FUNCTIONS OF EARLY B CELL FACTOR IN *XENOPUS* NEURONAL AND MUSCLE DEVELOPMENT

by

Yangsook Song Green

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Yangsook Song Green**

has been approved by the following supervisory committee members:

Monica L. Vetter	, Chair	**6/10/2010** Date Approved
Chi-Bin Chien	, Member	**6/10/2010** Date Approved
Richard I. Dorsky	, Member	**6/10/2010** Date Approved
Sabine Fuhrmann	, Member	**6/10/2010** Date Approved
Shannon J. Odelberg	, Member	**6/10/2010** Date Approved

and by **Monica L. Vetter**, Chair of the Department of **Neurobiology and Anatomy**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The development of an animal from a single cell to an organism requires that individual cells undergo several sequential processes. The final stages, termed commitment and differentiation, rely heavily on the cell-intrinsic activity of regulatory transcription factor networks. The early B cell factor (EBF) family transcription factors are known to have an important influence on commitment and differentiation in neurons, B cells and adipocytes in vertebrate animals, and muscle cells in invertebrate animals. The full range of their activity, though, is not understood. We have utilized a microarray screen in *Xenopus laevis* to identify an extensive list of candidate targets of EBF transcriptional activity, as a step toward expanding understanding of the scope of EBF functions. This thesis focuses on the functions of EBF proteins in neuron and muscle cell development. To expand current knowledge of EBF functions in neuronal development, we selectively chose candidate targets from the microarray screen that have expected function in neurons, and verified that their expression depends on EBF activity. These targets demonstrate several previously unknown functions of EBF proteins in neuronal cell commitment and differentiation. We also have discovered a new function of the EBF protein partner ZFP423 as a synergistic mediator of the critical Notch signaling pathway, and show that EBF proteins can promote neuronal commitment in part by blocking the function of ZFP423. We next demonstrate that EBF proteins are necessary for normal *Xenopus* skeletal muscle development, and that they act by controlling

expression of several genes critical for commitment and differentiation of muscle cells. This thesis significantly contributes to understanding of the function of EBF proteins in neuronal development. It also demonstrates for the first time an important role for EBF proteins in muscle cell development in vertebrates. Overall, this thesis expands our understanding of how EBF proteins participate in the complex transcriptional regulation of vertebrate development.

사랑, 희망 그리고 인내를 가르쳐 주신 부모님께 이 논문을 바칩니다.

This thesis is dedicated to my parents, who taught me love, hope and patience.

CONTENTS

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CHAPTER 1

INTRODUCTION

The mechanisms of animal development, though highly ordered, are exceedingly complex. One crucial aspect of this complexity is the progression of cells from an uncommitted state to, eventually, the highly differentiated states of the many tissue types of an adult animal. A common thread in the process of cell development is an early dependence on cell-extrinsic signals, followed by increasing dependence on cell-intrinsic signals, especially transcription factors.

We now know that a relatively limited number of cell-extrinsic paracrine signals and signaling pathways underlie a great number of the processes of early development. For example, TGF-beta, Hedgehog, FGF, and Wnt family members are used as paracrine signals in widely divergent tissue types and across vastly different animal species (Reviewed in Ciani and Salinas, 2005; Dessaud et al., 2008; Wu and Hill, 2009).

However, it is a less certain proposition that there could be extensively shared mechanisms involved in the more terminal events of cell development, as cells become dramatically different. A critical step toward an understanding of cell commitment and differentiation, then, is analysis of the precise transcriptional targets of the cell-intrinsic transcription factors that participate in these developmental events. The EBF (early B cell factor, also called Collier/Olf/Ebf (COE) and Olf/Ebf (O/E)) protein family is one such group of transcription factors, with involvement in a variety of processes in an intriguing array of tissue types. The aim of this thesis is to provide an extensive characterization of the transcriptional targets and an interacting protein partner of EBF transcription factors, and to use this knowledge to help explain how EBF proteins impact a wide array of developmental events.

Common processes in the development of different cell lineages

A fertilized egg develops into a complicated organism containing many cell types, including muscle cells, neuronal cells, epithelial cells, and connective tissue cells such as lymphocytes and adipocytes. The process of development of any cell type is a progression through multiple events (Figure 1.1). These are patterning (or regional specification), proliferation, commitment, and differentiation (reviewed in Slack, 1991; Wolpert, 1977). Some cells also migrate during development. Generally, the term patterning refers to early events such as production of the three primary germ layers of an animal, regional specification of areas like the neural plate, or formation of the body axes. Proliferation generates adequate numbers of cells for formation of a particular tissue, and is therefore independently regulated in different tissues. Commitment describes the further maturation of cells, whereby they adopt a particular fate. It includes a reversible phase, called specification, and an irreversible phase, called determination. Finally, the term differentiation usually is used to describe all aspects of the further development of a committed cell. Fully differentiated cells have a multitude of specialized cell products and functions (reviewed in Slack, 1991).

A combination of different extrinsic signals and intrinsic signals drives the specific developmental pathway for each lineage (Figure 1.1). While extrinsic signals are important for patterning and proliferation, intrinsic signals become more important for commitment and differention. Understanding more about how intrinsic signals work in their complex transcriptional networks will help us to understand the common yet different processes of commitment and differentiation of multiple lineages.

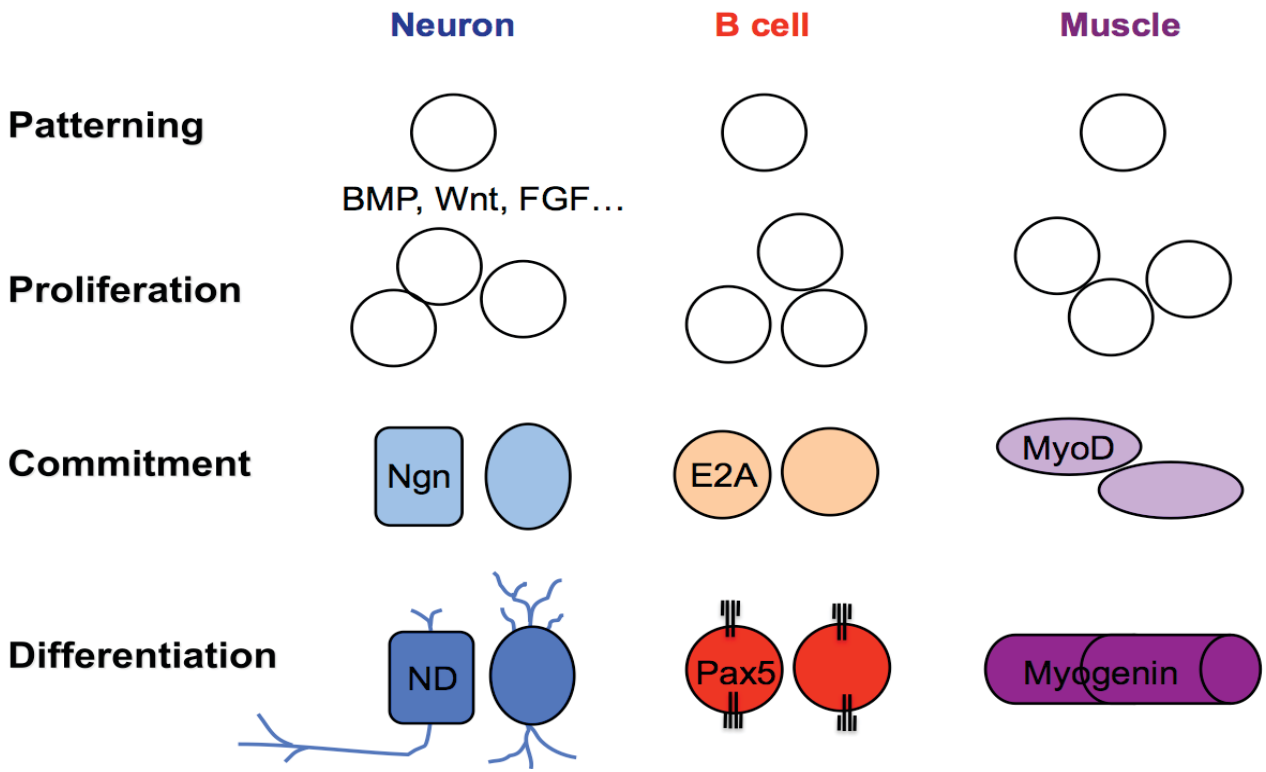

Figure 1.1: A common developmental progression generates cells of multiple tissue types. Progenitor cells first undergo patterning and proliferation, driven by extrinsic signals like BMP, Wnts and FGF. Gradually intrinsic signals including Neurogenin and NeuroD in neurons, E2a and Pax5 in B cells, and MyoD and Myogenin in muscle cells become more important. They drive the processes of commitment and differentiation. EBF proteins are also known to be important intrinsic signals for the commitment and differentiation of neurons and B cells, and for differentiation of muscle cells.

EBF molecular characterization, and functions in the development of connective tissue cells

EBF family members and structure

The EBF family of transcription factors is recognized for its multiple transcriptional roles in the development of a variety of tissues (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002; Lukin et al., 2008). This family includes EBF1, 2, 3 and O/E-4 in mammals (Garel et al., 1997; Hagman et al., 1993; Kudrycki et al., 1993; Malgaretti et al., 1997; Wang et al., 2002; Wang et al., 1997), XEBF2 and XEBF3 in *Xenopus* (Dubois et al., 1998; Pozzoli et al., 2001), and ZCOE2 in zebrafish (Bally-Cuif et al., 1998). Invertebrate members of this gene family include Collier in *Drosophila*, and UNC3 in *C. elegans* (Crozatier et al., 1996; Prasad et al., 1998).

EBF family proteins contain a DNA binding domain (a zinc finger coordination motif), which can also participate in dimerization and transactivation, an atypical helix-loop-helix (HLH) domain, which is critical for formation of homo- and heterodimers, and a C-terminal domain, which is also important for transactivation (Hagman et al., 1993; Hagman et al., 1995; Wang et al., 1997). The DNA binding domain of EBF family members recognizes the following sequence in promoters of targets: 5'-ATTCCCNNGGGAAT-3' (Hagman et al., 1993; Hagman et al., 1995; Wang and Reed, 1993). The DNA binding domain is itself atypical, containing a Zn finger region known as a zinc knuckle motif, and this motif is important for the specificity of EBF binding to target gene promoters (Fields et al., 2008; Hagman et al., 1995). The atypical HLH motif in vertebrates contains helix 1, a loop, and a duplicated helix 2 (Hagman et al., 1995), while Collier and unc-3 have only one helix 2 (Crozatier et al., 1996; Prasad et al., 1998).

EBF family members are expressed in nervous tissue, muscle tissue, B cells and adipocytes. Some functions of EBF proteins are conserved among the tissues, while others are tissue specific (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002; Lukin et al., 2008). EBF functions in B cell and adipocyte development will be discussed in the following section, while functions in neuron development and muscle development will be discussed in separate sections below.

EBF functions in B cell development

Mature B lymphocytes are derived from hematopoietic stem cells (HSCs) through a multi-step pathway of differentiation. HSCs become multipotential progenitors (MPP), which then become common lymphoid progenitors (CLP), then B-biased progenitors, then pro-B cells, and then pre-B cells, before becoming mature B lymphocytes. *Ebf1* knockout mice do not have mature B cells, and B cell development is arrested in the pro-B cell stage. They do not progress to the pre-B cell stage, as evidenced by the fact that the cells in knockout mice fail to express genes coding for proteins involved with B cell differentiation, including λ5, VpreB, CD19, mb-1, B29 and RAG1 (Lin and Grosschedl, 1995). During B cell development, the expression of *Ebf1* is controlled by PU.1, IL-7R and E2A, which are expressed from the MPP or CLP stages (reviewed in Hagman and Lukin, 2005; Singh et al., 2005; Smith and Sigvardsson, 2004). Interestingly, the bHLH E2A proteins (also called E proteins) E12 and E47 are upstream of *Ebf1* (Greenbaum and Zhuang, 2002; Kee and Murre, 1998; Kwon et al., 2008; Seet et al., 2004; Smith et al., 2002). Furthermore, E2A expression is dramatically upregulated at the time of *Ebf1* gene expression in B cells, but this upregulation does not occur in Ebf1 null mice (Zhuang et

al., 2004). These experiments suggest that there is a positive feedback loop between E2A and EBF. Also, mice double heterozygous for *E2A* and *Ebf1* have more severe defects of B cell development than single homozygous null mice of each genes, suggesting synergistic regulation of gene expression by E2A and EBF1 (O'Riordan and Grosschedl, 1999). For the target genes *λ5* and *VpreB*, this synergistic effect has been directly demonstrated (Sigvardsson, 2000; Sigvardsson et al., 1997). Ebf1 also can control the expression of *Pax5*, which is an important gene for B cell commitment (Lin and Grosschedl, 1995; O'Riordan and Grosschedl, 1999; Urbanek et al., 1994). For the expression of the B-cell differentiation marker *mb-1*, Ebf1 cooperates with E2A, Pax5 and Runx1 (also called Aml1). In particular, Ebf1 is required, for unclear reasons, for demethylation of the *mb-1* promoter, which is a critical step for the expression of *mb-1* (Gao et al., 2009; Maier et al., 2004; Sigvardsson et al., 2002). These results, taken together, demonstrate a significant role for Ebf1 in mouse B cell commitment and differentiation, with important target genes and complex relationships with other transcription factors. *Ebf3* is not expressed in B cells, and little is known of any potential role for Ebf2 in B cell development (Garel et al., 1997).

EBF functions in adipocyte development

Ebf1, Ebf2 and Ebf3 are expressed in mouse adult adipocytes, and in induced preadipocyte cells (Dowell and Cooke, 2002; Hagman et al., 1993; Jimenez et al., 2007). When Ebf1, Ebf2 and Ebf3 are ectopically expressed in the preadipocyte cells, they promote adipogenesis by controlling the expression of many genes involved in adipocyte differentiation and function. These targets include *C/EBPα*, *C/EBPδ*, *PPARγ* and *Glut4*

(Akerblad et al., 2002; Akerblad et al., 2005; Dowell and Cooke, 2002; Jimenez et al., 2007). Ebf1 in particular can control the expression of *PPARγ* and *C/EBPα* directly, and *C/EBPδ* through *C/EBPα* (Jimenez et al., 2007). C/EBPδ and another protein, C/EBPβ, are critical for adipogenesis, and are expressed very early (within two hours) when preadipocyte cell lines are induced to differentiate (Cao et al., 1991; Jimenez et al., 2007; Yeh et al., 1995). Among *C/EBPβ, C/EBPδ* double knockout mice, 85% of animals die perinatally for unknown reasons, and the surviving animals have severe defects in adipose tissue (Tanaka et al., 1997). The expression of *C/EBPα* and *PPARγ* is later than *C/EBPβ* and *C/EBPδ* (Cao et al., 1991; Jimenez et al., 2007; Yeh et al., 1995), but these two genes are also very important genes for adipocyte differentiation (Barak et al., 1999; Lin and Lane, 1992; Rosen et al., 1999). These results suggest that Ebf proteins may act downstream of C/EBPβ and C/EBPδ and upstream of PPARγ and C/EBPα, with a positive feedback loop to C/EBPδ, to reinforce the early phase of adipocyte differentiation (Jimenez et al., 2007).

Neuronal development and EBF proteins

Aspects of neuronal development relevant to the functions of EBF

The development of neurons follows the general progression through patterning, proliferation, commitment, and differentiation. In animals with three germ layers, neural tissue is derived from ectoderm. The process of patterning to form nervous tissue relies on a combination of signaling pathways, including FGF, Wnt, and BMP signaling. One common mechanism, which is best understood in *Xenopus*, involves the signaling molecules Noggin and Chordin. These signals are secreted by the Spemann organizer,

and inhibit BMP signaling in the ectodermal tissue. This inhibition allows ectoderm tissue to follow its default course toward neural patterning (reviewed in De Robertis and Kuroda, 2004). These events lead to intrinsic activity of molecules like SoxD, Zic1, Zic3, and Geminin, which act to drive neurogenesis in the neural plate (reviewed in Chitnis, 1999; Stern, 2005). Regions of ectoderm that do not receive this BMP antagonist signaling, due to their distance from the notochord, become epidermis.

Patterning is followed by extensive mitotic proliferation of neuroblasts, to generate appropriate neuronal cell numbers. Proliferation occurs in a tissue-specific manner, driven both by intrinsic signals like Pax6 and Emx2 (Estivill-Torrus et al., 2002; Galli et al., 2002), and by extrinsic Notch signaling (discussed below), and terminates with cell cycle exit.

The events of cell cycle exit and commitment of neurons are often coupled (Buttitta et al., 2007; Sakagami et al., 2009). A key event in neuronal commitment is the beginning of expression of members of the basic helix-loop-helix (bHLH) class of transcription factors, which act as important regulators of neurogenesis. The proneural bHLH transcription factors include an *Ash* group and an *Ath* group, defined based on their similarity to the *Drosophila AS-C* complex and the *Drosophila atonal* gene, respectively (reviewed in Vetter and Brown, 2001). The *Ath* group includes the Neurogenin family (Ngn1, 2, and 3) and the protein NeuroD. The Neurogenins and NeuroD are widely expressed in both the central and peripheral nervous systems (Sommer et al., 1996). The Neurogenins are involved in the commitment step of neuronal development, and mice lacking these genes have decreased cell numbers in spinal cord and in cranial and vertebral ganglia (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999;

Scardigli et al., 2001). Furthermore, Neurogenin-related-1 (NGNR-1) can drive expression of the Notch ligand Delta1, which participates in lateral inhibition of neurogenesis in neighboring cells (Ma et al., 1996). *NeuroD* is a key target of Neurogenins, and despite the limited defects of NeuroD null mice, its widespread expression and its potent pro-neural activity suggest that it is important in the initiation of neural differentiation events in many nervous system regions (Lee et al., 1995; Logan et al., 2005; Seo et al., 2007).

Another aspect of neuronal differentiation involves the outgrowth of axons and dendrites (collectively termed neurites). Actin microfilaments, microtubules, and intermediate filaments are all cytoskeletal elements involved in this process. In particular, actin filaments and microtubules are heavily involved in the growth and length of neurites, while intermediate filaments are involved in the diameter of neurites, stabilizing neurites, and the growth of neurites in certain neuronal populations (reviewed in Lariviere and Julien, 2004). These cytoskeletal elements are regulated both at the transcriptional level and by post-translational phosphorylation (reviewed in Sihag et al., 2007).

The abilities to regulate cellular homeostasis, form and strengthen appropriate synaptic connections, and migrate to their proper locations in the animal are all additional critical aspects of neuronal development, each requiring expression of large numbers of genes. To date, there is no complete picture of the transcription factor networks acting to regulate these complex processes of commitment, neurite growth, and other aspects of differentiation.

EBF function in neuronal development

EBF transcription factors are known to influence many aspects of neuronal development, and some of their target genes and mechanisms of action are known. They are known to influence multiple populations of developing neurons, and they affect commitment, differentiation, neurite development, and cell migration (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002). One of the best areas of understanding of EBF protein functions is in their roles in stabilizing cell commitment. Dubois et al. showed that EBF2 can affect neuronal progenitor cell commitment in early *Xenopus* embryos by reinforcing the expression of the proneural basic helix-loop-helix (bHLH) transcription factor NGNR-1, and by maintaining the expression of Delta1 (Dubois et al., 1998). Misexpressed mouse Ebf1 in chick spinal cord leads to upregulation of the proneural bHLH genes *Ngn1* and *Ngn2*, which also indicates that Ebf proteins stabilize neuronal commitment in this tissue (Garcia-Dominguez et al., 2003).

EBF proteins also have critical roles in neuronal cell differentiation. Misexpression of *Ebf1* in chick spinal cord leads to upregulation of the interneuron markers CRABP1 and Lim1 in motor neuron regions, which suggests an increase in interneurons (and a decrease in motor neurons). This suggests that EBF proteins can affect neuronal subtype specification (Garcia-Dominguez et al., 2003). In *Ebf1* null mouse striatum, early neuronal cells show abnormal expression of several genes, indicating that their differentiation process is disrupted (Garel et al., 1999). In this mouse, the expression levels of *CRABP-1* and *cadherin-8* genes are downregulated in the lateral ganglionic eminence (LGE) (Garel et al., 1999). EBF2 and EBF3 both are important factors for neuronal differentiation during early *Xenopus* neurogenesis. When

overexpressed in *Xenopus* embryos, *ebf2* and *ebf3* lead to ectopic expression of neuronal specific markers like *n-tubulin* and *nf-m* (Dubois and Vincent, 2001; Pozzoli et al., 2001). In *Drosophila* and *C. elegans* ventral nerve cord, the EBF orthologs Collier and UNC-3 control the fate of specific subsets of interneurons and motor neurons (Baumgardt et al., 2007; Prasad et al., 2008). In *Drosophila*, there are some known targets through which Collier can affect interneuron fate.

Several different experiments show a strong role for Ebf proteins in neurite formation and guidance. In *Ebf1* null mice, the thalamocortical fibers in the LGE fasciculate abnormally (Garel et al., 1999). In this region, *Sema6a* expression also is downregulated and this may lead to abnormal fiber growth (Garel et al., 2002). Olfactory axons in both *Ebf2* and *Ebf3* null mice show defects in their projection to the dorsal olfactory bulb surface. In *Drosophila*, Collier is an important factor for the control of dendrite length and branching in type IV dendritic arborization (da) neurons, and *Spastin* is a known target in this neuron type (Crozatier and Vincent, 2008; Hattori et al., 2007; Jinushi-Nakao et al., 2007). In *C. elegans*, the *unc-3* mutation causes modest defasciculation and pathfinding defects in motor neurons (Prasad et al., 1998).

Finally, EBF proteins also are important for neuronal cell migration. *Ebf2* null mice have defects of the migration of gonadotropin releasing hormone-synthesizing (GnRH) neurons from the olfactory epithelium to the hypothalamus, and defects of Purkinje neuron migration from the anterior cortical transitory zone to their final position beneath the external granular layer in cerebellar cortex (Corradi et al., 2003; Croci et al., 2006). In *Ebf1* null mice, facial branchiomotor (fbm) neurons migrate from rhombomere 4 (r4) not only to r6 but also to r5 in the early embryo (Garel et al., 2000). In the

migrating fbm neurons in r5, the expression level of *Tag-1* is slightly reduced, and the expression level of *Cadherin-8* is upregulated (Garel et al., 2000). When *Ebf1* is misexpressed in chick spinal cord, neuroepithelial progenitors migrate toward the mantle layer faster than normal, and the expression of *NF* and *R-cadherin* are upregulated (Garcia-Dominguez et al., 2003).

Experiments like those described above provide excellent clues to EBF protein functions in neuronal development in these systems. However, *Ebf* genes are strongly expressed in differentiating central and peripheral neurons throughout development (Davis and Reed, 1996; Garel et al., 1997), so it is important to ask whether additional important targets and mechanisms of EBF function exist.

