute for one another during neural crest development [34•]. Nevertheless, the delayed induction of neural crest progenitors in Sox8 morphants impairs neural crest migration and results in deficits in some derivatives. This study further found that the effects of Sox8 depletion at neurula stages could be rescued by either Sox9 or Sox10, providing additional support for functional equivalency of SoxE factors [33•].

Recently, in an analogous set of experiments in the mouse, the Sox10 open reading frame was replaced with that of Sox8 [32••]. This study found that Sox8 rescued the embryonic and perinatal lethality associated with Sox10-deficient mice. However, these mice died in their first postnatal week, suggesting an incomplete rescue. While Sox8 appeared to rescue neuron and glia formation in sensory and sympathetic ganglia fully, rescue of enteric

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nervous system defects and oligodendrocytes was only partial, whereas melanocyte formation was not rescued [32••]. From these findings it appears that Sox8 may be unable to fully replace the function of Sox10 in the mouse, implying that these highly related proteins might possess at least some distinct activities. Drawing such a conclusion would require data showing that mice with an analogous Sox10 knockin at the same locus develop normally.

Regulation of SoxE activity by SoxD proteins

All three SoxE factors are expressed in oligodendrocytes, the myelin-forming macroglia of the CNS [31], and Sox9 and Sox10 do appear to be functionally redundant in this cell type [35–37]. SoxE function appears to be regulated in oligodendrocytes by the context-dependent activities of co-expressed SoxD proteins. Two SoxD proteins, Sox5 and 6, are co-expressed with SoxE factors in these cells [38••], as they are in chondrocytes [39]. However, whereas in chondrocytes SoxD proteins cooperate with SoxE proteins to activate the collagen2a1 promoter [40], in oligodendrocytes they inhibit SoxE-dependent activation of myelin promoters [38••]. Importantly, this inhibitory activity depends on cellular context, as SoxD and SoxE proteins cooperate to activate the same myelin promoters in mesodermal cell types [38••].

Interestingly, another SoxD protein, LSox5, is co-expressed with SoxE proteins in peripheral glial cells as well as at the neural plate border, where it could play a role in SoxE-mediated patterning events [41]. This raises the possibility that SoxD-dependent control of SoxE function may be a more widely used regulatory mechanism. It is therefore important to understand how SoxD factors modulate SoxE function and how this regulation depends on cell type. SoxD and SoxE factors do not appear to interact physically; however, they do compete for binding to myelin gene promoters on which SoxD represses transcriptional activation by SoxE, and they may compete for binding to co-regulatory factors [38••]. Interestingly, SoxD proteins, such as Sox6, have been shown to be SUMOylated [42]. If SUMO modification is regulated in a promoter and/or cell type dependent manner, this could explain the contrasting outcomes of combined SoxD and SoxE activity on myelin versus collagen promoters, or on myelin promoters in different cell types [38••,40]. Importantly, SoxE factors have also been shown to be SUMOylated in early embryos, and this modification has distinct effects on the ability of SoxE factors to carry out different patterning roles, such as neural crest precursor versus otic placode formation [34•]. SUMOylation may, therefore, be a key regulatory mechanism for conferring context-dependent function on widely deployed transcription factors. Other post-translational modifications are also likely to contribute to the modulation of SoxE activity, independent of, or in combination with, SUMO and SoxD mediated regulation. For example, Sox9 has been shown to be phosphorylated by cAMP-PKA, and this modification may regulate Sox9 nuclear translocation and transactivation [43,44].

N-cadherin proteolysis and neural crest cell emigration

Whereas the above examples dealt with DNA binding regulatory molecules, exciting advances have also been made in understanding the signaling molecules that drive key events in neural crest development. A recent paper by Shoval et al highlights the importance of signal cross talk in understanding the onset of neural crest migration [45••]. These authors had previously shown that a noggin-regulated gradient of BMP activity along the rostrocaudal axis triggers the EMT of neural crest precursors in the dorsal neural tube by a Wnt-dependant process that included upregulation of Wnt1 expression [46,47]. In an elegant new study, these authors uncover an additional mechanism via which BMP modulates canonical Wnt signaling [45••]. They show that BMP signaling also triggers the gamma-secretase-mediated proteolysis of *N*-cadherin. Cleavage leads to the production of a soluble fragment, called CTF2 that translocates to the nucleus in a process reminiscent of Notch signaling. Interestingly, this fragment had previously been shown to function as a transcriptional repressor by binding to and promoting the proteasomal degradation of CBP [48]. However, this fragment has also been demonstrated to bind to beta-catenin [49], and in neural crest precursors release of CTF2 appears to promote transcription of Wnt/beta-catenin targets and trigger neural crest cell delamination [45••]. One possibility is that CTF2 acts as a chaperone to protect beta-catenin from degradation and facilitate its translocation to the nucleus, but it will be important to determine the precise mechanisms via which CTF2 signals. Such studies are likely to prove significant for multiple aspects of neural crest development since *N*-cadherin has recently been shown to control the pattern of the sympathetic ganglia as well as neural crest remodeling of the cardiac outflow tract [50,51]. It will also be of interest to determine whether other cadherins involved in neural crest development also undergo regulated intramembrane proteolysis.