EBF interactions with the protein ZFP423 and the Notch pathway

EBF forms heterodimers with ZFP423

EBF proteins can function as homodimers, but can also function as heterodimers with the protein ZFP423 (Zinc finger protein 423) (Hata et al., 2000; Tsai and Reed, 1997, 1998). ZFP423 (also known as OAZ (O/E associated zinc finger protein) and EBFAZ (EBF associated zinc finger protein)) is a 30 zinc finger domain nuclear protein. It has a DNA binding domain and protein interaction domains, and can function as a transcription factor by heterodimerizing with SMADs, in addition to EBF proteins. By interacting with the SMAD1-SMAD4 complex after BMP treatment, ZFP423 can control the expression of BMP target genes in cultured cells and in *Xenopus* (Hata et al., 2000; Ku et al., 2006; Shim et al., 2002).

There is a clear interaction between Zfp423 and EBF proteins in neuronal development in olfactory and cerebellar tissues. In rat olfactory neuron differentiation, ZFP423 prevents EBF1 and probably other EBF family members from binding to the EBF-binding sites of olfactory specific genes by forming heterodimers, which prevents EBF from promoting neuronal differentiation (Tsai and Reed, 1997, 1998). This involvement of both EBF and Zfp423 is seen in the finding that in both *Zfp423* null mice and *Ebf2* and *Ebf3* null mice, the axons of olfactory receptor neurons (ORNs) fail to reach the caudal region of the olfactory bulb (Cheng and Reed, 2007; Wang et al., 2004). Interestingly though, artificially sustained expression of *Zfp423* throughout ORN development leads to arrested ORN maturation at an early stage (Cheng and Reed, 2007). These results raise the possibility that inhibition of ZFP423 activity may also be important for neuronal commitment. Since EBF proteins are known to interact with ZFP423, they are good candidates for inhibitors of ZFP423 activity, but it is not known whether EBF proteins in fact have this function.

Zfp423 null mice also show profound hypoplasia of the cerebellar vermis (Alcaraz et al., 2006; Cheng et al., 2007; Warming et al., 2006). In these null mice, proliferation of granule cells is reduced, and several aspects of Purkinje cell (PC) development are disrupted, including reduced PC number, defective migration and reduced dendritic arborization. Interestingly, there are similar defects in cerebellar PC development in *Ebf2* null mice (Croci et al., 2006), suggesting that the interaction between ZFP423 and EBF may be important for cerebellar development as well. The reduced proliferation of cerebellar granule cells in *Zfp423* null mice suggests that ZFP423 must be involved in neuronal cell proliferation, but the mechanism is not known.

Notch signaling and EBF

Notch signaling is critical for a wide variety of events in development, with core mechanisms conserved across species separated by hundreds of millions of years (reviewed in Artavanis-Tsakonas et al., 1999; Kageyama et al., 2009). In neurogenesis, Notch signaling serves to delay neuronal commitment and differentiation (Figure 1.2). In doing so, it also maintains a progenitor population for the production of later-born neuronal and glial cell types (Dorsky et al., 1997, and reviewed in Kageyama et al., 2007).

The mechanism by which Notch signaling delays neuronal commitment begins with the Notch receptor binding transmembrane ligands of the Delta or Jagged/Serrate family, expressed on adjacent cells. This binding leads to cleavage of the Notch intracellular domain (Brou et al., 2000; Mumm et al., 2000), which then translocates to the nucleus, and initiates transcription of target genes such as *Drosophila Enhancer of Split*, or vertebrate *Hairy*, *Hes1* and *Hes5/ERS1* (Fryer et al., 2002; Hsieh et al., 1999; Jarriault et al., 1995; Jennings et al., 1994; Ohtsuka et al., 1999; Sasai et al., 1992; Wallberg et al., 2002; Wu et al., 2000). These genes code for proteins that block the activity of bHLH transcription factors, like Neurogenin and ATH5 (Kageyama et al., 2005; Schneider et al., 2001). The potency of Notch signaling is illustrated by the phenotype of *Hes1*/*Hes5* double mutants, which exhibit massive premature differentiation, especially in the dorsal neural tube (Hatakeyama et al., 2004).

The interactions between Notch signaling and EBF proteins have been studied in several tissues, including *Xenopus* nervous system, *Drosophila* muscle and mouse B cells. During primary neurogenesis in *Xenopus*, the function of EBF2 (but not EBF3) as a

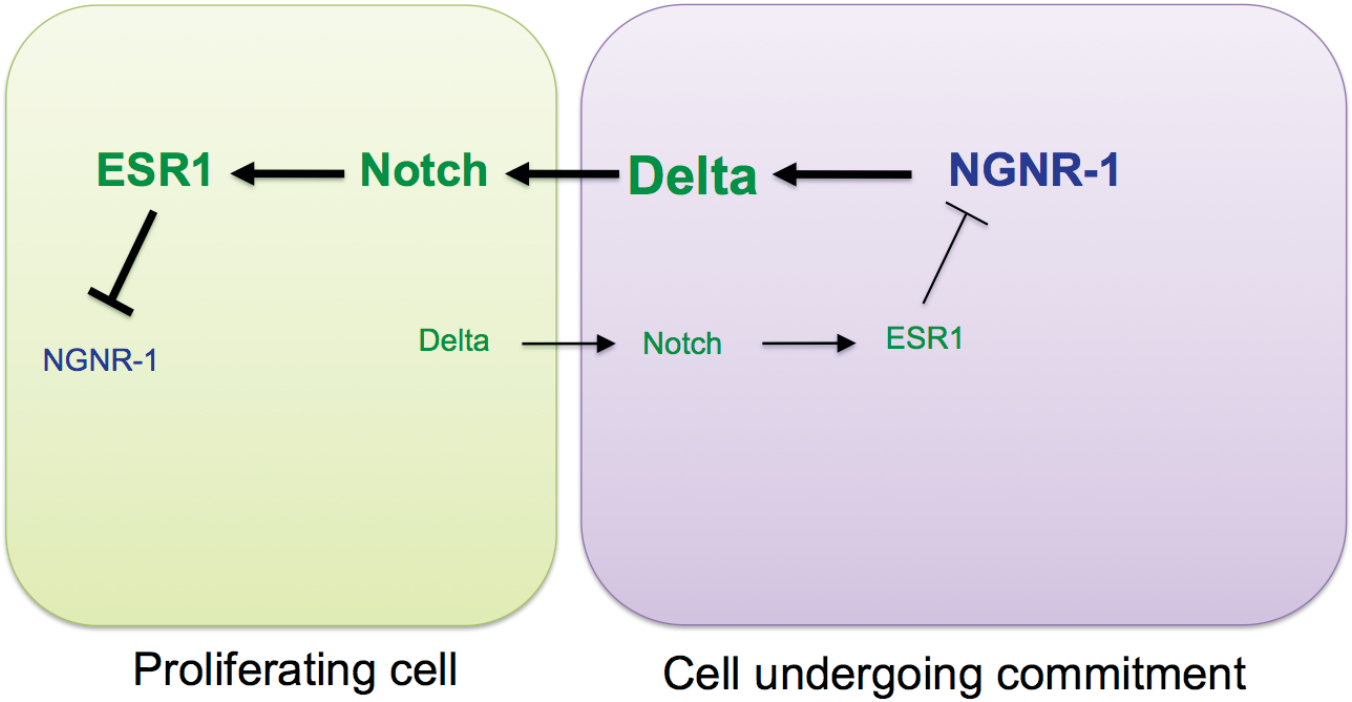

Figure 1.2: Notch/Delta signaling maintains cells in a proliferating state. In a proliferating cell, there is strong Notch/Delta signaling. The Delta ligand on a neighbor cell binds to the Notch receptor on the proliferating cell. This binding leads to cleavage of the Notch intracellular domain, which moves into the nucleus and transcribes the targets of Notch signaling. Targets including ESR1 block the expression of genes related to neuronal commitment and differentiation, such as Neurogenin. Through ESR1, Notch signaling can maintain cells in a proliferating state. Cells that receive less Notch/Delta signaling can undergo commitment and differentiation, because the expression of Neurogenin is not blocked by Notch targets.

neuronal differentiation factor is controlled by Notch signaling (Dubois et al., 1998; Dubois and Vincent, 2001; Pozzoli et al., 2001). When *ebf2* is coinjected with the dominant negative form of *delta* (XDeltadn, (Chitnis et al., 1995)), the ectopic expression of neuronal differentiation markers is strongly upregulated compared to the injection of *ebf2* alone or XDeltadn alone (Dubois et al., 1998).

Interestingly, although expression of *ebf2* is slightly later than the expression of the bHLH gene *ngnr-1* in *Xenopus* embryos, *ebf2* can drive expression of *ngn-r1* and *delta* (Dubois et al., 1998). These results suggest that there is an early period where Notch signaling blocks the expression of both *ngnr-1* and *ebf2* during neuronal progenitor development, followed, in some progenitors, by increased expression of *ngnr-1* and then *ebf2*, followed in turn by a period where EBF2 provides positive feedback to drive *ngnr-1* and *delta* expression, to stabilize and promote neuronal commitment and differentiation (Dubois et al., 1998; Dubois and Vincent, 2001). The aspects of Notch signaling that suppress Collier or EBF activity, and whereby Collier or EBF eventually overcome this negative regulation, are similarly important regulatory pathways for *Drosophila* muscle development and murine B cell development (Crozatier and Vincent, 1999; Smith et al., 2005; Souabni et al., 2002). While it is known that EBF proteins can increase delta levels cell-intrinsically, the full extent of interaction between EBF activity and Notch signaling is not known, and the involvement of EBF proteins in promoting cell commitment suggests the possibility of a more extensive interaction.

Muscle development and EBF proteins

Xenopus skeletal muscle development

In *Xenopus*, prior to metamorphosis, skeletal muscle arises from early mesoderm tissue, which is induced by the mesoderm inducing factors Nodal, BMP, Wnt and FGF (reviewed in Chanoine and Hardy, 2003; Elinson, 2007; Kimelman, 2006; Kumano and Smith, 2002). Mesoderm tissue becomes presomitic mesoderm, and somites are formed from this presomitic mesoderm through the process of somitogenesis. *Xenopus* somitogenesis is different from mouse somitogenesis. During *Xenopus* somitogenesis, a patch of vertically arrayed cells rotates together to assume a horizontal orientation, and after rotation, they form segmented somites (Hamilton, 1969; Youn and Malacinski, 1981). Most cells in a somite are myotome cells, which will become myoblasts, while other cells will become dermis and cartilage. Next, some cells in the ventral lip (hypaxial dermamyotome, far from the neural tube) of the somites will migrate ventrally, to form mature hypaxial muscle (Martin and Harland, 2001, 2006), while cells in the dorsal lip (epaxial dermamyotome, close to the neural tube) will eventually become epaxial back muscle.

In *Xenopus*, the myogenic regulatory factors (MRFs) MYF5 and MYOD are expressed early, in presomitic mesoderm during gastrulation (Dosch et al., 1997; Hopwood et al., 1989, 1991), while mouse MyoD and Myf5 are detected only from early somite stages (Kablar et al., 1998; Ott et al., 1991; Sassoon et al., 1989). In *Xenopus*, the mesoderm inducing factors FGF, TGFβ/activin, and Wnt8 can drive the expression of *myod* (and *myf5*) in presomitic mesoderm during gastrulation (Chen et al., 2003; Fisher et al., 2002; Hoppler et al., 1996; Isaacs et al., 1994; Kumano et al., 2001; Shi et al., 2002;

Standley et al., 2001). These early expression patterns suggest that MYOD and MYF5 can be involved in *Xenopus* muscle developmental steps earlier than in mouse. Functional evidence of this early involvement comes from experiments showing that when MYF5 function is blocked by morpholinos, normal development of presomitic mesoderm and somites is disrupted (Keren et al., 2005). In developing *Xenopus* somites, *myf5* is expressed in the dorsal lip and ventral lip and *myoD* is expressed throughout the somite (Martin and Harland, 2001; Martin et al., 2007).

While the cells of the dorsal lip are specified in a region close to the eventual placement of the muscles they will form, cells of the ventral lip must migrate substantially before maturation as muscle fibers. Myoblasts migrating ventrally from expanding hypaxial myotome compose what is termed the migrating hypaxial muscle anlagen, which will form abdominal muscles. LBX1 is an important homeobox transcription factor for this cell migration in *Xenopus* (Martin and Harland, 2006), but *myf5* and *myoD* are also expressed in this migrating hypaxial muscle anlagen (Martin and Harland, 2001).

Muscle fiber formation prior to metamorphosis in *Xenopus laevis* is unusual compared to muscle formation in other species (Chanoine and Hardy, 2003; Elinson, 2007). In *Xenopus*, a muscle fiber is formed by amitotic division of the nucleus of one myoblast, instead of the more typical process wherein multiple myoblasts undergo fusion to generate a multinucleated muscle fiber (Boudjelida and Muntz, 1987; Kielbowna, 1966). However during metamorphosis most of the muscle cells that were formed for the tadpole undergo apoptosis. These muscle cells are replaced with secondary muscle fibers,

formed by the more typical process of myoblast fusion, in a process that progresses from dorsomedial to ventrolateral (Nicolas et al., 1998; Nishikawa and Hayashi, 1994, 1995).

EBF function in muscle development

For several *Drosophila* muscles, including the muscle dorsal-achete-3 (DA3), the *Drosophila* EBF ortholog Collier is expressed from the stages of promuscular clusters, through muscle progenitors and founders, to the stage of fused muscle precursors. Collier is required for the fusion of neighboring myoblasts (Crozatier and Vincent, 1999). The *Drosophila* MYOD ortholog Nautilus is also necessary for formation of the muscle DA3 (Dubois et al., 2007; Keller et al., 1998). A cis-regulatory element of *collier* contains several muscle specific transcription factor binding sites, including a binding site for both Collier itself and for Nautilus, and both genes drive expression of *collier* synergistically (Dubois et al., 2007). In mouse, Northern blot analysis shows that *Ebf3* is expressed in adult skeletal muscle (Garel et al., 1997). In this tissue, Ebf proteins are known to bind to the negative regulatory element of the *Glut4* gene (Dowell and Cooke, 2002), which allows for insulin-mediated glucose uptake in muscle and adipocyte cells (Kahn, 1998). However the expression patterns, transcriptional targets, and functions of EBF genes in vertebrate muscle development are not understood.

Thesis overview

This thesis is an attempt to improve our understanding of how EBF transcription factors contribute to vertebrate development. The processes of cellular commitment and differentiation depend on transcriptional regulatory networks, and our understanding of

these networks depends greatly on knowledge of the targets and protein interactions of transcription factors like the EBF family. In the thesis, I describe findings of targets and functions of EBF transcription factors for neuronal and muscle cell development in *Xenopus*. To better understand how EBF transcription factors are involved in neuronal cell commitment and differentiation we performed a microarray screen for EBF targets. Chapter 2 describes this screen and the characterization of the neuronal targets we discovered. Chapter 3 describes how EBF and its protein partner ZFP423 contribute to processes of neuronal development, including proliferation and commitment of neuronal progenitors. Chapter 4 describes the discovery of a requirement for EBF proteins in *Xenopus* muscle development, and describes the characterization of some transcriptional targets that likely mediate EBF effects on commitment and differentiation of *Xenopus* skeletal muscle cells. The conclusions of the thesis are in Chapter 5.

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CHAPTER 2

EBF DRIVES EXPRESSION OF MULTIPLE CLASSES OF TARGET GENES, PARTICIPATING IN A TRANSCRIPTIONAL NETWORK OF REGULATION OF NEURONAL DEVELOPMENT

(Yangsook Song Green and Monica L. Vetter, in preparation for submission)

Abstract

EBF family members are transcription factors known to have important roles in several aspects of vertebrate neurogenesis, including commitment, migration and differentiation. Knowledge of how EBF family members contribute to neurogenesis is limited by the absence of a systematic analysis of their transcriptional targets. We performed a microarray screen in Xenopus animal caps to search for candidate targets of EBF transcriptional activity, and identified targets with multiple roles, including transcription factors of several classes. We determined that, among the most up-regulated candidate genes with expected neuronal functions, most require EBF activity for some or all of their expression, and all have overlapping expression with ebf genes. The identification of targets that are transcription factor genes, including *nscl-1*, *emx1* and *aml1*, improves our understanding of how EBF proteins participate in the hierarchy of transcription control during neuronal development, and suggests novel mechanisms by which EBF activity promotes migration and differentiation. Other targets, including *pcdh8* and *kcnk5*, expand our knowledge of the types of processes that EBF proteins regulate.

Introduction

Throughout animal development, many processes must occur coordinately, including patterning, commitment, differentiation and migration of progenitor cells. In the nervous system in particular, these processes are exceedingly complex and depend on the coordinated expression of many sets of genes. A detailed understanding of gene regulation, including knowledge of the types of genes that different transcription factors

target and the hierarchy of transcriptional activity, is therefore a critical foundation for understanding nervous system development. One group of transcription factors expressed strongly in the developing nervous system is the early B cell factor (EBF, also called Collier/Olf/Ebf (COE), and Olf/Ebf (O/E)) family of Zinc finger helix-loop-helix proteins.

The EBF family includes EBF1, 2, 3 and O/E-4 in mammals (Garel et al., 1997; Hagman et al., 1993; Kudrycki et al., 1993; Malgaretti et al., 1997; Wang et al., 2002; Wang et al., 1997), with EBF2 and EBF3 being the known family members in *Xenopus* (Dubois et al., 1998; Pozzoli et al., 2001), and ZCOE2 a family member in zebrafish (Bally-Cuif et al., 1998). Invertebrate members of this family include Collier in *Drosophila*, and UNC-3 in *C. elegans* (Prasad et al., 1998). EBF family proteins contain a DNA binding domain (a zinc finger coordination motif), which can also participate in dimerization and transactivation, an atypical helix-loop-helix domain, which is critical for formation of homo- and heterodimers, and a C-terminal domain, which is important for transactivation (Hagman et al., 1993; Hagman et al., 1995; Wang et al., 1997).

EBF proteins influence multiple processes in developing neurons (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002). One of their best-understood functions is a role in stabilizing cell commitment. For example, Dubois et al. showed that EBF2 can affect neuronal progenitor cell commitment in early *Xenopus* embryos by reinforcing the expression of the proneural basic helix-loop-helix (bHLH) transcription factor *ngnr*-1, and by maintaining the expression of *delta1* (Dubois et al., 1998). There is also strong evidence that EBF proteins stabilize neuronal cell commitment in developing chick spinal

cord, since electroporated mouse *Ebf1* drives expression of *Ngn1* and *Ngn2* (Garcia-Dominguez et al., 2003).

EBF proteins also have critical roles in neuronal cell differentiation. For example, overexpression of *ebf2* and *ebf3* leads to ectopic expression of neuronal specific markers like *n-tubulin* and *nf-m* in *Xenopus* embryos (Dubois et al., 1998; Pozzoli et al., 2001), suggesting that EBF2 and EBF3 may drive specific aspects of the neuronal differentiation program. Consistent with this, in *Ebf1* null mouse striatum, early neuronal cells show abnormal expression of several genes, indicating disruption of the process of differentiation (Garel et al., 1999). In this mouse, the expression levels of *CRABP-1* (a cellular retinoic acid binding protein) and *Cadherin-8* are downregulated in the lateral ganglionic eminence (LGE), providing further evidence of disruption of normal cellular differentiation (Garel et al., 1999). EBF proteins have also been shown to regulate aspects of cell differentiation in early chick spinal cord, where electroporated mouse *Ebf1* promotes expression of numerous neuronal markers (Garcia-Dominguez et al., 2003), and in both *Drosophila* and *C. elegans* ventral nerve cord (Baumgardt et al., 2007; Prasad et al., 2008).

Several studies have found a strong role for EBF proteins in neurite formation and axon guidance. For example, in *Ebf1* null mice, the thalamocortical fibers in the lateral ganglionic eminence fasciculate abnormally (Garel et al., 1999). In this region, *Sema6a* expression also is downregulated, which may lead to abnormal fiber growth (Garel et al., 2002). Olfactory axons in both *Ebf2* and *Ebf3* null mice show defects in their projection to the dorsal olfactory bulb surface (Wang et al., 2004). Other evidence of abnormal fiber growth comes from alterations in dendritic arborization of *Drosophila* type IV neurons in

Collier mutants (Crozatier and Vincent, 2008; Hattori et al., 2007; Jinushi-Nakao et al., 2007), and defasciculation and pathfinding defects in motor neurons of *C. elegans unc-3* mutants (Prasad et al., 1998).

Finally, EBF proteins also are critical for neuronal cell migration. Evidence for this comes from gonadotropin releasing hormone-synthesizing neurons, which fail to migrate normally from the olfactory epithelium to the hypothalamus in *Ebf2* null mouse embryos (Corradi et al., 2003). Furthermore, Purkinje neurons show defects in migration from the anterior cortical transitory zone to beneath the external granular layer in cerebellar cortex (Croci et al., 2006). In *Ebf1* null mice, facial branchiomotor (fbm) neurons migrate from rhombomere 4 (r4) not only to r6 but also to r5 in the early embryo (Garel et al., 2000). In the migrating fbm neurons in r5, the expression level of transient axonal glycoprotein (*TAG-1*) is slightly reduced, and the expression level of *Cadherin-8* is upregulated (Garel et al., 2000). When *Ebf1* is misexpressed in chick spinal cord, neuroepithelial progenitors migrate toward the mantle layer faster than normal, and the expression of *NF* and *R-cadherin* are upregulated (Garcia-Dominguez et al., 2003).

Ebf genes are strongly expressed in differentiating central and peripheral neurons throughout development (Davis and Reed, 1996; Garel et al., 1997), and the evidence described above provides strong indications of EBF protein function in neuronal development. However, it is not fully understood how these functions are executed since there has not previously been a systematic analysis of EBF transcriptional targets. The goals of this study were three-fold. First, we sought to identify the targets of EBF transcription factors, and to analyze *in vivo* the dependence of the discovered targets on EBF activity. We performed a microarray analysis to identify targets of EBF3 in the

developing *Xenopus* nervous system. The microarray results were confirmed with gain- and loss-of-function studies of EBF2 and EBF3 in *Xenopus* embryos. Second, we analyzed the expression of the candidate targets as compared to the *ebf* genes, to gain an understanding of where in the embryo these target genes may function. And third, we assessed which target genes are direct targets of EBF3, and which are indirect targets, to better understand the hierarchy of transcriptional control by EBF proteins. Many genes previously demonstrated to be required for neuronal development are strongly upregulated by EBF, but were not previously known to be targets of EBF transcriptional activity. These targets include transcription factors, cell structural proteins, an ion channel protein, and a gene involved in TGF-beta signaling. The variety of targets found expands our knowledge of the kinds of processes EBF proteins regulate, and reinforces the idea that EBF proteins can influence many aspects of neuronal development because they direct expression of several different functional classes of genes. The discovered targets open a new window to understanding the broader scope of EBF functions.

Materials and methods

Microinjection of RNA and morpholinos

The following constructs were used as DNA templates to make capped RNA: pCS2+Noggin (Richard Harland), pCS2+hGR-MT-Xebf2, pCS2+hGR-MT-Xebf3, pCS2+MT-DN-Xebf, and pCS2+nβgal (Chitnis et al., 1995). Capped RNA was generated *in vitro* using the Message mMachine kit (Ambion). Antisense morpholino oligonucleotides (MOs) were designed by Gene Tools, and directed against a region at or

near the translational start site of *ebf2* (5'-GCGCTTTGTCTCTCAAGGCAGTTCC-3') and *ebf3* (5'-GTATATTTTCCTGAATCCCAAACAT-3').

For microarray experiments, 1ng of hGR-XEBF3 mRNA and 0.2ng noggin mRNA were co-injected into *Xenopus* embryos at the one-cell stage. Alternatively, 0.4ng hGR mRNA and 0.2ng noggin mRNA were co-injected in control embryos. At stage 9, animal caps were dissected from the embryo, using either a Gastromaster or a hypodermic needle tip. Animal caps were treated with 30μM Dexamethasone (DEX) in 1x MMR for 4.5 hours before harvesting of total RNA.

For all other microinjections, a volume of 4nl containing capped RNA or morpholinos was injected into one blastomere of 2-cell stage embryos in the following amounts: hGR-XEBF2 (0.5ng), hGR-XEBFf3 (0.5ng), XEBF3ΔDΔI (2ng), MyoD-hGR (0.5ng), nβgal (30pg), XEBF2 MO (15ng) and XEBF3 MO (15ng). In the MO experiments, both EBF2 and EBF3 MOs were co-injected. For all injections nβgal capped RNA was co-injected as a tracer. Embryos were grown until neural plate or tail bud stages (Nieuwkoop and Faber, 1994). hGR-XEBF2, hGR-XEBF3 and MyoD-hGR injected embryos were treated with 30μM DEX from the gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). Embryos were then fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. After washing embryos 3 times with PBS, X-gal staining was performed as described (Turner and Weintraub, 1994), followed by post-fixation in 4% PFA for one hour at room temperature or overnight at 4°C.

Microarray analysis

Total RNA was isolated from animal caps with the RNeasy mini kit (Qiagen). This RNA was used to perform two-color microarray analysis on the *Xenopus* Agilent microarray by the University of Utah Microarray core facility. Fluorescently labeled cRNA, containing either cyanine 3-CTP or cyanine 5-CTP, was generated using the Agilent Two-Color Quick Amp Labeling kit (catalog # 5190-0444). Next, microarray hybridizations were performed using Agilent surehyb hybridization chambers. Slides were then scanned in an Agilent Technologies G2505B microarray scanner at 5μm resolution. Finally, TIF files were generated from the scanned microarray image, and loaded into Agilent Feature Extraction Software version 9.5.1. Data generated by the software were recorded as a tab-delimited text file. Genesifter was used for microarray data analysis. Four replicate experiments were performed, and significance was determined with a t-test, using a p-value of <0.05.

In situ hybridization

The following constructs were used to generate antisense RNA probes: pBS-Xebf2 (Pozzoli et al., 2001), pBS-Xebf3 (Pozzoli et al., 2001), pBS-Sox2 (Mizuseki et al., 1998), PCDH8 (IMAGE ID 6955713, ATCC), Peripherin (IMAGE ID 4959167, ATCC), GREB1 (IMAGE ID 5569934, ATCC), pBS-XNF-M (Pozzoli et al., 2001), KCNK5 (IMAGE ID 6863628, ATCC), NSCL-1 (IMAGE ID 5514274, ATCC), pBS-XNeuroD (Lee et al., 1995), AML1 (IMAGE ID 4963637, ATCC), Activin beta B (IMAGE ID 5440215, ATCC), Emx1 (IMAGE ID 6957219, ATCC). Antisense RNA probe was generated *in vitro* using SP6, T7 or T3 RNA polymerase (Ambion) and labeled

with digoxigenin-11-UTP (Roche). Whole mount *in situ* hybridization was performed on the fixed and X-gal stained embryos as described (Harland, 1991; Kanekar et al., 1997).

Real time quantitative PCR (RT-QPCR)

For RT-QPCR experiments, 1ng hGR-XEBF3 mRNA and 0.2ng noggin mRNA were co-injected into *Xenopus* cells at the one-cell stage and animal caps isolated at stage 9 as described above. The animal caps were divided into four groups. The control group received no treatment (-C-D). The second group was treated with 30μM DEX alone for 3 hour (-C+D), and the third group was treated with 5μg/ml cycloheximide (CHX) alone for 3.5 hours (+C-D). Finally the fourth group was treated with 5μg/ml CHX for 30 minutes and then 30μM DEX was added for 3 hrs (+C+D). Total RNA was purified from animal caps with Trizol (Invitrogen) and then genomic DNAs were removed with the RNeasy mini kit (Qiagen).

To make cDNA from the isolated total RNA from animal caps, the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) was used according to the manufacturer's instructions, and then quantitative PCR (QPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT Real Time PCR System (Applied Biosystems). Alternatively, the Superscript III Platinum two step qRT-PCR kit and SYBR Green (Invitogen) were used to make cDNA and to generate the PCR solution, and QPCR was performed on the same 7900HT Real Time PCR System (Applied Biosystems). MacVector Software was used to design the gene specific primers (Table 2.1). The relative gene expression level was determined by normalizing the threshold cycle (Ct) of each gene to the Ct of Histone H4. One Ct difference indicates a

Table 2.1: Primer sequences used for RT-QPCR.

Name	Sequence
protocadherin 8 forward	5′- AGGACAGCGGCAAAGGTGAC -3′
protocadherin 8 reverse	5′- GGCGGGGAGAGCAGATTTAG -3′
peripherin forward	5′- CCAAGCAAAGTCCAAAGAGCC -3′
peripherin reverse	5′- GGTTGTGCCTGAACGGTCAC -3′
greb1 forward	5′- TGACAAAAGGTTGGGCAGGG -3′
greb1 reverse	5′- AGGAAAACTATCGGCGGCTG -3′
nf-m forward	5′- GAACAGGTACGCCAAGCTGACTG -3′
nf-m reverse	5′- GCAGCAATTTCTATATCCAGAG -3′
kcnk5 forward	5′- CGGGTTTGGAGACTATGTGGC -3′
kcnk5 reverse	5′- ATCCTTTGGGTTGGTCATTGG -3′
nscl-1 forward	5′- TTCCATTGCTCCGTCAAGTTTC -3′
nscl-1 reverse	5′- GCCCATCGTGTCCATTGTTTTC -3′
neurod forward	5′- CCAGAAACCCAAAAGACGAGG -3′
neurod reverse	5′- ATGCGACGGCACATCCTGAC -3′
aml1 forward	5′- AACCAACCCAATCCAAGCAGTAG -3′
aml1 reverse	5′- CAGCAACCTGTCCTGTATGTTCC -3′
activin beta b forward	5′- ATGATTGTGGACGAGTGCGG -3′
activin beta b reverse	5′- TGCTTCTATCCCTTTGCCAGG -3′
emx1 forward	5′- CGCTCCATCTACAACAACCCG -3′
emx1 reverse	5′- ATGTCGCTGCCTTGAAATCTG -3′
histone h4 forward	5′- TGCGGGATAACATTCAGGGC -3′
histone h4 reverse	5′- CGGTCTTCCTCTTGGCGTG -3′

two-fold difference in the initial cDNA template amount. Finally, expression levels were normalized by setting the expression level in the condition of –C+D to 100.

Results

Identification of candidate targets of EBF3 in animal caps

To identify transcriptional targets of EBF3, we performed a microarray screen comparing the transcripts expressed in *Xenopus* animal cap ectoderm with and without active *Xenopus* EBF3 protein. To do this, we expressed a hormone-inducible fusion protein (hGR-XEBF3) that can be regulated by the hormone dexamethasone (DEX). In the absence of DEX, EBF3 remains inactive, while adding DEX to the explants induces EBF3 activity (Kolm and Sive, 1995). mRNA encoding hGR-XEBF3 was injected at the one-cell stage, animal caps were cut at the blastula stage then incubated with DEX for 4.5 hours to induce EBF3 activity, after which total RNA was isolated (see Methods).

Since EBF proteins are involved not only in neuronal development but also in the development of several other cell lineages, including B cell, adipocyte, and muscle cells (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002; Lukin et al., 2008), we attempted to refine our search for EBF targets related to neuronal development by coinjecting Noggin mRNA to neuralize the animal caps (Lamb et al., 1993). Agilent *Xenopus* microarrays were used to compare target gene expression levels in DEX-treated animal caps, with activated hGR-XEBF3, to those in control, DEX-untreated animal caps, in four independent experiments. To exclude genes that had their expression levels affected by the hormone DEX itself, we performed a separate, control microarray

analysis using animal caps treated with DEX expressing control hGR versus untreated animal caps expressing hGR-XEBF3.

Since we were most interested in positive transcriptional targets of EBF activity, we screened the array data for genes that are selectively upregulated when EBF3 activity is induced. This study specifically focuses on the genes that were most strongly upregulated by EBF3, with increases in expression of more than ten-fold, and that have potential roles in nervous tissue. Although we neuralized animal caps with Noggin, many genes were upregulated by hGR-XEBF3 that are known to be involved in the development of other tissue types, such as *myod*, *lmo2*, and *hex.* The targets with predicted function in muscle tissue are described in a separate report (Green et al., in preparation). The complete data set is publicly available on the GEO database (submission pending). In cases of incomplete annotation for the *Xenopus* microarray, we used NCBI UniGene or BLAST to identify homologs in other species and determine likely gene identity. An indication of the integrity of our screen is the strong upregulation of *nf-m*, a known EBF3 target gene (Pozzoli et al., 2001). Microarray results were confirmed for key genes by reverse transcriptase polymerase chain reaction (RT-PCR) (data not shown).

EBF2 and EBF3 are sufficient for the expression of candidate neuronal targets *in vivo*

We previously showed that the protein sequences of *Xenopus* EBF2 and EBF3, as well as their functions in neuronal development, are very similar (Pozzoli et al., 2001). For these reasons, we included both EBF2 and EBF3 in the experiments that follow. In order to both confirm the microarray data and to determine if EBF2 and EBF3 are

sufficient for the expression of the candidate target genes *in vivo*, we examined the expression level of candidates after overexpression of hGR-XEBF2 and hGR-XEBF3. For this analysis, we selected 14 genes predicted to be involved in neuronal development, based on expression patterns and functions known from the published literature, as well as on our own observations with whole mount *in situ* hybridization (WM-ISH) (discussed below). Overexpression was achieved by injection of mRNA for hGR-XEBF2 or hGR-XEBF3 into one cell of two-cell stage embryos, followed by treatment of the embryos with DEX from the gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). The expression level of candidate target genes was then examined by WM-ISH. We found that 10 of the 14 candidate target genes were upregulated by overexpression of EBF2 and by overexpression of EBF3 (Table 2.2 and Figure 2.1). These were *pcdh8* (*protocadherin* 8) (20/23 embryos by EBF2, 27/28 embryos by EBF3), *peripherin* (also called *xif3*) (15/15, 35/35), *greb1* (genes regulated by estrogen in breast cancer) (12/12, 10/10), *nf-m* (neurofilament-m) (15/15, 35/38), *kcnk5* (*potassium channel subfamily K member 5*, also called *task2*) (11/11, 39/40), *nscl-1* (also called *Xhen1* and *nhlh1*) (9/9, 33/40), *neurod* (20/21, 39/39), *aml1* (acute myeloid leukemia, also called *runx1*) (12/12, 11/11), *activin beta B* (also called *inhbb*) (11/11, 37/40), and *emx1* (11/13, 28/32) (Figure 2.1). However, four genes were not consistently upregulated by EBF2 or EBF3. The expression of *en-2* (10/19) and *hoxd10* (30/44) were downregulated, while the expression of *nr2f2* was upregulated (19/73) in some embryos but downregulated (30/73) in others, and the expression of *wnt3a* (24/24) was not changed by EBF3 (data not shown). We therefore believe that these four genes are unlikely to be *in vivo* targets of Ebf activity, and we have excluded them from the experiments that follow. The fact that the expression levels of 10

Table 2.2: Candidate targets of EBF activity. The genes chosen for additional analysis are shown with their known functional roles. Fold change refers to the increase in expression in the microarray, compared to control.

Gene name	Function	Fold change	GenBank
protocadherin 8	transmembrane protein	66	BC074360
peripherin	type III intermediate filament	37	BC056020
greb1	estrogen-regulated gene	32	BC043838
nf-m	type IV intermediate filament	28	BC078128
kcnk5	K+ ion channel subunit	27	BC084931
nscl-1	bHLH transcription factor	26	BC084434
neurod	bHLH transcription factor	26	BC072996
aml1	Runt-related transcription factor	22	BC057739
activin beta b	TGF-beta superfamily member	21	S61773
emx1	homeobox transcription factor	16	BC077629

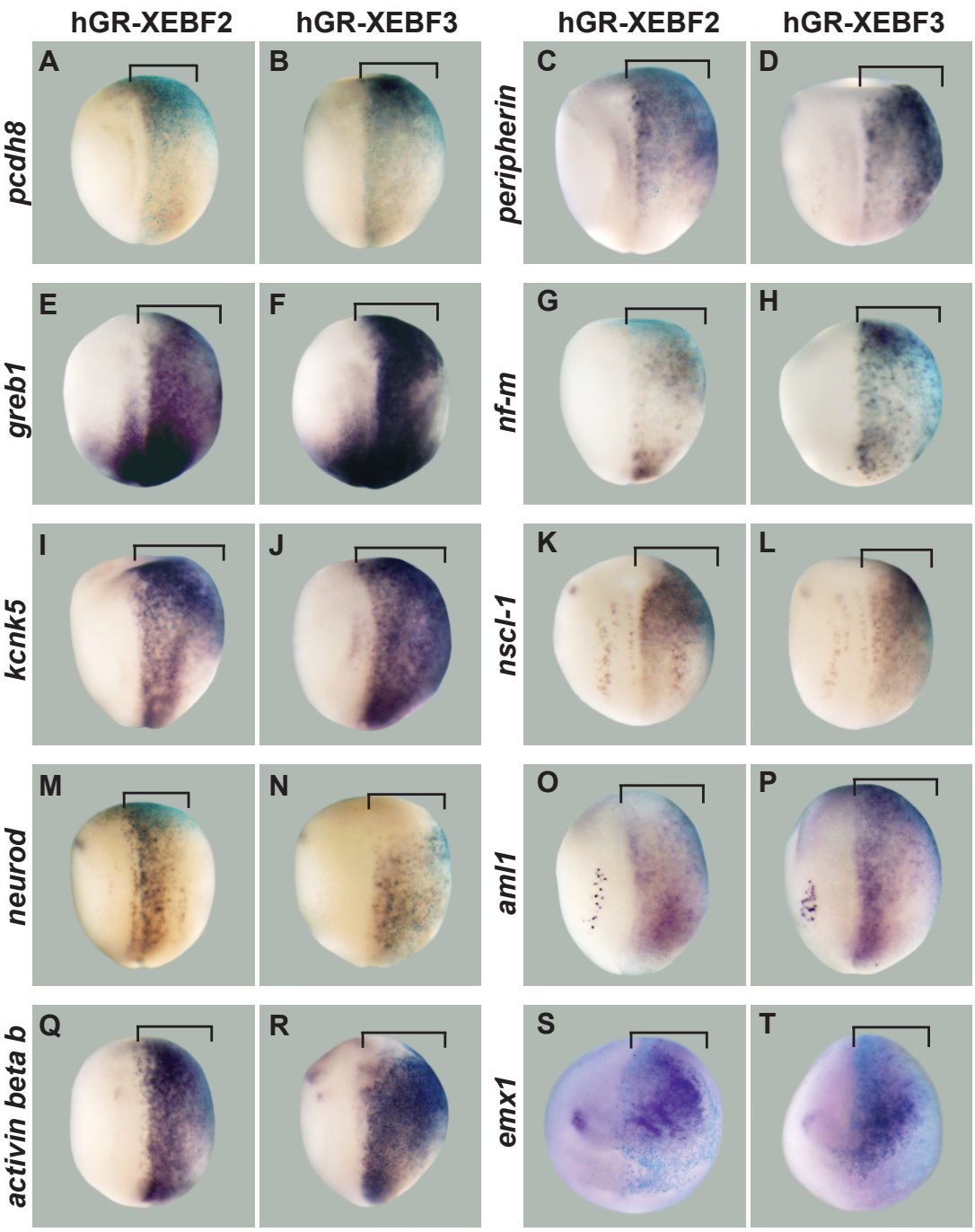


Figure 2.1: Target genes upregulated by overexpression of EBF2 or EBF3. hGR-XEBF2 or hGR-XEBF3 mRNA was injected into one cell of two-cell stage embryos, followed by DEX treatment from the late gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). β-galactosidase mRNA was coinjected as a marker of the injected side. In all panels the right side is the injected side, showing the light blue color of X-gal staining. The expression levels of *pcdh8* (A and B), *peripherin* (C and D), *greb1* (E and F), *nf-m* (G and H), *kcnk5* (I and J), *nscl-1* (K and L), *neurod* (M and N), *aml1* (O and P), *activin beta b* (Q and R), and *emx1* (S and T) are strongly upregulated by EBF2 and EBF3 (brackets). A-R show dorsal views, while S and T show anterior views.

genes among 14 candidates are upregulated by overexpression of EBF2 and EBF3 in the intact embryo supports the microarray data, and further shows that EBF2 and EBF3 activity are sufficient to drive expression of these candidate genes *in vivo*.

EBF2 and EBF3 are required for the expression of candidate targets *in vivo*

To determine if the expression of our identified candidate target genes is dependent on EBF2 and EBF3 *in vivo*, we examined the expression level of targets after knockdown of EBF2 and EBF3 expression using translation blocking antisense morpholinos (MO). EBF2 MO and EBF3 MO were coinjected into one cell of two-cell stage embryos and the expression level of endogenous candidate target genes was examined at the neural fold stage (stage 15/16) or tailbud stage (stage 25-28), when expression of candidate target genes is apparent (Figures 2.2 and 2.3). Expression of the neural plate marker *sox2* was first examined to determine if knockdown of EBF2 and EBF3 affects global neuronal development in the early embryos. *sox2* expression did not change (10/10 embryos; Figure 2.2B), indicating early global neuronal development was unaffected. After coinjection of EBF2 MO and EBF3 MO, the expression of *nscl-1* (10/11), *neurod* (13/15), *aml1* (7/12), *emx1* (12/15), *pcdh8* (12/13), *peripherin* (11/11), *greb1* (11/12), *nf-m* (14/16), *kcnk5* (10/15) and *activin beta b* (5/10) were downregulated (Figures 2.2 and 2.3). Control MO did not change the expression levels of these genes (Figures 2.2 and 2.3).

To confirm the MO results, we generated a dominant negative *Xenopus* EBF3 construct (DN-EBF). This DN-EBF lacks the DNA binding domain in the N-terminal region, but it has an intact dimerization domain (amino acids 349-598). (Dubois et al.,

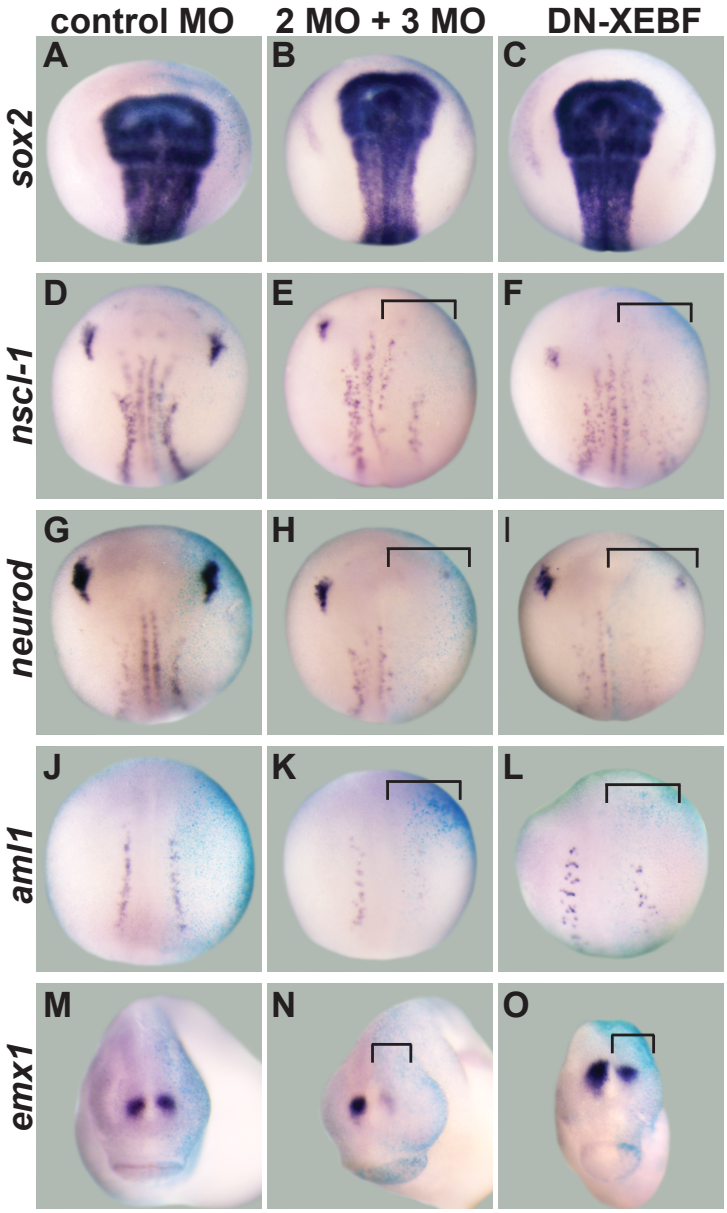


Figure 2.2: Downregulation of transcription factor targets after knockdown of EBF2 and EBF3. One cell of two-cell stage embryos was injected with either dominant negative Xenopus EBF3 (DN-XEBF) mRNA, both EBF2 MO and EBF3 MO (2MO + 3MO), or control MO. β-gal mRNA was coinjected as a marker of the injected side. In all panels the right side is the injected side, showing the light blue color of X-gal staining. The expression of *sox2* does not change in all three conditions (A-C). The expression of *nscl-1* (E and F), *neurod* (H and I), *aml1* (K and L), and *emx1* (M and O) is downregulated by EBF2 MO and EBF3 MO, and by DN-EBF (brackets), while control MO does not change their expression level (D, G, J, and M). A-L show dorsal views of neurula stage embryos (stage 15/16), and M-O are anterior views of tail bud stage embryos (stages 25-28).