Conclusions

Emerging evidence suggests that neural crest formation relies on a complex network of proteins that undergo considerable cross-regulatory interactions and display some degree of functional overlap [52]. Moreover, the proteins that comprise this network are not dedicated regulators of neural crest cells, and mostly appear to play multiple distinct roles in the development of the neural crest. It is essential to move beyond the ''parts lists'' phase toward an understanding of how these factors function in combination and how their activities are directed to particular developmental events and cellular processes. Progress has been made in understanding the regulation of key factors, such as the Snail and SoxE

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families, and the challenge will be to extend such studies to other components of the network and to investigate the conservation of elucidated regulatory mechanisms across species.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Dupin E, Creuzet S, Le Douarin NM: **The contribution of the neural crest to the vertebrate body**. *Adv Exp Med Biol* 2006, **589**:96-119.

2. Raible DW: **Development of the neural crest: achieving specificity in regulatory pathways**. *Curr Opin Cell Biol* 2006, **18**:698-703.

3. Nieto MA: **The snail superfamily of zinc-finger transcription factors**. *Nat Rev Mol Cell Biol* 2002, **3**:155-166.

4. Barrallo-Gimeno A, Nieto MA: **The Snail genes as inducers of cell movement and survival: implications in development and cancer**. *Development* 2005, **132**:3151-3161.

5. Dale JK, Malapert P, Chal J, Vilhais-Neto G, Maroto M, Johnson T, Jayasinghe S, Trainor P, Herrmann B, Pourquie O: **Oscillations of the snail genes in the presomitic mesoderm coordinate segmental patterning and morphogenesis in vertebrate somitogenesis**. *Dev Cell* 2006, **10**:355-366.

6. Isaac A, Sargent MG, Cooke J: **Control of vertebrate left-right asymmetry by a snail-related zinc finger gene**. *Science* 1997, **275**:1301-1304.

7. •• Murray SA, Gridley T: **Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice**. *Proc Natl Acad Sci U S A* 2006, **103**:10300-10304.

The authors show that neural crest forms and migrates normally in mice harboring conditional inactivation of Snai1 in a Snail2$^{-/-}$ background, at least up to E9.5. The work also shows that Snail1 is required for normal left-right asymmetry.

8. Nakayama H, Scott IC, Cross JC: **The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor**. *Dev Biol* 1998, **199**:150-163.

9. LaBonne C, Bronner-Fraser M: **Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration**. *Dev Biol* 2000, **221**:195-205.

10. del Barrio MG, Nieto MA: **Overexpression of Snail family members highlights their ability to promote chick neural crest formation**. *Development* 2002, **129**:1583-1593.

11. Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA: **Snail blocks the cell cycle and confers resistance to cell death**. *Genes Dev* 2004, **18**:1131-1143.

12. Tribulo C, Aybar MJ, Sanchez SS, Mayor R: **A balance between the anti-apoptotic activity of Slug and the apoptotic activity of msx1 is required for the proper development of the neural crest**. *Dev Biol* 2004, **275**:325-342.

13. Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J: **The transcriptional control of trunk neural crest induction, survival, and delamination**. *Dev Cell* 2005, **8**:179-192.

14. Zhang C, Carl TF, Trudeau ED, Simmet T, Klymkowsky MW: **An NF-kappaB and slug regulatory loop active in early vertebrate mesoderm**. *PLoS ONE* 2006, **1**:e106.

15. •• Lim HY, Tomlinson A: **Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis**. *Development* 2006, **133**:3529-3537.

This paper demonstrates for the first time a role for the Snail family in the promotion of cell death in the eye of Drosophila that can, in some cases, be non-cell autonomous. Before this, most data supported a cell autonomous anti-apoptotic role for these factors.

16. Vernon AE, LaBonne C: **Slug stability is dynamically regulated during neural crest development by the F-box protein Ppa**. *Development* 2006, **133**:3359-3370.

17. Thiery JP, Sleeman JP: **Complex networks orchestrate epithelial-mesenchymal transitions**. *Nat Rev Mol Cell Biol* 2006, **7**:131-142.