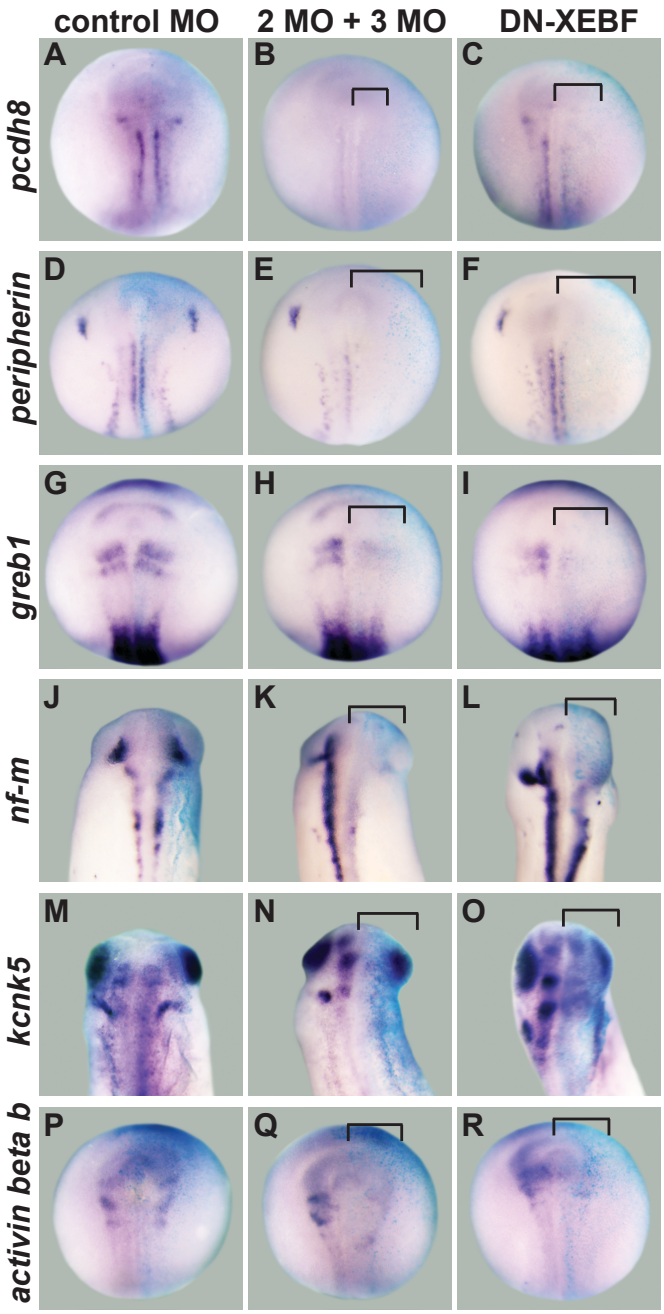


Figure 2.3: Downregulation of non-transcription factor target genes after knockdown of EBF2 and EBF3. One cell of two-cell stage embryos was injected with either EBF2 MO and EBF3 MO together (2MO + 3MO), or dominant negative Xenopus EBF3 (DN-XEBF) mRNA, or control MO. β-gal mRNA was coinjected as a marker of the injected side. In all panels the right side is the injected side, showing the blue color of X-gal staining. The expression of *pcdh8* (B and C), *peripherin* (E and F), *greb1* (H and I), *nf-m* (K and L), *kcnk5* (M and O), and *activin beta b* (Q and R) is downregulated by EBF2 MO and EBF3 MO, and by DN-EBF (brackets), while control MO does not change their expression level (A, D, G, J, M and P). A-I and P-R are neurula stage embryos (stage 15/16), and J-O are tail bud stage embryos (stages 25-28). All panels show dorsal views.

1998; Hagman et al., 1993; Hagman et al., 1995). Since EBF1, 2, and 3 can form homodimers or heterodimers in vitro (Hagman et al., 1993; Hagman et al., 1995; Wang et al., 1997), this DN-EBF is predicted to block the function of both EBF2 and EBF3 by forming non-functional dimers. Similar to our MO data, injection of RNA encoding DN-EBF led to downregulation of the expression of *nscl-1* (5/13 embryos), *neurod* (12/18), *aml1* (6/14), *emx1* (7/15), *pcdh8* (8/18), *peripherin* (8/13), *greb1* (8/14), *nf-m* (10/19), *kcnk5* (7/19) and *activin beta b* (8/17) (Figure 2.2 and Figure 2.3) while *sox2* expression was not changed by DN-EBF at the neural plate stage (13/14) (Figure 2.2C). However, the level of downregulation was weaker than that obtained by MO injection, perhaps because some EBF protein is able to form normal dimers even in the presence of DN-EBF. In addition, a majority of embryos became bent toward the injected side at the tailbud stage because this side was smaller than the uninjected side (data not shown). It is therefore possible that some of the downregulation we observed in these dominant negative knockdown experiments was due to changes in development of other tissues. Taken together, these function-blocking experiments with MOs and DN-EBF suggest that EBF2 and EBF3 are required for the expression of our candidate neuronal targets *in vivo*.

Classes of candidate target genes

The EBF gene candidate target genes that we identified by microarray, and confirmed by EBF gain and loss of function experiments, were classified based on their known or predicted functions, and these are summarized in Table 2.2. There are several transcription factors, including NSCL-1, NeuroD, AML1, and EMX1. NSCL-1 and NeuroD are basic helix loop helix (bHLH) transcription factors (Bao et al., 2000; Lee et

al., 1995). AML1 is a Runt-related transcription factor (Tracey et al., 1998), and EMX1 is a homeobox transcription factor (Brox et al., 2004; Patarnello et al., 1997). Activin beta B is a ligand involved in TGF-beta signaling (Dohrmann et al., 1993). Three genes code for cell structural proteins. PCDH8 is a transmembrane protein (Strehl et al., 1998), and Peripherin and NF-M are intermediate filament proteins. KCNK5 is a potassium ion channel (Reyes et al., 1998). *greb1* is a gene highly upregulated in breast cancer and by the hormone estrogen, but its function is not yet known (Ghosh et al., 2000). In our system, we found that about half of the EBF protein targets are transcription factors. The fact that so many transcription factors are strongly upregulated by EBF proteins suggests that there are multiple levels of transcriptional control that involve the activity of EBF proteins. The other half of the targets are involved in cell structure and neuronal function, reinforcing the idea that EBF proteins are involved in neuronal differentiation during development, as well as performing various functions in mature neurons.

Comparison of the expression patterns of EBF2, EBF3 and their targets in the *Xenopus* nervous system

To determine if the functional relationships we identified above are likely to be meaningful during *Xenopus* development, and to determine if the targets have expression patterns consistent with a role in neuronal development, we compared the expression domains of *ebf2* and *ebf3* with those of target genes by WM-ISH at four different stages in early *Xenopus* embryos: stage 12.5 (data not shown), 15, 23 and 28 (Figures 2.4 and 2.5). We chose these stages because the expression of *ebf2* is clearly visible from stage 12.5 and the expression of *ebf3* is clearly visible at stage 15, by WM-ISH (Dubois et al., 1998; Pozzoli et al., 2001), and their expression continues beyond stage 28. *ebf2* and *ebf3*

Figure 2.4: Neuronal expression for *ebf* genes and transcription factor target genes. *Ebf2* (A-C) and *ebf3* (D-F) are expressed in multiple regions of the developing nervous system including the trigeminal ganglia (yellow arrows), olfactory placodes (black arrows), some domains in the brain, the spinal cord (white arrows), and neural crest derivatives like the branchial arches (Dubois et al., 1998; Pozzoli et al., 2001). *nscl-1* (G-I) and *neurod* (J-L) are expressed in the trigeminal placodes (yellow arrows) and three stripes of primary neurons in the neural plate (arrow heads) at stage 15, and are strongly expressed in the trigeminal placodes (yellow arrow), olfactory placodes (black arrow), and spinal cord (white arrows) at stage 23. At stage 28, *nscl-1* is expressed in the olfactory placode, some domains in the midbrain/hindbrain, spinal cord and cranial ganglia IX and X. At stage 28, *neurod* is expressed in the olfactory placodes, retina, otic placodes, cranial ganglia, spinal cord and some domains in the brain. These expression patterns overlap with *ebf2* and *ebf3*. *aml1* (M-O) is expressed in the lateral primary neuron stripe at stage 15, sensory neurons of the spinal cord (white arrowheads) at stage 23, and the olfactory placodes (black arrow) and otic placodes. *emx1* (P-R) is expressed in the dorsal forebrain at all three stages. Stage 15 embryos show dorsal views except P (anterior view). Stage 23 and 28 embryos show lateral views.

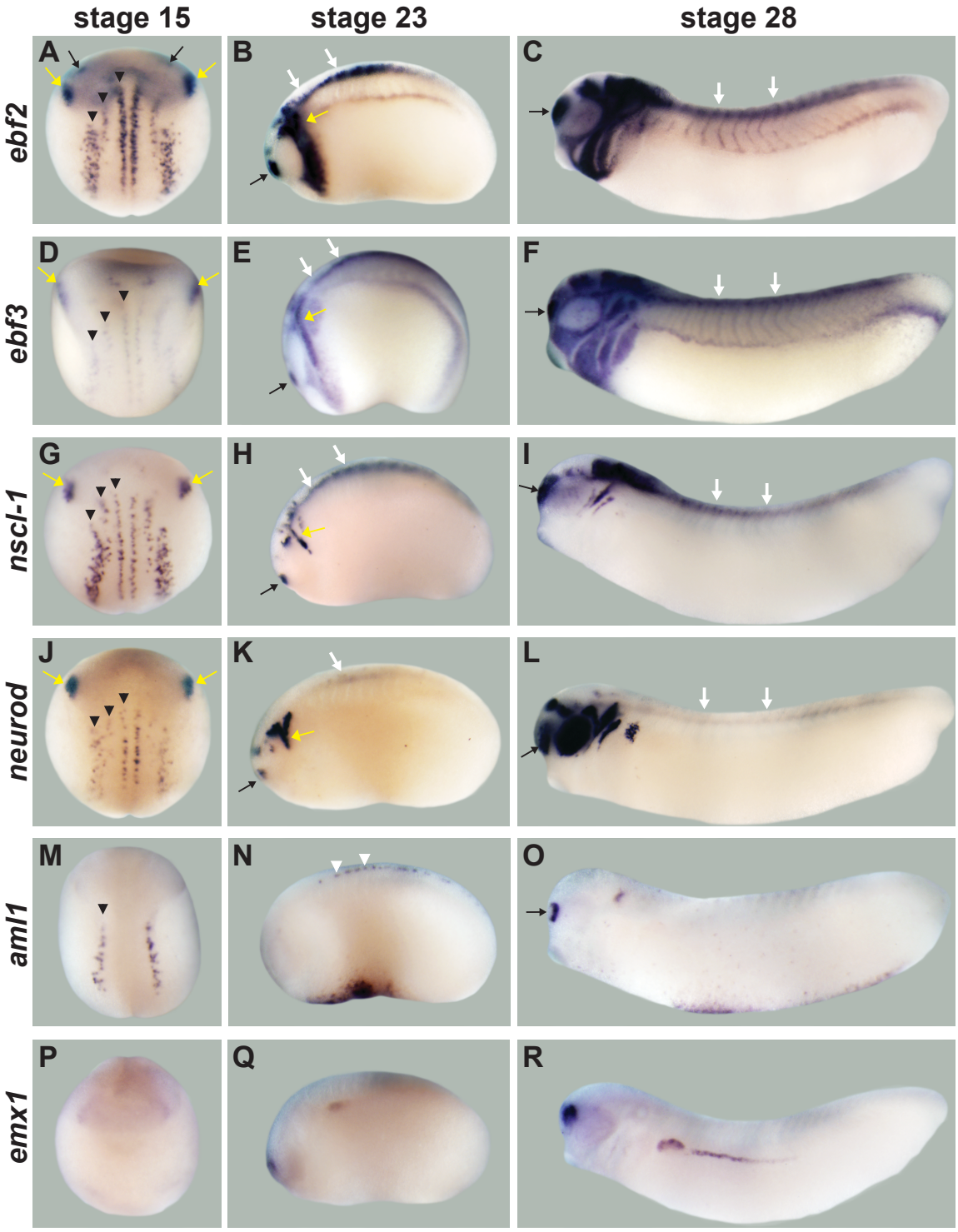
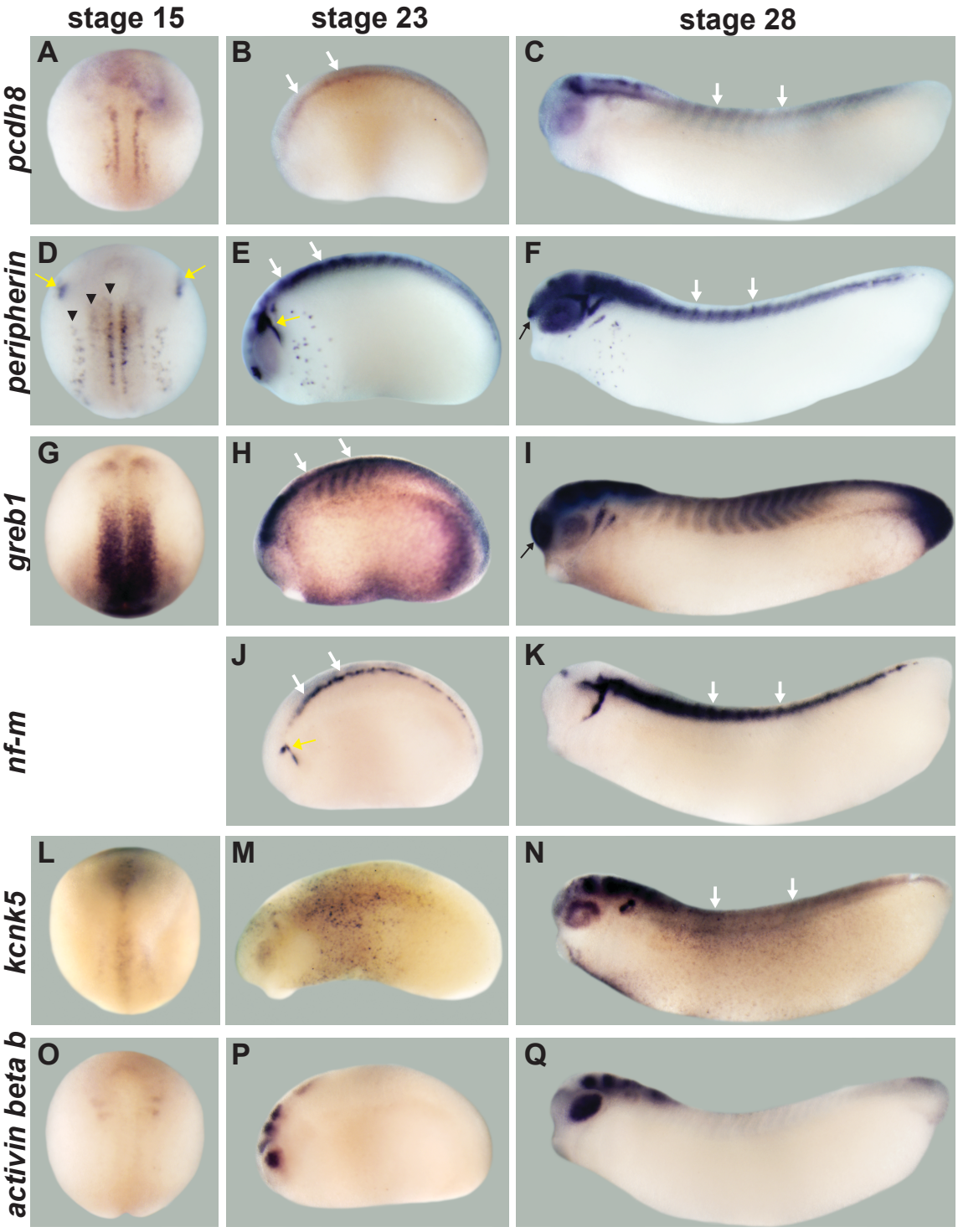

stage 15
stage 23
stage 28
ebf2
ebf3
nscl-1
neurod
aml1
emx1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R

Figure 2.5: Neuronal expression for non-transcription factor target genes. *pcdh8* (A-C) is expressed in two stripes and in the anterior domain of the neural plate at stage 15, and the spinal cord and some neuronal domains in the brain at stages 23 and 28. Like *ebf2* and *ebf3*, *peripherin* (D-F) is expressed in the trigeminal placodes (yellow arrows) and three stripes of primary neurons in the neural plate (arrow heads) at stage 15, and is strongly expressed in the trigeminal placodes (yellow arrow), olfactory placodes (black arrow), and spinal cord (white arrows) at stages 23 and 28. *greb1* (G-I) is expressed as a band in the prospective midbrain/hindbrain region at stage 15, and its expression becomes strong in the midbrain/hindbrain region and spinal cord (white arrows) at stages 23 and 28, and in the olfactory placodes (black arrow) at stage 28. *nf-m* (J and K) is not expressed at the neural plate stage (not shown), but at stages 23 and 28 it is expressed in the trigeminal placodes (yellow arrow) and spinal cord (white arrows), similar to the expression patterns of *ebf2* and *ebf3*. *kcnk5* (L-N) is weakly expressed in two stripes and in the anterior neural plate at stage 15, and in the optic vesicle, otic placode and some domains in the brain at stage 23. It is expressed in several domains in the brain, retina, otic placode and spinal cord at stage 28. At all three stages, *kcnk5* is expressed in spots distrubuted over the body. Activin beta B (J and K) is expressed in two bands in the prospective midbrain/hindbrain region and diffusely throughout the anterior neural plate at stage 15, and it is expressed in the optic vesicle and some brain domains at stages 23 and 28. Stage 15 embryos show dorsal views, and stage 23 and 28 embryos show lateral views.


stage 15
stage 23
stage 28
pcdh8
peripherin
greb1
nf-m
kcnk5
activin beta b
A
B
C
D
E
F
G
H
I
J
K
L
M
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are expressed in very similar neuronal tissues (Figure 2.4A-F, Dubois and Pozzoli). At stage 15, both are expressed in the three stripes of primary neurons and trigeminal placodes. At stage 23, both are expressed in the olfactory placodes, spinal cord, and neural crest derivatives, including branchial arches. By stage 28, their expression expands to encompass much of the developing brain.

First, we compared the expression patterns of *ebf* genes and the targets that are known transcription factors. The bHLH transcription factors NSCL-1 (Figure 2.4G-I, and Bao et al., 2000) and NeuroD (Figure 2.4J-L, and Lee et al., 1995) are expressed in the three stripes of primary neurons at stage 12.5, but their expression at this stage is much weaker than that of *ebf2*. They are expressed in the three stripes and trigeminal placodes at stage 15, and in the olfactory placodes, trigeminal placodes and spinal cord at stage 23 and 28, overlapping strongly with *ebf2* and *ebf3* at these stages. The Runt-related transcription factor AML1 is expressed in sensory neurons including dorsal root ganglia (DRG) in mouse (Kramer et al., 2006; Yoshikawa et al., 2007). In *Xenopus* embryos, *aml1* (Figure 2.4M-O, and Park and Saint-Jeannet, 2010; Tracey et al., 1998) is expressed only in the lateral stripe of primary neurons at stages 12.5 and 15, which gives rise to sensory neurons. At stage 23, this gene is expressed only in sensory neurons in the spinal cord, and this expression pattern overlaps with *ebf2* and *ebf3* (Figure 2.4N, and Park and Saint-Jeannet, 2010). At stage 28, expression of *aml1* is strong in olfactory placodes. The *aml1* expression in the lateral stripe at stage 15 and in olfactory placode at stage 28 overlaps with the expression of *ebf2* and *ebf3*. The homeobox transcription factor gene *emx1* (Figure 2.4P-R, and Brox et al., 2004; Pannese et al., 1998; Patarnello et al., 1997) is expressed in primordium of the forebrain region at stage 15. At stage 23

and 28, this gene is expressed in the dorsal forebrain region, and this expression overlaps with the expression of *ebf2* and *ebf3*.

Second, we compared the expression patterns of *ebf* genes and the targets that do not have transcriptional activity. The protocadherin PCDH8 (Figure 2.5A-C) is expressed in two stripes, but they do not appear to be the three stripes of primary neurons at stage 15. However, the expression of *pcdh8* does overlap with that of *ebf2* and *ebf3* at stage 23 and 28, in spinal cord, midbrain, and hindbrain. One neuronal intermediate filament gene *peripherin* (Figure 2.5D-F, and Gervasi et al., 2000), shows a strongly overlapping expression pattern with *ebf2* and *ebf3* from early embryonic stages, while another neuronal intermediate filament gene, *nf-m* (Figure 2.5J and 2.5K), also shows strong overlapping expression with that of *ebf2* and *ebf3* at stages 23 and 28. The partially overlapping expression regions for *greb1* (Figure 2.5G-I) and *ebf2* and *ebf3* are in the spinal cord and some domains in the brain at stages 23 and 28. *kcnk5* (Figure 2.5L-N) is expressed in two stripes but they also do not appear to be the three stripes of primary neurons at stage 15. Its expression does overlap with *ebf2* and *ebf3* in some domains in the brain and in the spinal cord at stage 28. The expression of *activin beta b* (Figure 2.5O-Q, and Dohrmann et al., 1993) in some domains in the brain at stages 23 and 28 partially overlaps with *ebf2* and *ebf3* expression.

In summary, the expression patterns of bHLH transcription factor genes (*nscl-1* and *neurod*) and intermediate filament genes (*peripherin* and *nf-m*) strongly overlap with those of *ebf2* and *ebf3*. The transcription factor genes *aml1* and *emx1* are expressed in a more limited domain within the nervous system, but their expression also overlaps with areas of *ebf* expression. *Pcdh8*, *greb1*, and *kcnk5* have not previously been strongly

implicated in *Xenopus* neuronal development, but we find that they are expressed in *Xenopus* neuronal tissues, and all three have at least partial overlap with *ebf* genes, suggesting potential importance for them in neuronal development downstream of EBF activity.

Identification of direct targets for EBF3 in animal caps by RT-QPCR

To better understand the transcriptional interaction between EBF3 and its candidate target genes, we sought to identify which genes are direct transcriptional targets and which are indirect transcription targets. We used an approach similar to the microarray analysis, with DEX treatment of animal caps to drive activation of hGR-XEBF3, but we added cycloheximide (CHX) to block protein synthesis, so that only direct EBF3 targets should be transcribed. Animal caps were collected at stage 9 after injection of hGR-XEBF3, and divided into four groups: untreated controls (-C-D), DEX alone (-C+D), CHX alone (+C-D), and both CHX and DEX (+C+D). All animal caps were collected after a 3.5 hour incubation. CHX treatment lasted the entire 3.5 hours, while DEX treatment started after a 30 minute delay to time for CHX to take effect. The expression level of each target was examined by reverse transcriptase quantitative polymerase chain reaction (RT-QPCR) (Figures 2.6 and 2.7). We normalized the expression level of target genes with that of *histone h4*, and set the normalized expression level in the condition of –C+D to 100 percent.

After treatment with both CHX and DEX, targets that had expression levels of less than 10% of the level in animal caps treated with DEX alone were considered to be indirect targets. These indirect targets are *pcdh8*, *kcnk5*, *activin beta b*, *neurod* and *greb1*

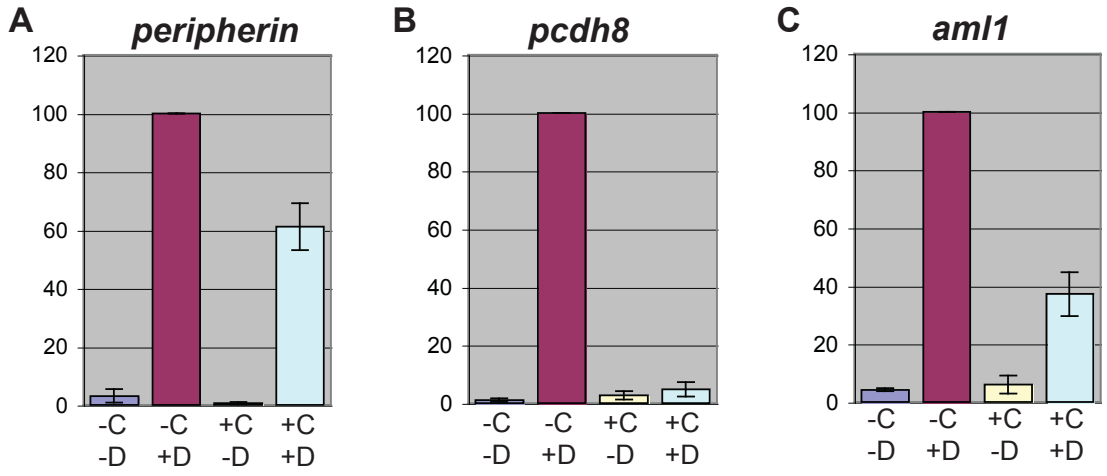

Figure 2.6: The identification of direct and indirect targets of EBF3 by RT-QPCR. hGR-XEBF3 mRNA and Noggin mRNA were injected into one-cell stage embryos, and animal caps were collected at the blastula stage (stage 9). The animal caps were divided into four groups, based on Cycloheximide and Dex treatment: -C-D, -C+D, +C-D, and +C+D. After a 3.5 hour incubation with CHX and/or a 3 hour incubation with DEX, total RNAs were isolated from each animal cap group. RT-QPCR was conducted with the isolated total RNAs. The expression level was normalized with the expression level of *histone h4* and then normalized to the expression level of –C+D, for each gene, at 100 arbitrary units. The expression levels of controls (–C-D and +C-D) in all target genes are very low compared to the treated condition of –C+D (A-C). The expression level of *peripherin* in +C+D (61%) is slightly lower than in –C+D, indicating that the majority of its expression is controlled by EBF3 directly (A). The expression level of *pcdh8* in +C+D (5%) is much lower than in –C+D and is similar to the levels of the control conditions, indicating that it is an indirect target (B). The expression level of *aml1* in +C+D (37%) is lower than the expression level in –C+D but higher than levels of the control conditions, indicating that its expression is controlled by EBF3 both directly and indirectly (C). Error bars represent SEM. The results for the remaining target genes are shown in Figure 2.7.

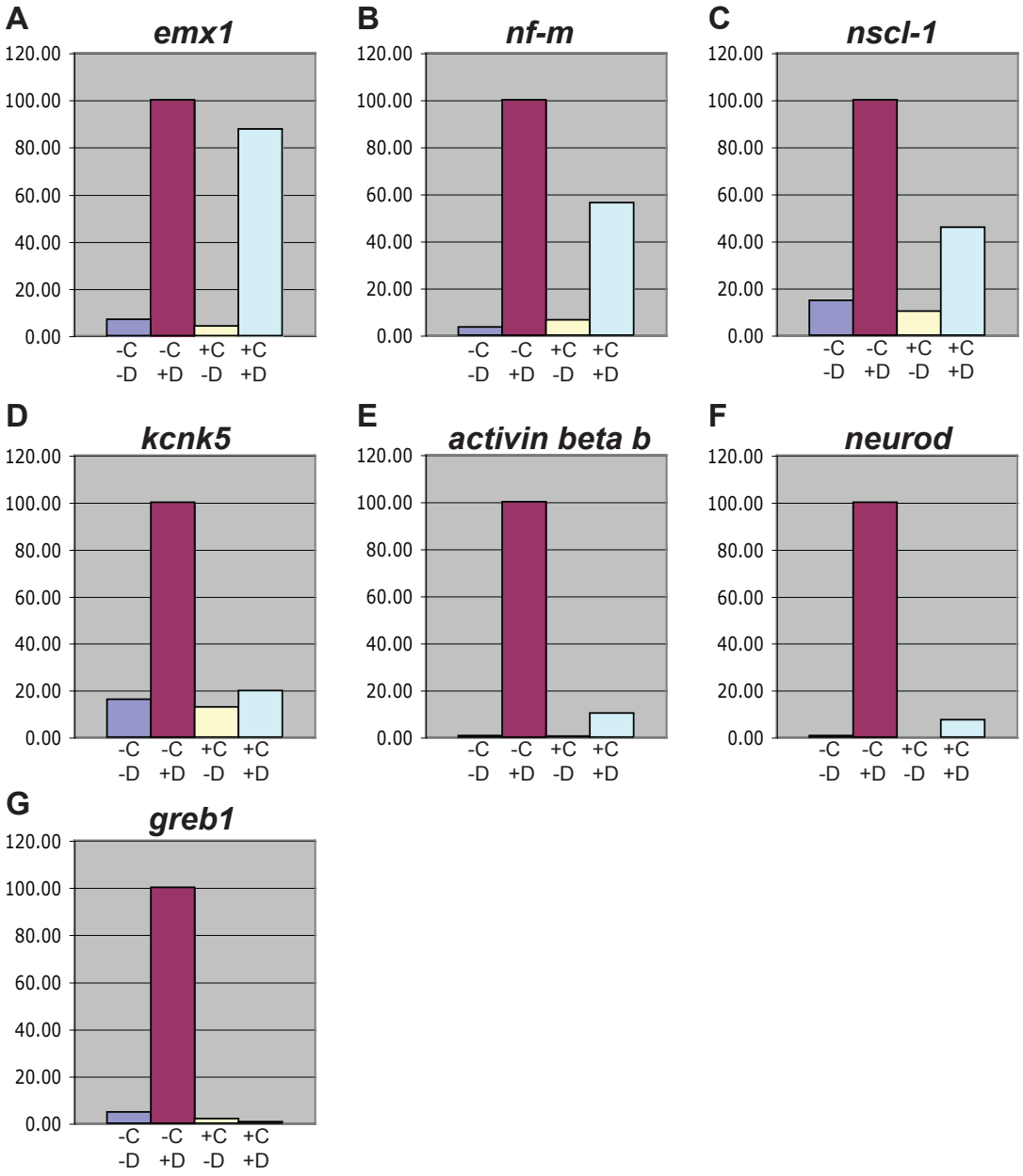

Figure 2.7: Additional identification of direct and indirect targets of EBF3 by RT-QPCR. Expression levels for the remaining target genes tested by RT-QPCR after CHX and DEX treatment (those not shown in figure 2.6). The expression levels of emx1 (88%) and nf-m (56%) in the +C+D are slightly lower than in –C+D, indicating that the majority of their expression is controlled by EBF3 directly (A and B). The expression level of nscl-1 (46%) in +C+D is lower than in –C+D but higher than that in the two controls, indicating that its expression is under both direct and indirect control of EBF3 (C). The expression levels of kcnk5 (20%), activin beta b (10%), neurod (8%) and greb1 (1%) in +C+D are similar to control levels (D) or much lower (less than 10%) than in –C+D (E-G).

(Figures 2.6B and 2.7D-G). Target genes with expression levels of greater than 50% of the levels in animal caps treated with DEX alone are targets for which the majority of their expression is directly controlled by EBF3. These include *peripherin*, *emx1* and *nf-m* (Figures 2.6A and 2.7A and B). Finally, there are genes with expression levels between 10% and 50% of the level in animal caps treated with DEX alone. These genes, including *aml-1* and *nscl-1*, likely have some expression that is under direct regulation by EBF3, but also have significant expression that is indirectly regulated (Figures 2.6C and 2.7C). Interestingly, the genes that appear to be indirect targets tended to be those that have the least overlap with Ebf genes in their WM-ISH expression patterns (with the exception of *neurod*, which does have significant overlap, probably due to the fact that NeuroD acts upstream of *ebf* genes (Dubois et al., 1998; Logan et al., 2005; Pozzoli et al., 2001; Seo et al., 2007) (Figures 2.4 and 2.5). The direct targets had more overlap in expression (Figures 2.4 and 2.5), and included the genes that code for axonal structural proteins (*peripherin* and *nf-m*) and one homeobox transcription factor (*emx1*). The targets with both direct and indirect regulation by EBF3 (*aml-1* and *nscl-1*) were transcription factors, suggesting that their expression is controlled not only by EBF3 directly but also through other targets of EBF3.

Discussion

To better understand the range of activities that are driven by EBF transcription factors, we used a systematic approach to identify target genes of EBF activity in *Xenopus*. In this study, we emphasize candidate targets with potential functions in neuronal development. Most significantly, we have identified target genes that participate

in processes that were previously not known to be driven by EBF activity, and we have generated an extensive list of previously unknown targets that can aid future research into EBF functions.

Our microarray screen for targets of EBF transcriptional activity revealed one gene (*nf-m*) that was a previously known target of EBF proteins (Pozzoli et al., 2001), but also revealed many genes that were previously not known to be targets of EBF activity. Some of these, like *pcdh8*, *peripherin*, *greb1*, *kcnk5*, *nscl-1*, *aml1*, *activin beta b* and *emx1*, were among the most strongly upregulated genes. Fourteen of the most strongly upregulated genes from the microarray were chosen as potentially important genes in neuronal development. The fact that ten of these fourteen genes were also upregulated in whole embryos in response to EBF overexpression shows that EBF activity is sufficient to drive expression of these targets *in vivo*. The fact that expression of all ten of these genes was decreased or eliminated in EBF loss-of-function experiments shows that EBF activity is at least partially required for their in vivo expression. In addition, since all of these genes have at least partially overlapping expression patterns with *ebf* genes, we believe that all these ten are *bona fide* targets of EBF proteins. Since some of these genes are direct targets, some are partially direct, and some indirect, and since most of them have only partially overlapping expression with *ebf* genes, it is clear that EBF proteins are part of a more complex transcriptional regulatory network involved in driving their expression. Individually, and as a group, the genes we have characterized give new insight into the range of functions driven by EBF transcriptional activity in the nervous system. The target genes we identified, but did not characterize further, also have the potential to reveal involvement of EBF proteins in additional activities.

EBF regulation of multiple transcription factor genes suggests involvement in extensive transcriptional networks for neuronal development

Our finding that several transcription factors are among the strongest targets of EBF activity expands the potential routes by which EBF activity could exert its many effects on neuronal development, and suggests some interesting new potential functions. *nscl-1* encodes a basic helix-loop-helix transcription factor that is strongly expressed in both the central and peripheral nervous system during development (Bao et al., 2000; Begley et al., 1992; Lipkowitz et al., 1992; Murdoch et al., 1999). In *Xenopus* embryos, NSCL-1 can drive expression of the proneural bHLH transcription factor NGNR-1, which is important for neuronal cell commitment (Bao et al., 2000; Ma et al., 1996). In chick and mouse, NSCL-1 can promote neuronal cell differentiation, and migration of cellular populations including GnRH-1 neurons (Kruger et al., 2004; Schmid et al., 2007; Xie et al., 2004). Interestingly, *Ebf2* knockout mice show a migration defect of GnRH-1 neurons (Corradi et al., 2003). We find that the expression of *nscl-1* is at least partially under the direct control of EBF activity, and that the expression patterns of *nscl-1* and *ebf* genes strongly overlap. This suggests that EBF activity may act through NSCL-1 to regulate neuronal cell commitment, differentiation or migration.

The proneural basic helix-loop-helix transcription factor NeuroD is also strongly expressed in the central and peripheral nervous systems throughout development (Lee et al., 1995; Lee et al., 2000; Osorio et al., 2010; Schlosser and Northcutt, 2000). This study and previous studies show that *neurod* expression is very similar to that of *ebf* genes (Dubois et al., 1998; Lee et al., 1995; Pozzoli et al., 2001). The well-known functions of NeuroD in multiple species show that it is involved primarily in differentiation, but also acts to regulate cell fate, cell migration and cell survival (Ge et al., 2006; Kim et al.,

2001; Lee et al., 1995; Liu et al., 2000; Seo et al., 2007). Previous studies showed that *neurod* is both downstream of EBF2 and upstream of *ebf2 and ebf3* in *Xenopus* embryos (Dubois et al., 1998; Logan et al., 2005; Pozzoli et al., 2001; Seo et al., 2007). Our present data suggest that *neurod* is also an indirect target of EBF3. Together, these results support and expand the concept of multiple transcriptional interactions between EBF proteins and NeuroD (Dubois et al., 1998; Logan et al., 2005; Pozzoli et al., 2001; Seo et al., 2007).

AML1 is most known for its affects on production of T lymphocytes (Ono et al., 2007; Taniuchi et al., 2002). However, it is also known to be expressed in neurons, including cortical progenitors, olfactory receptor progenitors and neurons in the dorsal root ganglia (DRG) and to be involved in differentiation and cell type specification of several types of sensory and motor neurons, including neurons in the DRG (Chen et al., 2006; Kramer et al., 2006; Theriault et al., 2005; Theriault et al., 2004; Yoshikawa et al., 2007). Interestingly, AML1 is known to cooperate with EBF proteins in B cell development (Maier et al., 2004). We find that *aml1* is partially under the direct control of EBF activity, and that the expression patterns of *aml1* and *ebf* genes overlap strongly in the nervous system. Thus AML1 and EBF proteins may also act cooperatively in promoting neuronal differentiation.

We show that the homeobox transcription factor Emx1 has a strong direct dependency on EBF activity for its expression. In multiple species, *emx1* is strongly expressed in the developing forebrain, and the EMX1 protein is present in the axons of the olfactory neurons (Briata et al., 1996; Brox et al., 2004; Pannese et al., 1998; Patarnello et al., 1997; Simeone et al., 1992). Compared to *Emx2* knockout mice, *Emx1*

knockout mice show only minor defects in brain development (Bishop et al., 2003; Mallamaci et al., 2000; Pellegrini et al., 1996; Qiu et al., 1996; Yoshida et al., 1997). However, *Emx1* and *Emx2* double mutant mice show more severe defects than *Emx2* knockout mice, including defects of neuronal differentiation and thalamocortical pathfinding (Bishop et al., 2003), similar to those found in *Ebf1* knockout mice (Garel et al., 1999). Since we find *emx1* to be a strong, direct target of EBF proteins, and *emx1* and *ebf* genes are both strongly expressed in the forebrain, EBF proteins may control cell differentiation and axon growth in part by driving expression of *emx1*.

EBF proteins drive expression of candidate targets involved in multiple aspects of neuronal differentiation

The candidate targets that are not transcription factors illuminate some of the ways that EBF activity could help regulate late steps of neuronal differentiation and neuronal function. Peripherin and NF-M are important components of neuronal intermediate filaments, which help to form the cytoskeleton in the cell body and neurites of neurons (Belecky-Adams et al., 2003; Fiumelli et al., 2008; Garcia et al., 2003; Gervasi et al., 2000; Helfand et al., 2003; Lin and Szaro, 1995; Smith et al., 2006). We find through CHX experiments that the majority of expression of *peripherin* and *nf-m* is controlled directly by EBF3, and that their expression strongly overlaps with that of *ebf* genes. These discoveries correlate with previous evidence showing thalamocortical and olfactory receptor neuron axonal pathfinding defects in *Ebf* null mice, pathfinding defects of motor neurons in *C. elegans* UNC-3 mutants, and problems with dendritic arborization in *Drosophila* Collier mutants (Crozatier and Vincent, 2008; Garel et al., 1999; Garel et al., 2002; Hattori et al., 2007; Jinushi-Nakao et al., 2007; Prasad et al., 1998; Wang et al.,

2004). Furthermore, we have identified *peripherin* as a new strong, direct target of EBF proteins with an expression pattern that strongly overlaps *ebf* expression. This finding both supports a role for EBF proteins in axon growth and provides a potential additional route for exploration of how EBF proteins can affect this important process.

PCDH8 is a transmembrane calcium-dependant adhesion molecule that is expressed in several regions of the CNS in the mouse and ferret (Etzrodt et al., 2009; Makarenkova et al., 2005). The rat homolog Arcadlin affects the number of dendritic spines in cultured hippocampal neurons (Yasuda et al., 2007) and is required for activity-induced long term potentiation (Yamagata et al., 1999). We find in our microarray screen and EBF gain of function experiments that EBF proteins positively regulate the expression of a gene that is likely the *Xenopus pcdh8* homolog (based on sequence similarity and similar range of gene expression with mouse *Pcdh8* in midbrain, hindbrain and spinal cord (Makarenkova et al., 2005)). We show that *pcdh8* is an indirect target of EBF activity, and that *pcdh8* and *ebf* expression patterns overlap in the brain and spinal cord, suggesting that EBF proteins may be involved in synaptic plasticity by controlling the expression of *pcdh8*. If true, this would be a new function for EBF proteins in the nervous system.

KCNK5 is a K+ channel that is sensitive to extracellular pH, and is expressed in rat kidney cells, where it functions to stabilize bicarbonate transport and control cell volume (Barriere et al., 2003; Reyes et al., 1998; Warth et al., 2004). It is also expressed in the mouse brainstem, where it appears to be involved in maintaining the membrane potential of chemoreceptor cells (Gestreau et al., 2010). We find that *Xenopus kcnk5* is indirectly upregulated by EBF activity. In addition, we find overlap between *ebf* and

kcnk5 gene expression in the midbrain and hindbrain at the tailbud stage. Regulation of this gene represents a previously unknown function for EBF transcriptional activity.

Activin beta B forms homodimers, or heterodimers with Activin beta A. Activins are ligands of the TGF-beta superfamily, which are involved in differentiation in tissues from many systems including the reproductive system (Itman et al., 2006; Knight and Glister, 2006; Matzuk et al., 1995a; Matzuk et al., 1995b). Activin beta B is expressed in the developing brain and retina (Belecky-Adams et al., 1999; Dohrmann et al., 1993; Roberts et al., 1996) and our data), but its function in neuronal development is not yet clear. Our study shows that the *activin beta b* gene is likely an indirect target of EBF proteins, and that its expression precedes that of *ebf* genes in midbrain, hindbrain and retina. These results suggest that EBF activity may maintain the expression of *activin beta b* instead of initiating its expression.

GREB1 is thought to be involved in the estrogen-induced growth of breast cancer cells. Its expression is strongly upregulated by estrogen treatment in estrogen receptor positive breast cancer tissue (Ghosh et al., 2000; Rae et al., 2005). In our study, we find that the *greb1* gene is expressed in several tissues, including neurons and muscle cells, during *Xenopus* development. Overlapping expression with *ebf* genes is limited to the spinal cord and a few brain regions at tailbud stages, and we find that the expression of *greb1* is controlled by EBF proteins indirectly. We do not know which neuronal processes may be governed by GREB1, but our findings demonstrate a potential relationship between these genes and a possible role for GREB1 in neuronal development.

Our study shows that more than 50% of the expression of the target genes *emx1*, *peripherin* and *nf-m* depends directly on EBF3 transcriptional activity. Consistently, their expression patterns strongly overlap with those of *ebf* genes. The expression of *nscl-1* and *aml1* depends in part directly, and in part indirectly, on EBF3 transcriptional activity, and their expression also very strongly overlaps with the expression of *ebf* genes. Genes having indirect dependency on EBF activity show less overlap in expression patterns. These results suggest that expression of the direct target genes we have described is likely to be heavily dependent on EBF activity.

EBF2 and EBF3 appear to share most targets during early *Xenopus* development

The known functions of EBF2 and EBF3 are very similar during early *Xenopus* development (Dubois et al., 1998; Pozzoli et al., 2001). They are both important for neuronal differentiation, including control of the expression of the neuronal specific markers N-tubulin, N-CAM, and NF-M. *Ebf2* knockout mice and *Ebf3* knockout mice have similar phenotypes for olfactory axon growth (Wang et al., 2004). Supporting the similarity of the roles of these two genes, we find that the ten targets of EBF3 that were upregulated by hGR-XEBF3 in *Xenopus* animal caps and *in vivo*, could also be upregulated by hGR-XEBF2 *in vivo*. Although there is interesting evidence for some differences in expression patterns and functions of EBF2 and EBF3 (Dubois et al., 1998; Pozzoli et al., 2001), our results support the idea that at the transcriptional level EBF2 and EBF3 have largely redundant functions.

Conclusion

In conclusion, we have found multiple candidate EBF targets with a systematic approach in *Xenopus* embryos. The expression patterns of direct targets of EBF3 have strong overlap with *ebf* gene expression, while targets having largely indirect dependency on EBF3 are expressed in less overlapping patterns, suggesting more complex modes of regulation. The novel candidate target genes suggest new potential routes for EBF transcription factors to carry out their previously known functions of neuronal cell commitment, differentiation, neurite formation and migration, and also suggest some new potential functions of EBF activity.

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CHAPTER 3

ZINC FINGER PROTEIN 423 (ZFP423) COORDINATES NOTCH AND BONE MORPHOGENETIC PROTEIN (BMP) SIGNALING, SELECTIVELY UPREGULATING *HAIRY-ENHANCER OF SPLIT 5* (*HES5*) GENE EXPRESSION

(Giacomo Masserdotti, Aurora Badaloni, Yangsook Song Green, Laura Croci, Valeria Barili, Giorgio Bergamini, Monica L. Vetter and G. Giacomo Consalez, Journal of Biological Chemistry, Submitted)

The work I contributed is shown in Figure 3.4, Figure 3.5 and Figure 3.8.

Abstract

Zinc finger protein 423 encodes a 30 Zn-finger transcription factor involved in cerebellar and olfactory development. ZFP423 is a known interactor of SMAD1-SMAD4 and of Collier/Olf-1/EBF proteins, and acts as a modifier of retinoic acid-induced differentiation. In the present paper, we show that ZFP423 interacts with the Notch1 intracellular domain in mammalian cell lines and in *Xenopus* neurula embryos, to activate the expression of the Notch1 target *Hes5* / *ESR1*. This effect is antagonized by EBF transcription factors, both in cultured cells and in *Xenopus* embryos, and amplified *in vitro* by BMP4, suggesting that ZFP423 acts to integrate BMP- and Notch signaling, selectively promoting their convergence onto the *Hes5* gene promoter.