18. Langeland JA, Tomsa JM, Jackman WR Jr, Kimmel CB: **An amphioxus snail gene: expression in paraxial mesoderm and neural plate suggests a conserved role in patterning the chordate embryo**. *Dev Genes Evol* 1998, **208**:569-577.

19. Yook JI, Li XY, Ota I, Hu C, Kim HS, Kim NH, Cha SY, Ryu JK, Choi YJ, Kim J *et al.*: **A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells**. *Nat Cell Biol* 2006, **8**:1398-1406.

20. Yook JI, Li XY, Ota I, Fearon ER, Weiss SJ: **Wnt-dependent regulation of the E-cadherin repressor snail**. *J Biol Chem* 2005, **280**:11740-11748.

21. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC: **Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition**. *Nat Cell Biol* 2004, **6**:931-940.

22. Dominguez D, Montserrat-Sentis B, Virgos-Soler A, Guaita S, Grueso J, Porta M, Puig I, Baulida J, Franci C, Garcia de Herreros A: **Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor**. *Mol Cell Biol* 2003, **23**:5078-5089.

23. Locascio A, Manzanares M, Blanco MJ, Nieto MA: **Modularity and reshuffling of Snail and Slug expression during vertebrate evolution**. *Proc Natl Acad Sci U S A* 2002, **99**:16841-16846.

24. Moreno-Bueno G, Cubillo E, Sarrio D, Peinado H, Rodriguez-Pinilla SM, Villa S, Bolos V, Jorda M, Fabra A, Portillo F *et al.*: **Genetic profiling of epithelial cells expressing e-cadherin repressors reveals a distinct role for snail, slug, and e47 factors in epithelial-mesenchymal transition**. *Cancer Res* 2006, **66**:9543-9556.

25. Murray SA, Oram KF, Gridley T: **Multiple functions of Snail family genes during palate development in mice**. *Development* 2007.

26. Vernon AE, LaBonne C: **Tumor metastasis: a new twist on epithelial-mesenchymal transitions**. *Curr Biol* 2004, **14**:R719-R721.

27. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA: **The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression**. *Nat Cell Biol* 2000, **2**:76-83.

28. Taneyhill LA, Coles EG, Bronner-Fraser M: **Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest**. *Development* 2007, **134**:1481-1490.

29. Martinez-Estrada OM, Culleres A, Soriano FX, Peinado H, Bolos V, Martinez FO, Reina M, Cano A, Fabre M, Vilaro S: **The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells**. *Biochem J* 2006, **394**:449-457.

30. Wang Z, Wade P, Mandell KJ, Akyildiz A, Parkos CA, Mrsny RJ, Nusrat A: **Raf 1 represses expression of the tight junction protein occludin via activation of the zinc-finger transcription factor slug**. *Oncogene* 2007, **26**:1222-1230.

31. Kelsh RN: **Sorting out Sox10 functions in neural crest development**. *Bioessays* 2006, **28**:788-798.

Please cite this article in press as: Taylor KM, LaBonne C, Modulating the activity of neural crest regulatory factors, Curr Opin Genet Dev (2007), doi:10.1016/j.gde.2007.05.012

32. Kellerer S, Schreiner S, Stolt CC, Scholz S, Bosl MR, Wegner M:
•• **Replacement of the Sox10 transcription factor by Sox8 reveals incomplete functional equivalence**. *Development* 2006, **133**:2875-2886.
The authors employ targeted mutagenesis in the mouse to test the functional equivalency of Sox8 and Sox10. They find that when Sox8 is knocked in to the Sox10 locus, the embryonic and perinatal lethality associated with Sox10-deficient mice was rescued, but defects remained in melanocyte and enteric nervous system development, as well as in oligodendrocytes.

33. O'Donnell M, Hong CS, Huang X, Delnicki RJ, Saint-Jeannet JP:
• **Functional analysis of Sox8 during neural crest development in *Xenopus***. *Development* 2006, **133**:3817-3826.
The authors show that morpholino mediated depletion of Sox8 in *Xenopus* delayed the onset of expression of markers of neural crest precursors. The expression of these factors was later recovered; however, the embryos showed defects in neural crest cell migration and differentiation. The authors also show that depletion of Sox8 can be rescued by either Sox9 or Sox10.

34. Taylor KM, Labonne C: **SoxE factors function equivalently
• during neural crest and inner ear development and their activity is regulated by SUMOylation**. *Dev Cell* 2005, **9**:593-603.
The authors demonstrate that Sox9 and Sox10 can function equivalently with respect to the formation of neural crest precursors, as well as later neural crest derivatives. It is also shown that SUMOylation state of these factors plays a key role in determining whether SoxE factors mediate neural crest or inner ear formation.