Introduction

A small set of regulatory factors, mostly secreted or surface molecules, modulates neural development, from neural induction through synaptogenesis. Morphogens, acting instructively, permissively, or through inhibitory interactions, control various aspects of neurogenesis, eliciting different responses in target cells, dependent upon their evolving windows of competence. The integrated effects of various morphogens regulate a range of developmental switches, controlling – among other aspects - regional identity, fate determination and the timing of neuronal commitment and differentiation. Although the activities of extracellular factors have been intensively studied, many of their cell-intrinsic effectors have yet to be discovered and characterized.

The Notch pathway exerts regulatory activities in a diverse array of developmental contexts (reviewed in Artavanis-Tsakonas et al., 1999; Kageyama et al.,

2008). In neurogenesis, Notch signaling suppresses the neurogenic cascade, which is promoted and sustained by proneural basic helix-loop-helix (bHLH) transcription factors. When the Notch single pass receptor is bound by the Delta or Jagged/Serrate family of transmembrane ligands expressed by adjacent cells, the Notch intracellular domain (NICD) is cleaved proteolytically (Brou et al., 2000; Mumm et al., 2000) and translocates into the nucleus. There, it interacts with the DNA binding protein $CSL^{CBF1/Su(H)/LAG-1}$ (Jarriault et al., 1995), displacing a co-repressor complex (Hsieh et al., 1999) and recruiting a transcriptional activation complex (Fryer et al., 2002; Wallberg et al., 2002; Wu et al., 2000). This leads to the transcription of various immediate target genes, including *Drosophila enhancer of Split*, and its vertebrate homologs *Hairy*, *Hes1*, and *Hes5* (Jennings et al., 1994; Ohtsuka et al., 1999; Sasai et al., 1992). These genes encode transcriptional repressors of the basic Helix-Loop-Helix family which act as inhibitors of neuronal differentiation. In the developing nervous system, *Hes1*, *Hes5* double mutants feature a loss of mitotic progenitors and a massive premature differentiation, particularly in the dorsal neural tube (Hatakeyama et al., 2004).

While this pathway effectively delays neuronal commitment and differentiation, it also promotes diversity in neuronal development by actively preserving a pool of uncommitted and mitotic neural progenitors, sustaining the birth of successive waves of distinct neuronal and glial types (reviewed in Kageyama et al., 2007). To perform this broad array of modulatory effects at different developmental stages and in distinct morphogenetic domains, the Notch signaling cascade is tightly regulated, from the cell surface to the nucleus (reviewed in Bray, 2006; Kadesch, 2004).

The *Zfp423* gene is expressed alongside the dorsal midline of the embryonic mouse neural tube, at the border with the roof plate, particularly in the hindbrain and cerebellum (Cheng et al., 2007). The roof plate provides critical signals for cerebellar development (Chizhikov et al., 2006). The gene encodes a 30 Zn-finger domain nuclear protein involved in cerebellar and olfactory development. Interestingly, *Zfp423* null mice develop a profound hypoplasia of the cerebellar vermis (Alcaraz et al., 2006; Cheng and Reed, 2007; Warming et al., 2006), reminiscent of the Dandy-Walker malformation (reviewed in Millen and Gleeson, 2008), and a premature differentiation of olfactory neuron progenitors (Cheng and Reed, 2007), although the underlying molecular mechanisms remain unclarified.

ZFP423 is known to interact with the SMAD1-SMAD4 complex, which transduces Bone Morphogenetic protein (BMP2/4/7) signaling (reviewed in Liu and Niswander, 2005) into the nucleus, upregulating *Xvent2* transcription in *Xenopus laevis* gastrulae and mammalian cells (Hata et al., 2000). However, no information is available to date as to the functional significance (if any) of the interaction of ZFP423 and receptor-dependent SMADs in mammalian neural development.

ZFP423 has also been found to complex with EBF ^{COE} proteins (Tsai and Reed, 1997, 1998). EBF ^{COE} TFs are important players in the context of neuronal differentiation and migration (Corradi et al., 2003; Dubois et al., 1998; Garcia-Dominguez et al., 2003; Pozzoli et al., 2001), olfactory neurogenesis (Davis and Reed, 1996; Wang and Reed, 1993; Wang et al., 1997), cerebellar PC migration and survival (Croci et al., 2006, and Croci *et al.*, submitted) and cerebellar cortical patterning (Chung et al., 2008; Croci et al., 2006). Finally, a recent paper described the role of ZFP423 as a modifier of retinoic acid-

induced differentiation(Huang et al., 2009). Thus, ZFP423 is poised to interact with multiple signaling pathways and transcriptional effectors, likely integrating their function during development.

In the present paper, we analyze some of the functional and molecular interactions established by ZFP423 *in vitro* and *in vivo*. We demonstrate that ZFP423 interacts functionally and molecularly with the Notch intracellular domain (NICD) in mammalian cell lines and in *Xenopus* neurula embryos, to activate the expression of the Notch target *Hes5*/*ESR1*. A small proximal region of the *Hes5* promoter is sufficient to reproduce this cooperation *in vitro*. This effect is enhanced in BMP4 treated cells. By triggering *Hes5* expression and by modulating BMP signaling cell autonomously, ZFP423 may help maintain a pool of *Hes5* positive neurogenic progenitors in the developing neural tube.

Materials and methods

Animal Care

All experiments described in this paper were conducted in agreement with the stipulations of the San Raffaele Scientific Institute Animal Care and Use Committee, and the University of Utah Institutional Animal Care and Use Committee guidelines.

Tissue preparation

Pregnant mice were anesthetized with Avertin (Sigma). For in situ hybridization on sagittal sections, embryos were fixed overnight by immersion with 4% PFA, cryoprotected in 30% sucrose overnight, embedded in OCT (Bioptica), and stored at –80°C, before sectioning on a cryotome (20 μm). For whole mount in situ hydridization

and whole mount LacZ staining embryos were fixed with 4% PFA 6 hours or 10 minutes, respectively. *Zfp423* expression at embryonic day 10.5 was re-examined by LacZ staining using a transgenic line obtained from the German Genetrap Consortium (Id: W008G09, (Skarnes et al., 2004)) carrying a LacZ gene inserted by gene trapping within the *Zfp423* gene. LacZ staining was performed as described (Croci et al., 2006).

Xenopus embryo microinjection

Mouse *Zfp423* from pCDNA3-*Zfp423* was subcloned into the pCS2+ expression vector and used to make capped mRNA *in vitro* using the Message mMachine kit (Ambion). Also the following constructs were used as DNA templates to make capped mRNA : pCS2+X-Delta^{stu} (Chitnis et al., 1996), pCS2+MT-XotchΔE (Referred to here as N^{act}) (Coffman et al., 1993), pCS2+Xebf2 (Pozzoli et al., 2001), pCS2+Xebf3 (Pozzoli et al., 2001), pCS2+nβgal (Chitnis, 1999) and pCS2+GFP (Chalfie et al., 1994). The full length *Xenopus Zfp423* (XZfp423) cDNA including part of 5'UTR was acquired by 5'RACE (Roche) using the sequence of image clone 6636947, and then by RT-PCR with Superscript II Reverse Transcriptase (Invitrogen) and PfuUltraII fusion HS DNA polymerse (Stratagene) (GenBank accession No. GQ421283). The sequence of our XZfp423 morpholino (Gene Tools) is TCCACTGTACCTCAAAACTAACCCC, which is complementary to nucleotides –26 to –2. mRNA and morpholino were injected into one blastomere of 2-cell stage embryos in the following amounts: *Zfp423* (1ng for single injection and co-injection with *Delta^{stu}*, 600pg for co-injection with *XotchΔE*), *Delta^{stu}* (400pg), *XotchΔE* (100pg), *Xebf2* (100pg), *Xebf3* (100pg), *nβgal* (50pg) and *XZfp423* morpholino (30ng). mRNA was injected into one dorsal blastomere of 16-cell stage

embryos in the following amounts: *Zfp423* (300pg) and *Gfp* (200pg). mRNA for *nβgal* or *Gfp* was co-injected into all embryos as a tracer. Embryos were grown until neural plate stages (Nieuwkoop and Faber, 1994) and fixed in MEMFA for 30 minutes (Harland, 1991). X-gal staining was performed on the embryos injected with nβgal as described (Turner and Weintraub, 1994). GFP expressing embryos were sorted under fluorescence based on which side was the injected side, and fixed for 30 more minutes.

In situ hybridization

For whole mount *in situ* hybridizations of *Xenopus* embryos, the following constructs were used to generate antisense RNA probes: pCMV-sport6-XZfp423 (Image clone 6636947, ATCC), pBS-ESR1 (Wettstein et al., 1997), pBS-Hairy1 (Dawson et al., 1995), and pBS-Nrarp (Lamar et al., 2001). For mouse experiments, digoxygenin-labeled riboprobes were transcribed from plasmids containing *Hes1*, *Hes5* and *Zfp423* cDNAs. Antisense RNA probes were generated *in vitro* using T7 or T3 RNA polymerase (Roche) and labeled with digoxigeninin-11-UTP (Roche). In situ hybridizations of whole-mount mouse embryos and embryonic sections were performed as described by Pringle and Richardson (www.ucl.ac.uk/~ucbzwdr/double in situ protocol.htm).

Cell Culture and DNA Transfection

The P19 cell line was maintained in MEM-alpha (Invitrogen) supplemented with 10% FBS (Invitrogen). The C2C12 myoblastic cells (American Type Culture Collection), COS7 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, EuroClone). P19, HEK293

and COS7 cells were transfected with Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). P19 cells were grown in MEM ALPHA medium (Invitrogen), 5% FBS and neuralized with 10^{-6} M retinoic acid (Sigma) treatment for 24 hours. C2C12 cells were transfected using Lipofectamine2000 and Plus reagent, according to the manufacturer's instructions (Invitrogen), and treated with 100 ng/ml BMP4 (R&D Systems) when specified.

Plasmids and constructs

To generate pCDNA3-6Myc-ZFP423, we subcloned ZFP423 from pXY-ZFP423 (RZPD, IRAK MGC full length cDNA, clone 961, Berlin, Germany), in pC2+6Myc. 6Myc-ZFP423 was excised and cloned into pCDNA3.1 vector (Clontech). pCDNA3-Flag-Notch-Intracellular-Domain (Flag-NICD) was a kind gift of Georg Feger (Serono). 1Kb and smaller fragment of the *Hes5* promoter were amplified from wild type mouse genome, cloned into pBluescript SK and sequenced. The fragments were subcloned into the pGL3 vector (Promega). To generate pCDNA3-6myc-ZFP423Δ9-20, pCDNA3-6myc-ZFP423 was digested with the enzymes SacI and PvuII and the fragment – 1381 bps – was cloned into pBluescript SK, previously opened with PvuII. A second fragment – 2120 bps – obtained from pCDNA3-6myc-ZFP423 by SacI-SacII digestion and blunt, was cloned downstream of the first fragment in pBKS. 6myc-ZFP423Δ9-20 was excised with the enzymes HindIII-NotI and subcloned into pCDNA3.1 (Invitrogen).

RT-PCR

Total RNA was extracted with RNeasy MicroKit (Qiagen), according to the manufacturer's instructions. Total RNA (1-1,5 μg) was retro-transcribed using first strand cDNA MMLV-Retrotranscriptase (Invitrogen) and random primers. Each cDNA was diluted one to 10, and 3μl were used for each real-time reaction. mRNA quantitation was performed with LightCycler480 SYBR Green I Master Mix (Roche) on a LightCycler480 instrument (Roche) following the manufacturer's protocol. The following primers were used: *Gapdh* (Vincent et al., 2002); *Hes5*, *Hes1* (Jensen et al., 2000); *Blbp* (Lowell et al., 2006); *Zfp423* (Hata et al., 2000); flagNICD F: 5'-ATGGACTACAAAGACGATGAC, flagNICD R: 5'-CAAACCGGAACTTCTTGGTC

RNA Interference

Single-stranded DNA oligos encoding the pre-miRNAs were annealed according to the manufacturer's instructions (Invitrogen). Pre-miRNA double-stranded oligos were cloned in pCDNA^{TM}6.2-GW/-EmGFP-miR vector (Invitrogen). A Pre-miRNA containing a non-targeting sequence (Invitrogen) was used as a negative control. The pre-miRNAs were transfected via Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. To test the efficacy of the pre-miRNA, HEK293 cells were transfected with pcDNA3-6Myc-ZFP423 and either a nontargeting pre-miRNA or ZFP423-specific pre-miRNA vector, and cell lysates were analyzed via Western blot. Among the specific pre-miRNA tested, we selected the most effective one in abolishing protein expression. Transfected P19 cells were sorted for GFP expression and lysed for RNA extraction with RNeasy MicroKit (Qiagen), according to the manufacturer's instructions.

Coating assay

To generate a soluble form of Notch1 ligand, 293T cells grown in a 10-cm Petri dish were transfected using Fugene HD (Roche), according to the manufacturer's instructions, with either 5 μg Fc-TRAIL-Receptor 4 (Fc-control) or 5 μg Fc-Jagged1 expression plasmids (Courtesy of Tom Kadesch, (Buas et al., 2009)). After 48 hours, the growth medium was collected and filtered through a 0.45-μm syringe filter. Fc-TRAIL-R4 or Fc-Delta4 fusion protein was immobilized by incubating polystirene plates for 2 hours at room temperature with 10 μg/mL rabbit anti–human IgG Fc antibody (Jackson ImmunoResearch Laboratories), and then incubated for 2 hours with respective filtered supernatant. The pre-miRNAs were transfected in P19 cells via Lipofectamine 2000. After 48 hours, the cells were split and grown for 24 hours on plates coated with Fc-TRAIL-R4 (control) or Fc-Jagged1. Transfected P19 cells were sorted for GFP expression and RNA was extracted with RNeasy MiniKit (Qiagen), according to the manufacturer's instructions.

Promoter-reporter assays

The day before transfection, C2C12 cells were plated in 12-well plates and grown in DMEM supplemented with 10% FBS. Luciferase assays were carried out 24 hours after transfection using Dual Luciferase Assay kit according to the manufacturer's instructions (Promega). Each result is the average of three independent measurements, and each experiment was repeated at least three times.

Chromatin immunoprecipitation (ChIP)

P19 cells ($2X10^6$) were treated with 1% paraformaldehyde in 1X PBS by rotation for 10 minutes at RT. Fixation was stopped by addition of glycine to a final concentration of 125 mM. Cells were washed two times in 1X PBS and centrifuged at 2,000 rpm for 2 minutes. The pellet was resuspended in lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40, and protease inhibitors cocktail) and incubated on ice for 10 minutes. After centrifugation at 4,000 rpm for 5 minutes at 4°C, the pellet was resuspended in sonication buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 0.1% SDS, and protease inhibitors cocktail). Sonication was performed five times with 20 second pulses using a microprobe at 40% output. Equal amounts of chromatin, precleared with blocked protein A-Sepharose (GE Healthcare), were incubated by overnight rotation with rabbit anti-OAZ (ZFP423) antibody (5 µg, H-105, Santa Cruz Biotechnology). Protein A-Sepharose was added to each sample and incubated at 4°C with rotation for 3 hours. Beads were spun at 14,000 rpm for 5 minutes, washed six to eight times with wash buffer (10mM Tris-HCl pH 8, 0.5 mM EGTA, 1mM EDTA 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), and eluted with 1% SDS in 50 mM $NaHCO_3$. Bound fractions were de-cross-linked by adding 200 mM NaCl and by incubation at 65°C for 6–8 hours. De-cross-linked samples were treated with RNase (0.03 mg/ml) and Proteinase K (0.3 mg/ml) at 55°C for 2 hours. DNA was precipitated with 2.5 volumes of absolute ethanol and purified using Qiagen PCR Purification kit (Qiagen). Cross-link-reversed chromatin was used as a PCR control. For qPCR, each primer pair was assessed for amplification efficiency on serial dilutions of genomic DNA. PCR Primer sequences: *Hes5* promoter (b), F: 5'-TTCCCACAGCCCGGACATT; R: 5'- GCGCACGCTAAATTGCCTGTGAA

T; *Hes5* sequence a, F: 5'- TCAACTACTGTCCCTTCGCCCAGA; R: 5'- GGATTGGA GTCCTCTAGTTTGCCT; *Hes 5* sequence c: F: 5'-CTTGGTCATGTGGGAGAACA; R: 5'- GGCTGCTAAGGACAGACGAG; *Hes5* sequence d: F: 5'- TAGCTTACCACAGGA GCAGCAGAA; R: 5'-ACCCAGCAACTTCAGTCCCTGTAA; *Mrps15* (mitochondrial ribosomal protein S15), F: 5'-CTGGGACATAGTGGGTGCTT; R: 5'-GAGCCTAGAG ATGGGCTGTG.

Immunoprecipitation and Western blots

All biochemical procedures were conducted as described (Ausubel et al., 1995). In particular, COS7 and HEK293 cells were harvested 24 hours after transfection and centrifuged; pellets were frozen at -80°C. For co-immunoprecipitation experiments, cell pellets were thawed at room temperature and lysed in 5 volumes of extraction buffer (10 mM Hepes pH 7.9; 400 mM NaCl; 5% glycerol, PMSF 1mM, Leupeptin 0,5mM, NaF 50mM, Pepstatin 1mM). Samples were centrifuged at 34,000 rpm for 30 minutes at 4°C, and supernatants were collected. Protein concentration was determined by the BCA assay (Pierce). Part of the lysate (20%) was kept as a positive input control. Lysates were incubated overnight with 10 µg of the indicated antibodies; 30 µl of protein G-Sepharose (GE Healthcare) were added for 4 hours at 4°C. The resin was washed five times with extraction buffer. Protein complexes were eluted by addition of sample buffer (Tris-Cl 125 mM pH 6.8; 0.1 M 2-mercaptoethanol; 2% SDS; 20% glycerol; 25 mg/ml Bromphenol Blue), boiled for 15 minutes and separated on an 8% SDS-polyacrylamide gel. Proteins were transferred on a PVDF membrane (Millipore). Western Blots were performed with the following antibodies: mouse anti-myc (9E10, Santa Cruz

Biotechnology), rabbit anti-Notch (C-20, Santa Cruz Biotechnology), rabbit anti-OAZ (ZFP423) (H-105 Santa Cruz Biotechnology), mouse anti-βactin (Sigma). As secondary antibodies, a goat anti-rabbit HRP-conjugated antibody (Bio-Rad), and a sheep anti-mouse HRP-conjugated antibody (Amersham) were used. Blots were developed with the LiteAblot substrate (EuroClone).

Statistical Analysis

Statistical significance was determined by the Student *t* test with a threshold for significance set to $p = 0.05$. All results are plotted as the mean + standard deviation.

Results

Zfp423 expression in the cerebellar primordium

Since *Zfp423* mutants feature a severe cerebellar midline deletion (Alcaraz et al., 2006; Cheng et al., 2007; Warming et al., 2006), we re-examined the expression of *Zfp423* at the onset of cerebellar neurogenesis. To this end, we used a transgenic line (Id: W008G09, (Skarnes et al., 2004)) carrying a *LacZ* gene inserted by gene trapping within the *Zfp423* gene. *Zfp423* expression in the neural plate is detectable by whole mount *in situ* hybridization as early as E7.5 (not shown). *Zfp423* strongly labels the rhombencephalon and mesencephalon at E8.5 and E9.5 (not shown, (Cheng et al., 2007)), and the hindbrain and cerebellum thereafter (Figure 3.1A). *In situ* hybridization of embryonic tissue sections (Figure 3.1B-E) revealed that the gene is expressed in both germinative epithelia of the cerebellar primordium, i.e. the rhombic lip (RL, arrow), that will give rise to all glutamatergic progenitors, and the cerebellar ventricular zone (VZ,

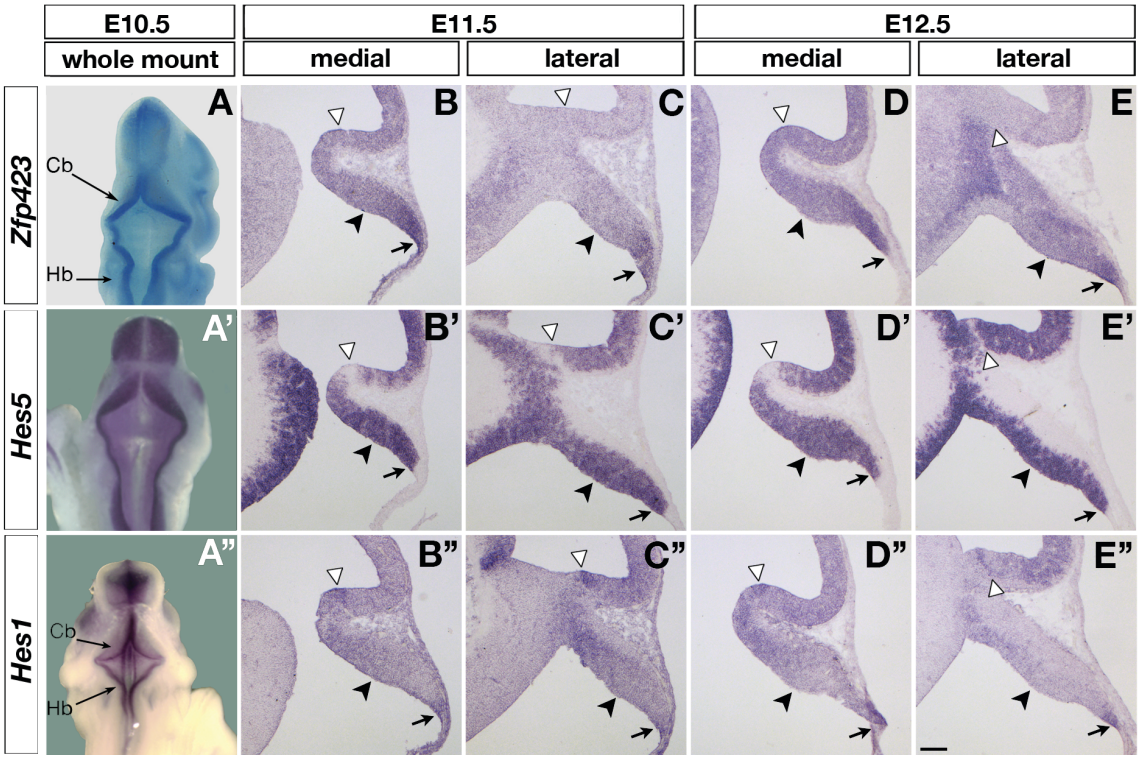


Figure 3.1: Colocalization of *Zfp423* and *Hes5* in the medial cerebellar primordium. A) Whole mount LacZ staining of an E10.5 embryo carrying a gene trap insertion in the *Zfp423* locus. A', A") Whole mount E10.5 embryos hybridized with Hes5 and Hes1, respectively. Cb, cerebellar primordium; Hb, hindbrain. Note *Zfp423* expression at the border with the roof plate. B-E) E11.5 and E12.5 sagittal sections from medial and lateral territories of the cerebellar primordium, hybridized with *Zfp423*. Solid arrowhead: ventricular zone (VZ); arrow: rhombic lip (RL). B'-E' and B"-E") as above, hybridized with *Hes5* and *Hes1*, respectively. Empty arrowhead in B" indicates the isthmic organizer (IO). Notably, *Hes5* is expressed in VZ and RL and sharply silenced in the IO, whereas Hes1 is transcribed in the RL and IO, and silenced in most of the VZ.

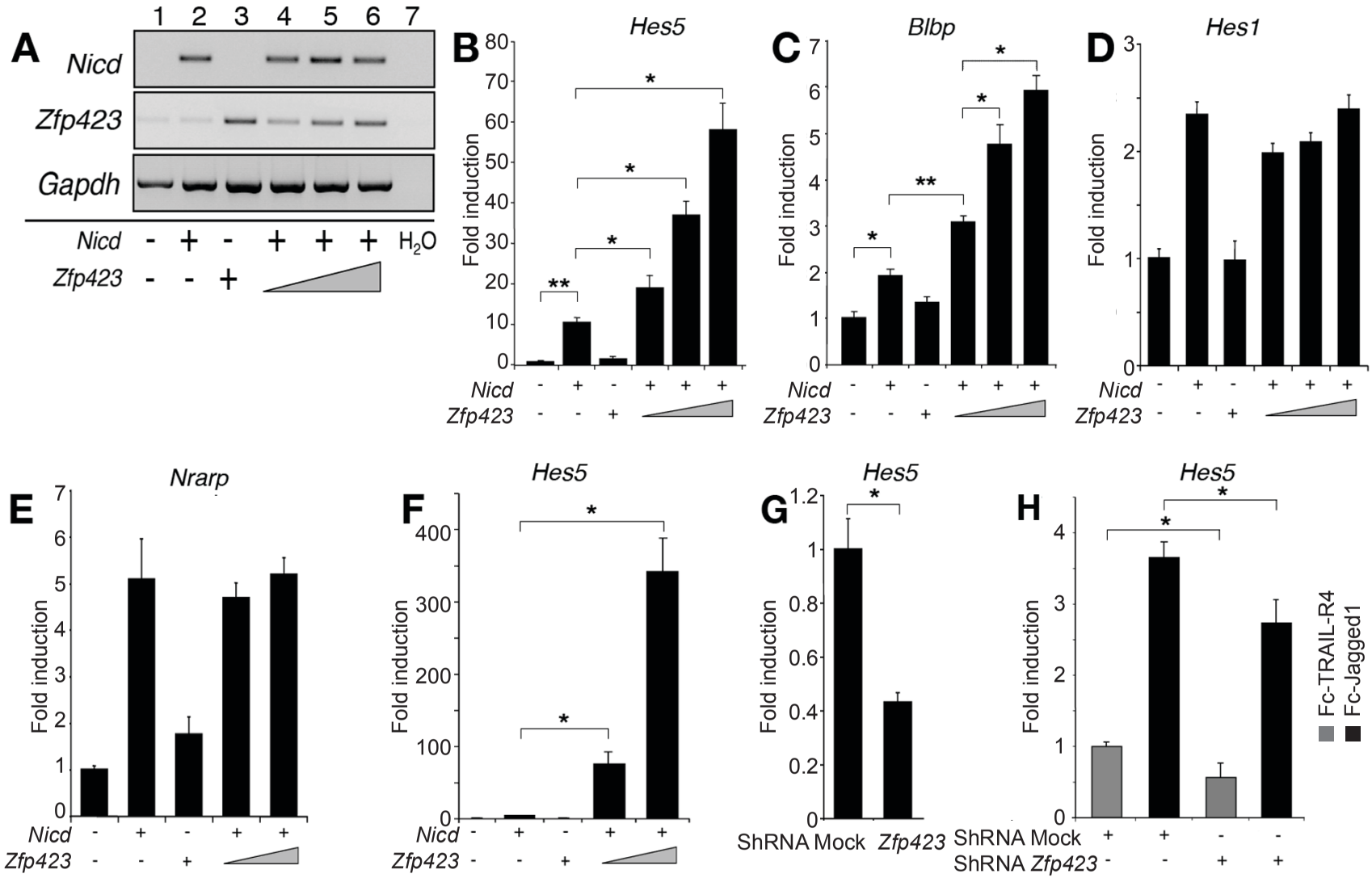
solid arrowhead), that harbors all GABAergic progenitors. Since *Zfp423* mutants feature a severe cerebellar midline deletion (Alcaraz et al., 2006; Cheng et al., 2007; Warming et al., 2006), we asked if the gene is expressed at this site (Figure 3.1B,D). Interestingly, in the E12.5 cerebellar primordium, *Zfp423* is expressed at high levels flanking the midline, where it colocalizes with *Hes5* (Figure 3.1B'-D'), an immediate transcriptional target of the Notch1 signaling pathway expressed in the VZ and RL. In contrast, *Hes1* is more restricted at this stage, labeling the isthmic organizer (empty arrowhead) and rhombic lip (arrow), adjacent to the cerebellar roof plate, while it is considerably downregulated in most of the VZ (Figure 3.1A"-E").

Functional and molecular interactions between ZFP423 and Notch signaling

Based on the above observations, we sought to determine whether ZFP423 acts in the context of the Notch signaling pathway. To this end, we overexpressed the corresponding gene in neuralized P19 cells (see Experimental Procedures) together with a cDNA encoding NICD (Schroeter et al., 1998). Shown in Figure 3.2A is a sample semi-quantitative RT-PCR analysis of P19 cells transfected with a fixed amount of *Nicd* and increasing concentrations of *Zfp423*, illustrating the conditions achieved in subsequent experiments.

We measured the levels of four known direct targets of Notch signaling: *Hes5* and *Hes1* (Ohtsuka et al., 1999), *Nrarp* (Krebs et al., 2001), and *Blbp* (Anthony et al., 2005). As regards *Hes5* (Figure 3.2B), while overexpression of *Nicd* alone produces a significant increase in *Hes5* gene expression over mock transfected cells, the overexpression of *Zfp423* alone has no effect. However, combined overexpression of *Nicd* and *Zfp423*

Figure 3.2: Cooperative activation of *Hes5* and *Blbp* gene expression by NICD and ZFP423 in cell lines. A) Semiquantitative RT-PCR analysis of exogenous *Nicd* and *Zfp423* levels in transfected P19 cells. Note increasing *Zfp423* levels in lanes 4-6. This is representative of the amounts of *Nicd* and *Zfp423* cDNAs used in B-F. **B-G)** Quantitative RT-PCR analysis of P19 (B-E, G) and C2C12 (F) cells treated as indicated below. **B,C)** *Nicd* and *Zfp423* coexpression in neuralized P19 cells upregulates endogenous *Hes5* and *Blbp* expression, respectively, to a level greater than cells transfected with *Nicd* alone. Induction of *Hes5* and *Blbp* transcription is dependent on *Zfp423* dosage. **D,E)** Cotransfection of P19 cells with *Nicd* and *Zfp423* fails to activate *Hes1* and *Nrarp* gene expression to a level greater than *Nicd* alone. **F)** Transfection of C2C12 cells with *Nicd* and *Zfp423* induces *Hes5* gene expression in a *Zfp423* dose-dependent fashion. In this line, *Hes5* expression is strictly dependent on the addition of exogenous *Zfp423*. **G)** *Zfp423* RNA interference in P19 cells reduces endogenous *Hes5* expression. **H)** *Zfp423* RNA interference reduces *Hes5* expression in P19 cells grown on Fc-Jagged1-coated plates compared to cells plated onto Fc-TRAIL-R4-coated plates. *, $p \leq 0.05$; **, $p < 0.01$.


A
1 2 3 4 5 6 7
Nicd
Zfp423
Gapdh
Nicd - + - + + + H2O
Zfp423 - - +
B
Hes5
Fold induction
Nicd - + - + + +
Zfp423 - - +
C
Blbp
Fold induction
Nicd - + - + + +
Zfp423 - - +
D
Hes1
Fold induction
Nicd - + - + + +
Zfp423 - - +
E
Nrarp
Fold induction
Nicd - + - + +
Zfp423 - - +
F
Hes5
Fold induction
Nicd - + - + +
Zfp423 - - +
G
Hes5
Fold induction
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H
Hes5
Fold induction
ShRNA Mock + + - -
ShRNA Zfp423 - - + +
Fc-TRAIL-R4
Fc-Jagged1

elicits a strong cooperative interaction, leading to a significant increase in *Hes5* transcript levels that is dependent on *Zfp423* DNA dosage. Likewise, *Blbp* gene expression (Figure 3.2C) is cooperatively upregulated by NICD and ZFP423, whereas *Hes1* and *Nrarp* transcript levels (Figure 3.2D and 3.2E, respectively) are unaffected by *Zfp423* overexpression in addition of *Nicd*. Because neuralized P19 cells express endogenous *Hes5* and *Zfp423* (Figure 3.1A, and Hata et al., 2000), we moved to a system in which neither gene is expressed: the C2C12 myoblastoid cell line (Hata et al., 2000). In this system, overexpression of *Nicd* upregulates *Hes1* and *Hey* (Dahlqvist et al., 2003), but activates *Hes5* transcription very weakly (our observation). Our results indicate that in C2C12 cells *Hes5* expression is strictly dependent upon co-expression of *Nicd* and *Zfp423* and that the levels of *Hes5* expression are again *Zfp423*-dose dependent (Figure 3.2F). Next, we asked whether ZFP423 contributes to *Hes5* gene regulation even at physiological levels of expression. To address this point, we used RNA interference (RNAi). The efficiency of *Zfp423* knockdown was tested and validated at the protein level (Figure 3.3). RNAs extracted from P19 cell lysates were analyzed by RT-qPCR for *Hes5* gene expression. In unstimulated P19 cells, transfected with a Zfp423 shRNA, *Hes5* transcription was clearly downregulated (Figure 3.2G). Next, we activated endogenous Notch signaling by growing P19 cells, transfected with the *Zfp423* shRNA, onto plates coated with a secreted form of the Notch ligand Jagged (Fc-Jagged) (Buas et al., 2009) or with an inactive control (Fc-TRAIL-R4). Our results indicated that in Jagged-activated P19 cells, *Hes5* is upregulated (Figure 3.2H), and that *Zfp423* RNAi causes a significant downregulation of the same gene.

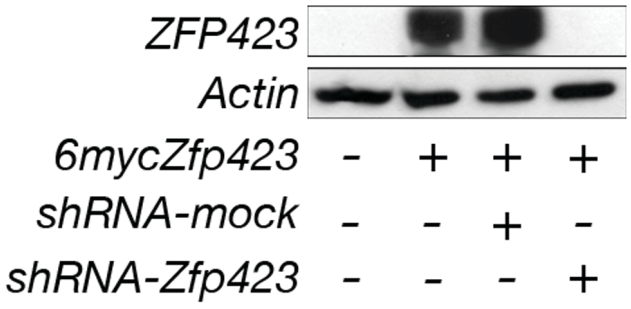
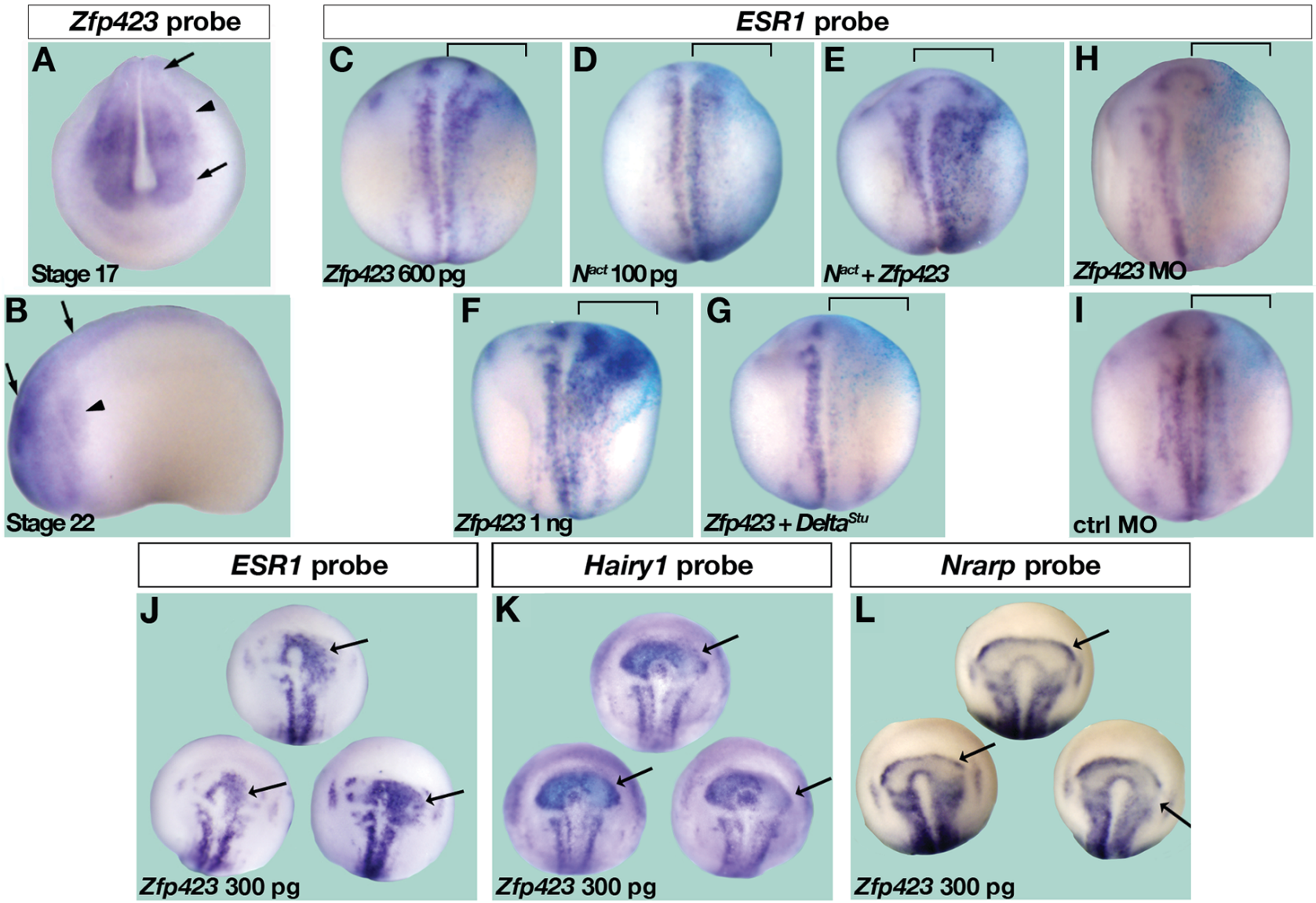


Figure 3.3: A specific ShRNA effectively abolishes ZFP423 protein expression. HEK293 cells were transfected with avector encoding 6mycZFP423 and a vector either expressing unrelated shRNA or a ZFP423-specific shRNA. 48 hours after the transfection, cells were collected and lysed in RIPA buffer, and lysates loaded on an 8% acrylamide gel. The corresponding western blot was immunostained first with an anti-myc Ab to reveal ZFP423, then with a beta-actin antibody for normalization.

Next, we asked if the functional cooperation found to occur between ZFP423 and NICD also takes place *in vivo*. To address this question, we used *Xenopus laevis* neurula embryos. We identified a *Xenopus* expressed sequence tag clone (Image clone 6636947, GenBank accession: BU911031) very similar (83% identity at the nucleotide level) to *Gallus gallus ZFP423* (see Experimental Procedures). This clone was used as an *in situ* probe to analyze *Zfp423* distribution in neurula and tailbud stage embryos. The gene is expressed in the head and spinal cord (Figure 3.4A,B; see also stages 28 and 34 in Figure 3.5). Subsequently, two-cell *Xenopus* embryos were injected unilaterally with mouse *Zfp423* and/or mRNA for *Xenopus Notch* Δ*E* (N^{act}); *beta galactosidase* mRNA was included to mark the injected side. N^{act} encodes an N-terminally deleted, constitutively active *Xenopus* NOTCH protein (Coffman et al., 1993). Both N^{act} and *Zfp423* mRNA concentrations were titrated so that either construct would produce moderate changes in target gene expression when overexpressed alone. As a first target, we analyzed the *Hes5* ortholog *ESR1* (Davis and Turner, 2001; Lamar and Kintner, 2005). Our results (Figure 3.4C-E) indicate that embryos injected with *Zfp423* (600 pg) alone show a moderate activation of *ESR1* expression (Figure 3.4C); likewise, injection of low amounts of N^{act} alone (100 pg) promoted a low-level expansion of the *ESR1* domain (Figure 3.4D); however, the simultaneous overexpression of N^{act} (100 pg) and *Zfp423* (600 pg) sharply increased *ESR1* expression on the injected side (Figure 3.4E). Furthermore, we asked whether the effect on *ESR1* expression produced by the injection of *Zfp423* alone (600 pg, Figure 3.4C; 1 ng, Figure 3.4F) requires endogenous Notch pathway activation. To address this question, *Zfp423* (1 ng) was coinjected with a dominant negative Delta ligand (DeltaStu, Chitnis et al., 1995). $Delta^{Stu}$ coinjection

Figure 3.4 - Selective, cooperative activation of *ESR1* gene expression by N^{act} and ZFP423 in *Xenopus* embryos. A,B) Whole mount *in situ* hybridization analysis of *Xenopus Zfp423* gene expression in stage 17 (frontal view) and stage 22 (lateral view, head to the left) embryos, respectively: the gene is widely expressed starting in the neural plate/tube first (arrow), and then moving to the cranial neural crest (arrowhead). **C-E)** Whole mount *in situ* hybridization analysis of *ESR1* gene expression in embryos injected unilaterally (bracket) with *Zfp423* (600 pg) and/or N^{act} (100 pg), as indicated. *LacZ* (blue stain) serves as an indicator of the injected side. Note strong *ESR1* expression after coinjection of N^{act} and *Zfp423*. **F)** Embryo injected unilaterally (bracket) with 1 ng of *Zfp423*. **G)** Embryo injected unilaterally (bracket) with 1 ng *Zfp423* and 400 pg $Delta^{Stu}$, encoding a dominant negative Notch ligand. Note disappearance of *ESR1* signal on the co-injected side. **H,I)** Unilateral injection (bracket) of a *Zfp423* morpholino oligonucleotide (H) and of a control morpholino (I) (see Experimental Procedures). Note disappearance of *ESR1* signal on the injected side in embryos injected with *Zfp423*-specific morpholino unlike those injected with the control morpholino. **J-L)** *Zfp423* overexpression selectively upregulates *ESR1* but not *Hairy1* or *Nrarp* in *Xenopus* embryos. Whole mount *in situ* hybridization analysis of *ESR1, Hairy1, and Nrarp* gene expression in embryos injected unilaterally with *Zfp423* (300pg, arrow) in a dorsal blastomere. Note expanded *ESR1* expression domain (**J**), but reduced/unaffected expression of *Hairy1* (**K**) and *Nrarp* (**L**) on the injected side. *Gfp* was coinjected with *Zfp423* as a lineage tracer (not shown).


Zfp423 probe
ESR1 probe
A
Stage 17
B
Stage 22
C
Zfp423 600 pg
D
Nact 100 pg
E
Nact + Zfp423
H
Zfp423 MO
F
Zfp423 1 ng
G
Zfp423 + DeltaStu
I
ctrl MO
ESR1 probe
Hairy1 probe
Nrarp probe
J
Zfp423 300 pg
K
Zfp423 300 pg
L
Zfp423 300 pg

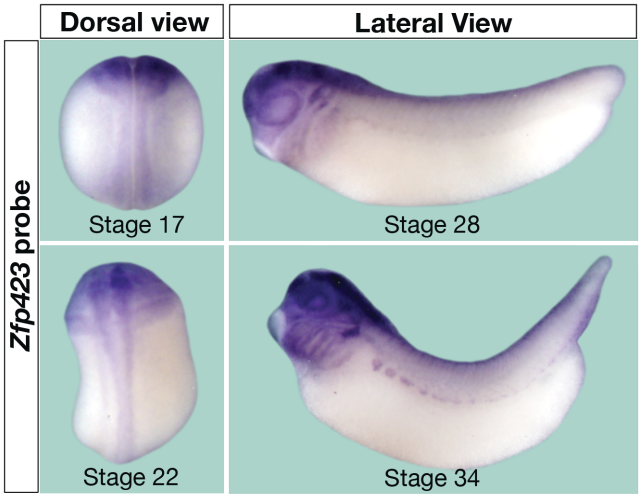


Figure 3.5: *Zfp423* expression during *Xenopus laevis* development. Whole mount in situ hybridization analysis of *Zfp423* gene expression in embryos at different stages of development (stage 17 and 22, dorsal view; stage 28 and 34, lateral view).

ablated the expansion of *ESR1* consequent to *Zfp423* overexpression (Figure 3.4G), indicating that this response is strictly dependent upon endogenous Notch signaling activation. Finally, we asked whether the endogenous expression of *ESR1* depends upon the presence of endogenous ZFP423. We injected 2-cell embryos with a ZFP423-specific morpholino antisense oligonucleotide and found a significant downregulation of *ESR1* on the injected side (Figure 3.4H) while the control morpholino had no effects on *ESR1* expression (Figure 3.4I).