35. Stolt CC, Lommes P, Friedrich RP, Wegner M: **Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy**. *Development* 2004, **131**:2349-2358.

36. Stolt CC, Schmitt S, Lommes P, Sock E, Wegner M: **Impact of transcription factor Sox8 on oligodendrocyte specification in the mouse embryonic spinal cord**. *Dev Biol* 2005, **281**:309-317.

37. Wegner M, Stolt CC: **From stem cells to neurons and glia: a Soxist's view of neural development**. *Trends Neurosci* 2005, **28**:583-588.

38. Stolt CC, Schlierf A, Lommes P, Hillgartner S, Werner T, Kosian T,
•• Sock E, Kessaris N, Richardson WD, Lefebvre V *et al.*: **SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function**. *Dev Cell* 2006, **11**:697-709.
The authors show that SoxD factors are co-expressed with SoxE factors in oligodendrocytes and that they inhibit the ability of SoxE factors to activate myelin promoters. They further show that this inhibition is cell type dependent because these factors co-operate to activate myelin promoters when expressed in mesodermal cells.

39. Lefebvre V, Li P, de Crombrugghe B: **A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene**. *EMBO J* 1998, **17**:5718-5733.

40. Lefebvre V, Behringer RR, de Crombrugghe B: **L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway**. *Osteoarthritis Cartilage* 2001, **9(Suppl. A)**:S69-S75.

41. Perez-Alcala S, Nieto MA, Barbas JA: **LSox5 regulates RhoB expression in the neural tube and promotes generation of the neural crest**. *Development* 2004, **131**:4455-4465.

42. Fernandez-Lloris R, Osses N, Jaffray E, Shen LN, Vaughan OA, Girwood D, Bartrons R, Rosa JL, Hay RT, Ventura F: **Repression of SOX6 transcriptional activity by SUMO modification**. *FEBS Lett* 2006, **580**:1215-1221.

43. Huang W, Zhou X, Lefebvre V, de Crombrugghe B: **Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer**. *Mol Cell Biol* 2000, **20**:4149-4158.

44. Malki S, Nef S, Notarnicola C, Thevenet L, Gasca S, Mejean C, Berta P, Poulat F, Boizet-Bonhoure B: **Prostaglandin D2 induces nuclear import of the sex-determining factor SOX9 via its cAMP-PKA phosphorylation**. *EMBO J* 2005, **24**:1798-1809.

45. Shoval I, Ludwig A, Kalcheim C: **Antagonistic roles of full-length
•• *N*-cadherin and its soluble BMP cleavage product in neural crest delamination**. *Development* 2007, **134**:491-501.
The authors reveal a novel mechanism for regulating canonical Wnt signaling during neural crest emigration. BMP signaling promotes γ-secretase mediated cleavage of the *N*-cadherin cytoplasmic domain. The cleavage product, CTF2, binds beta-catenin and promotes its accumulation and nuclear translocation leading to activation of Wnt/beta-catenin targets.

46. Sela-Donenfeld D, Kalcheim C: **Localized BMP4-noggin interactions generate the dynamic patterning of noggin expression in somites**. *Dev Biol* 2002, **246**:311-328.

47. Sela-Donenfeld D, Kalcheim C: **Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration**. *Development* 2000, **127**:4845-4854.

48. Marambaud P, Wen PH, Dutt A, Shioi J, Takashima A, Siman R, Robakis NK: **A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of *N*-cadherin is inhibited by PS1 FAD mutations**. *Cell* 2003, **114**:635-645.

49. Uemura K, Kihara T, Kuzuya A, Okawa K, Nishimoto T, Bito H, Ninomiya H, Sugimoto H, Kinoshita A, Shimohama S: **Activity-dependent regulation of beta-catenin via epsilon-cleavage of *N*-cadherin**. *Biochem Biophys Res Commun* 2006, **345**:951-958.

50. Kasemeier-Kulesa JC, Bradley R, Pasquale EB, Lefcort F, Kulesa PM: **Eph/ephrins and *N*-cadherin coordinate to control the pattern of sympathetic ganglia**. *Development* 2006, **133**:4839-4847.

51. Luo Y, High FA, Epstein JA, Radice GL: ***N*-cadherin is required for neural crest remodeling of the cardiac outflow tract**. *Dev Biol* 2006, **299**:517-528.

52. Meulemans D, Bronner-Fraser M: **Gene-regulatory interactions in neural crest evolution and development**. *Dev Cell* 2004, **7**:291-299.

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