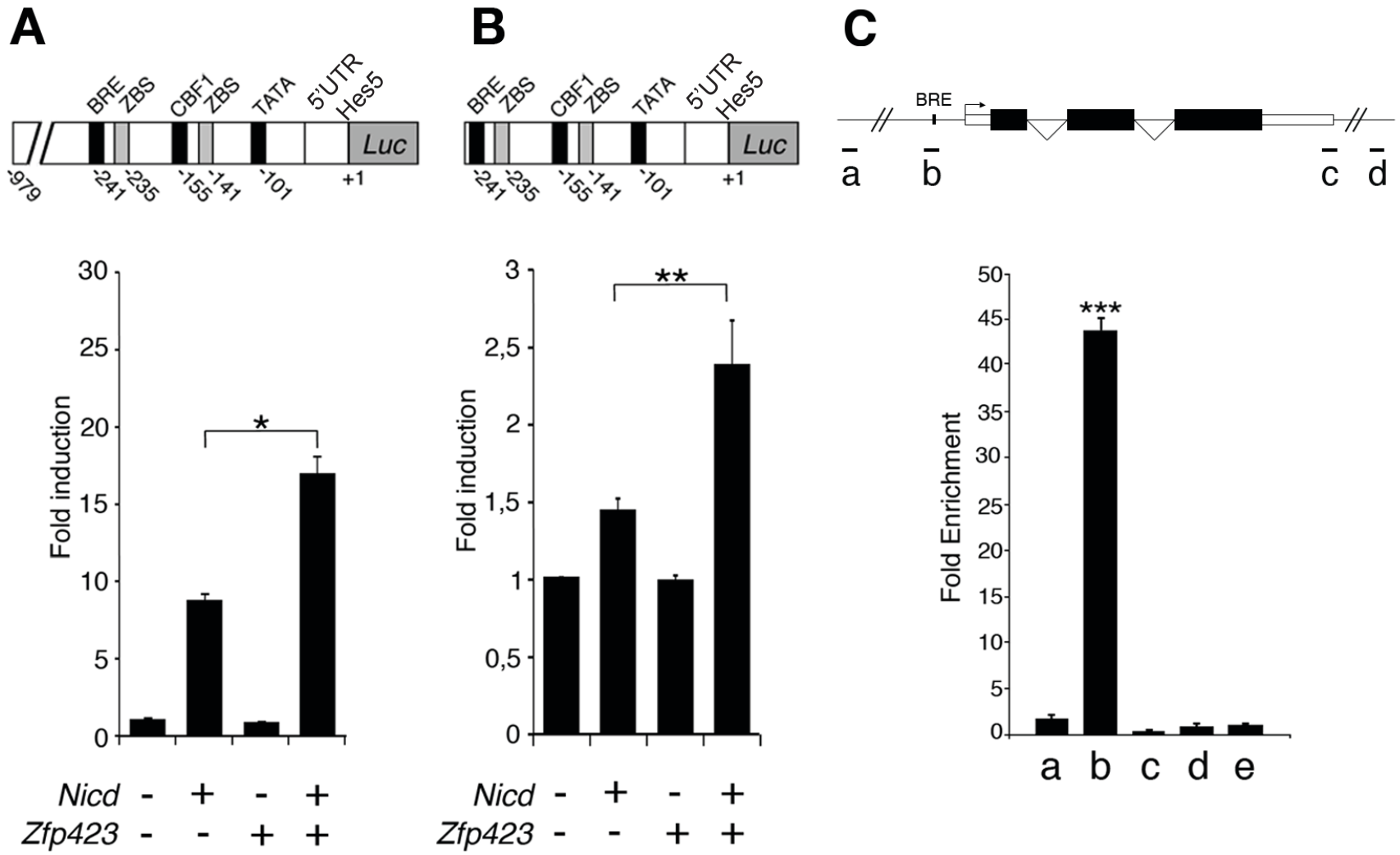
Because of the previously reported role of ZFP423 as a SMAD cofactor in the context of mesodermal patterning (Hata et al., 2000), the overexpression experiments were repeated by targeting unilaterally a dorsal blastomere at the 16-cell stage to prevent a possible interaction of exogenous ZFP423 with the BMP effector complex p-SMAD1-SMAD4 during gastrulation. The results of this experiment further corroborated the notion that *Zfp423* overexpression promotes *ESR1/Hes5* upregulation on the injected side (Figure 3.4I). In the same experiment, the *Hes1* ortholog *Hairy1* (Dawson et al., 1995; Jennings et al., 1994) was either unchanged or slightly downregulated on the injected side (Figure 3.4J) and *Nrarp* (Krebs et al., 2001; Lamar et al., 2001) transcript levels were also either left unchanged or slightly downregulated (Figure 3.4K) in response to *Zfp423* overexpression, indicating that *Zfp423* promotes a dissociation in the response to NICD *in vivo*, favoring the expression of *Hes5* over *Hes1* or other targets. Taken together, our *in vivo* results are consistent with those previously obtained in cell lines and point to a role for *Zfp423* as a cell-autonomous modifier of Notch signal transduction.

Hes5 promoter analysis

To identify and map *Hes5* promoter sequences mediating the transcriptional response of *Hes5* to ZFP423 and NICD, we performed luciferase assays in C2C12 cells, using different extents of the *Hes5* 5' flanking region, as described (Ohtsuka et al., 2006; Takizawa et al., 2003). NICD binds to the *Hes5* promoter by forming a complex with CBF1/RBPjκ/CSL on a CBF1 recognition site(Jarriault et al., 1995), located 153 bp upstream of the murine *Hes5* ATG. At first, we used a luciferase (luc) reporter containing 1051 bp of the *Hes5* 5' flanking sequence + 5' UTR (Sketched in Figure 3.6A). Our results show that coexpression of *Nicd* and *Zfp423* leads to a significant (twofold) increase in luc expression compared to *Nicd* alone (Figure 3.6A). Similar results were obtained using a reporter construct containing a shorter stretch of the *Hes5* promoter / 5' UTR (267 bp) (Sketched in Figure 3.6B), although the absolute activity levels were lower (Figure 3.6B). These results indicate that while distal promoter regions contain elements that are important for *Hes5* gene activation, those regions are however not essential for the cooperation between NICD and ZFP423 to occur.

Next, we set out to establish whether ZFP423 interacts *in vivo* with the *Hes5* promoter. To this end, we performed a chromatin immunoprecipitation (ChIP) experiment using neuralized P19 cells (Figure 3.6C) (see Materials and methods). P19 cell chromatin was immunoprecipitated using a ZFP423 Ab. For PCR amplification, we developed *Hes5* promoter-specific primers amplifying a 94 bp product spanning the BMP-responsive element (BRE) located within the *Hes5* promoter region depicted in Figure 3.6B, an extremely GC-rich, PCR unfriendly sequence. We performed a qPCR using the *Hes5* primer pair and different control primers corresponding to an upstream 5'

Figure 3.6 – *ZFP423 binds to and activates the most proximal 267bp of the Hes5 promoter.* A,B) Promoter-reporter assays performed in C2C12 cells using a long and a short version of the *Hes5* gene promoter fused to luciferase. The two variants of the *Hes5* promoter are sketched above each histogram. **A)** A 1 kb wt 5' sequence responds to cotransfection with *Zfp423* by upregulating luciferase compared to levels reached with *Nicd* alone. **B)** Likewise, a 267 bp proximal element is cooperatively activated by *Nicd* and *Zfp423*, albeit to a lower level with respect to the experiment in **A**.**p*<0.05; ***p*<0.01. **C)** A chromatin immunoprecipitation was conducted on neuralized P19 cells. Sheared chromatin immunoprecipitated with anti-ZFP423 was purified and amplified by quantitative PCR. a-d, primer pairs spanning the *Hes5* gene (b is the primer pair spanning the BRE, see text). e, primer pair amplifying the syntenic gene *Mrps15*. Data are plotted as fold enrichment relative to the abundance of the *Mrps15* qPCR product (e). ****p*<0.0004.


A
BRE
ZBS
CBF1
ZBS
TATA
5'UTR
Hes5
Luc
-979
-241
-235
-155
-141
-101
+1
B
BRE
ZBS
CBF1
ZBS
TATA
5'UTR
Hes5
Luc
-241
-235
-155
-141
-101
+1
C
BRE
a
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Nicd - + - +
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Fold induction
3
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Nicd - + - +
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a b c d e

flanking sequence (-2500 bp) and to two downstream sequences (+1600 bp and + 2500 bp). In addition, we analyzed a syntenic gene (*Mrps15*) located megabases away from *Hes5* (Henke et al., 2009). The histogram in Figure 3.6C shows a massive fold-enrichment for the *Hes5*-promoter-specific product with respect to flanking *Hes5* sequences and to the syntenic *Mrps15* gene.

Molecular interactions between ZFP423 and NICD

Since our results indicated that *Zfp423* and *Nicd* cooperate functionally *in vitro* and *in vivo*, we investigated whether the corresponding proteins interact at the molecular level. First, COS7 cells were cotransfected with constructs encoding 6xmycZFP423 and flagNICD. Lysates were immunoprecipitated with an irrelevant anti-GFP, or with an anti-myc monoclonal antibody. Immunoprecipitates were analyzed by WB using Abs for ZFP423 and for NICD, revealing an NICD-specific band only in the myc-immunoprecipitated lane (Figure 3.7A). To exclude the possibility that NICD might bind aspecifically to the beads or the myc antibody, the experiment was repeated and lysates from single-transfected cells were immunoprecipitated as negative controls (Figure 3.7C). NICD coimmunoprecipitated only in the cell lysates containing both factors.

EBF TF overexpression abolishes the cooperation of ZFP423 with NICD

In previously published work (Pozzoli et al., 2001), we showed that *Nicd* overexpression was capable of reducing or abolishing the ability of *XEbf2* to induce *Nfm* gene expression in *Xenopus* neurulas. This result suggested that Notch and EBF2 might act antagonistically in neuronal differentiation, through a mechanism independent of *Ebf2*

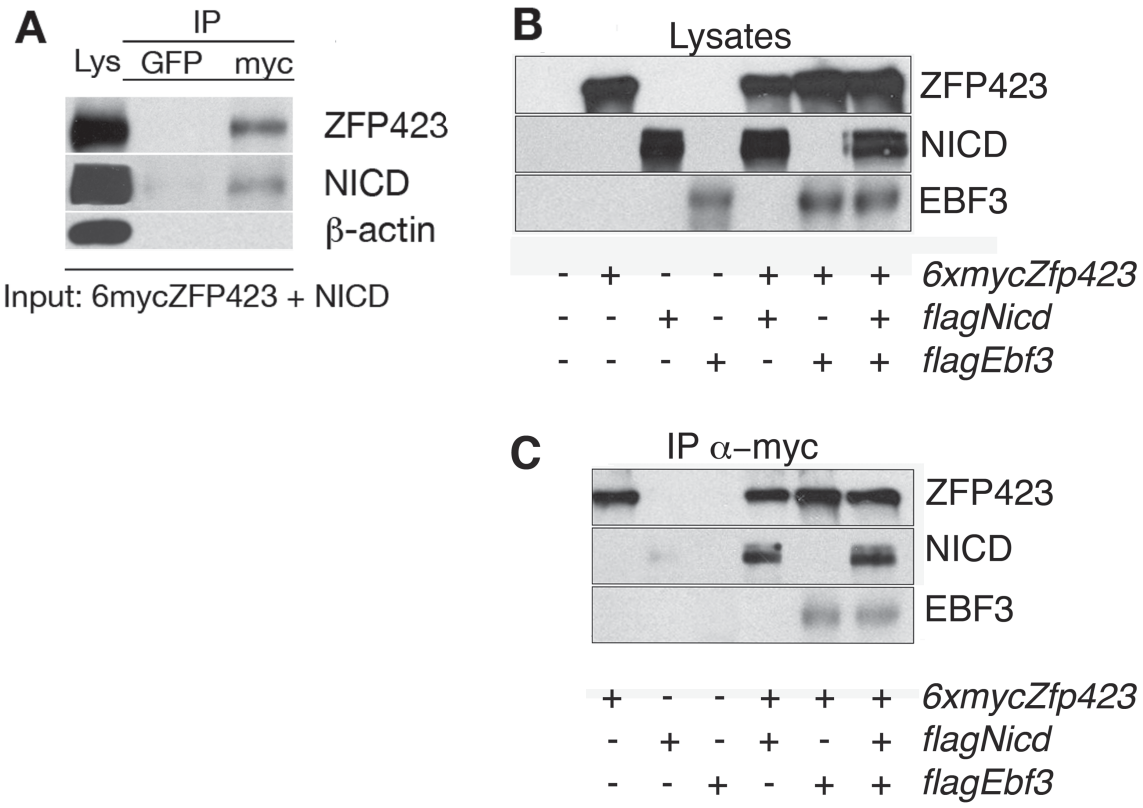


Figure 3.7: Molecular interaction between ZFP423 and NICD. In A), COS7 cells transfected with the indicated constructs, were subjected to immunoprecipitation using anti-GFP as an irrelevant antibody, or anti-myc to precipitate 6xmycZFP423. Filters were cut and stained for ZFP423, NICD, and actin (unrelated protein). Only NICD coprecipitated in the fraction immunoprecipitated with anti-myc. B) Lysates of COS7 cells, transfected with the indicated constructs, were blotted and immunostained as shown. C) The lysates shown in B were immunoprecipitated with the anti-myc antibody. Filters were immunostained for ZFP423, NICD and EBF3. Notably, NICD coprecipitated equally with ZFP423 in the presence or absence of EBF3.

gene transcription. Since ZFP423 is a molecular interactor of EBF TFs, we repeated the experiment described in figure 3.2B and added *Ebf1*, *Ebf2*, or *Ebf3* to the transfection mix. Transfected cells were harvested, lysed and analyzed by RT-qPCR for *Hes5* mRNA levels. Our results indicate that *Ebf1-3* overexpression alone has no detectable effect on *Hes5* transcription. However, cotransfecting cells with *Nicd*, *Zfp423* and either *Ebf1*, *Ebf2*, or *Ebf3* antagonizing the cooperative effect of ZFP423 and NICD on *Hes5* gene transcription (Figure 3.8A). The experiment was repeated *in vivo*, by injecting 2-cell *Xenopus* embryos unilaterally with the same combination of mRNAs. Again, our *in vivo* results faithfully recapitulated those obtained in P19 cells: coinjection of either *XEbf2* (C) or *XEbf3* (Figure 3.8D) reduced *ESR1* activation induced by exogenous *Zfp423* (Figure 3.8B). *XEbf2* or *XEbf3* injection led to a low level of ectopic *ESR1* expression (Figure 3.8E,F). This might be caused by increased *X-Delta-1* expression, driven by XEBF2 (Dubois et al., 1998), or to expression in differentiating neurons outside of the neural plate (Garcia-Dominguez et al., 2003). Since our data indicate that EBFs interfere with the cooperation of ZFP423 and NICD, we asked if EBF overexpression can antagonize the assembly of the ZFP423-NICD molecular complex. To address this point, COS7 cells were cotransfected with 6xmycZFP423, flagNICD and flagEBf3. Lysates were immunoprecipitated with anti-myc and analyzed by WB using Abs for ZFP423, NICD and EBF3. Both NICD-specific and EBF specific bands were present in the anti-myc immunoprecipitates (Figure 3.7C). Again, lysates from single-transfected cells were used as negative controls. These results suggest that the three proteins form a complex exhibiting a reduced ability to activate *Hes5* gene expression.

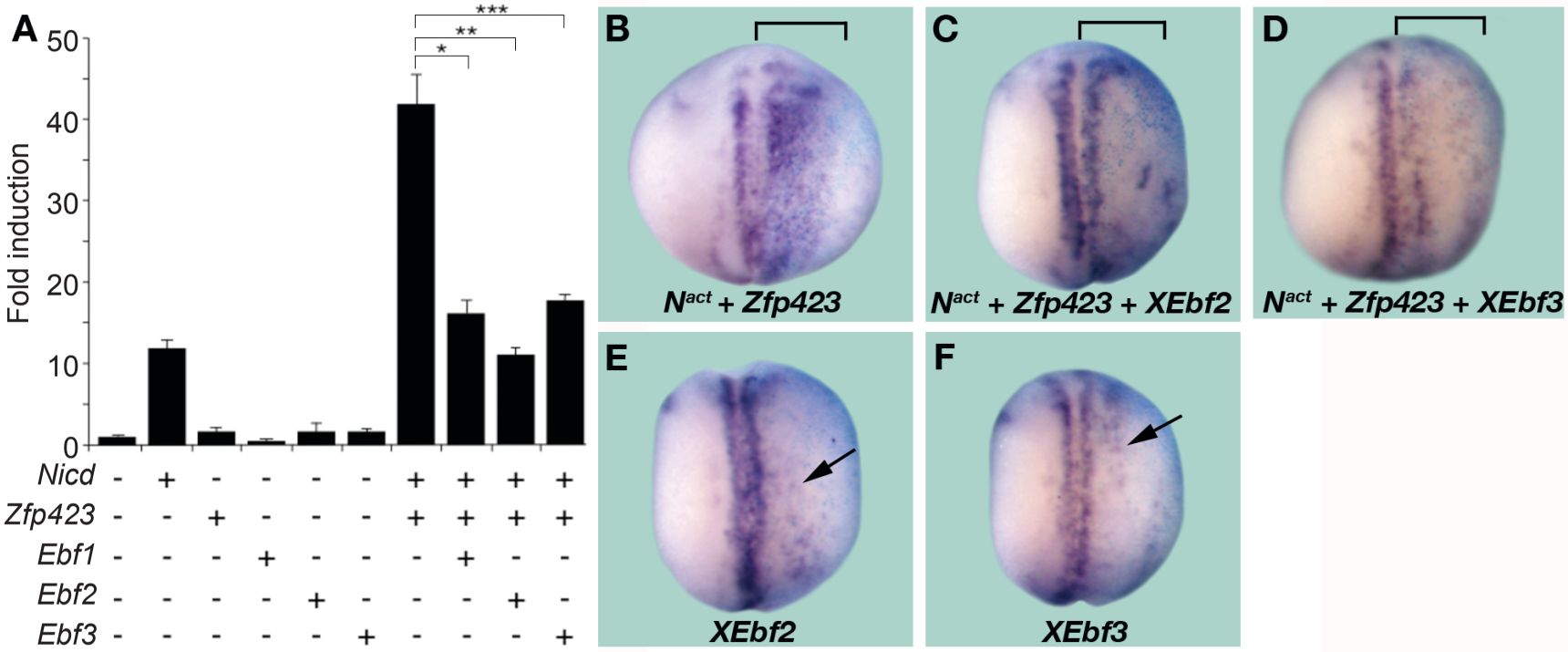


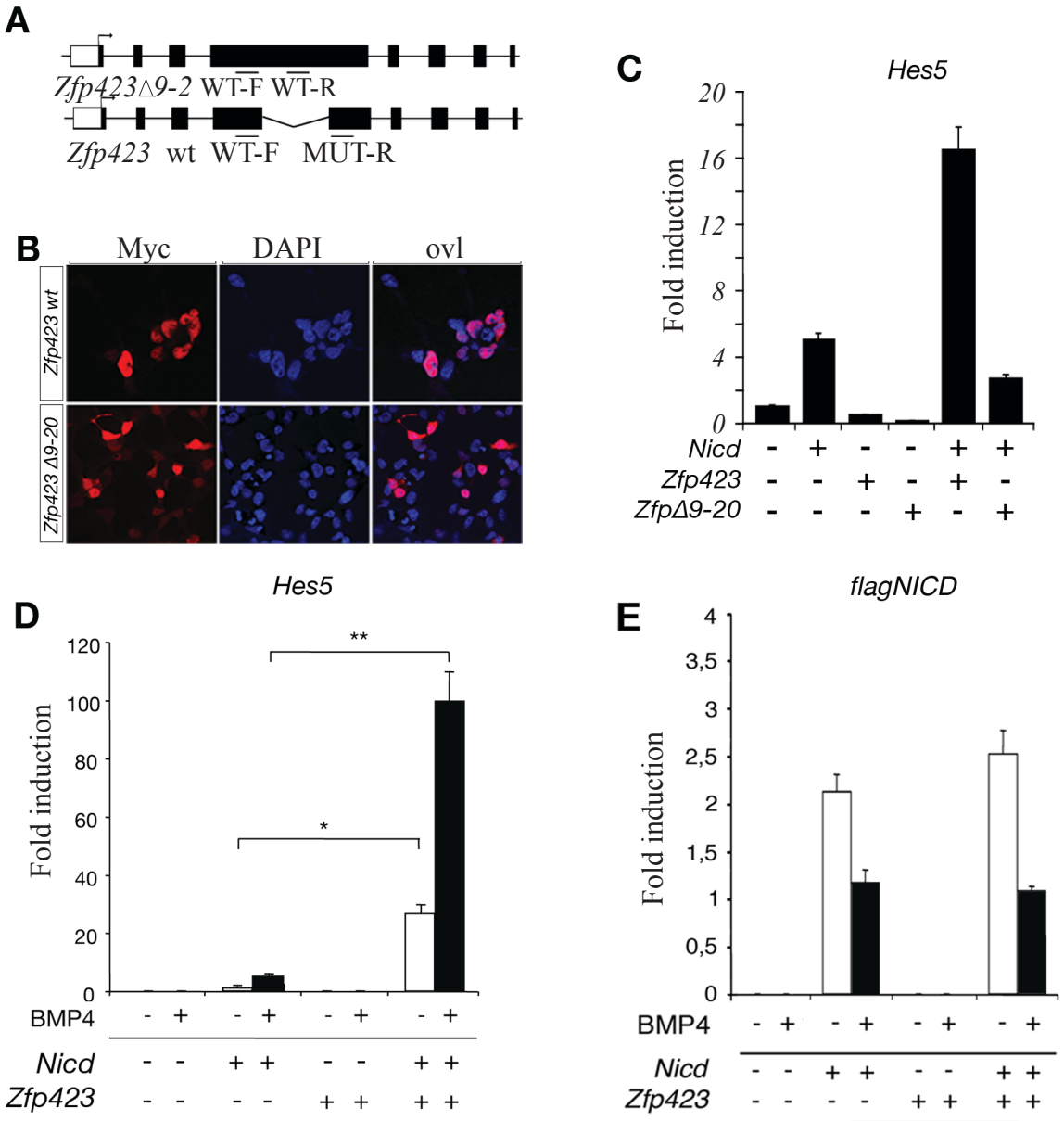
Figure 3.8: EBF TFs reduce ZFP423-NICD-mediated *Hes5* gene expression *in vitro* and *in vivo*. A) RT-qPCR analysis of *Hes5* gene expression in P19 cells transfected with the indicated constructs. Note that *Ebf1*, *Ebf2* or *Ebf3* alone does not affect basal *Hes5* expression. B-D) Whole mount *in situ* hybridization analysis of *ESR1* gene expression in embryos injected unilaterally (bracket) with *Nact* (100 pg) and *Zfp423* (600 pg) and/or *XEbf2* (100 pg) or *XEbf3* (100 pg). E, F) Whole mount *in situ* hybridization analysis of *ESR1* gene expression in embryos injected unilaterally with *XEbf2* (100 pg) or *XEbf3* (100 pg), as indicated. Note slightly-increased *ESR1* positive cells in *XEbf2* and *XEbf3* injected embryos (arrow). B-F) LacZ (blue stain) serves as an indicator of the injected side.

Zfp423 enhances the cooperation between NICD and BMP/SMAD signaling

Previous studies (Hata et al., 2000) showed that ZFP423 interacts with the SMAD1/SMAD4 complex in the nucleus to activate the *Xvent2* promoter in response to BMP signaling activation. Moreover, other authors have described the existence of interactions between BMP and Notch signaling pathways, resulting in repression of neurogenesis (Takizawa et al., 2003) and myogenesis (Dahlqvist et al., 2003), while others have proposed BMP as a rhombic lip (RL)-inducing signal in cerebellar development, antagonized by Notch signaling, which sets the RL's ventral limit (Machold et al., 2007). Results obtained by other authors indicate that ZFP423 is a functional and molecular interactor of the SMAD1-SMAD4 complex. That interaction occurs thanks to a domain constituted by Zn fingers 9-20, encoded by exon 4 (Hata et al., 2000; Ku et al., 2006). We asked if Zfp423 uses the same domain to interact with NICD. To address this question we generated a deleted construct missing Zn-fingers 9-20 (sketched in Figure 3.9A). First, we overexpressed the construct in COS7 cells to determine the subcellular localization of the corresponding protein. Δ9-20 ZFP423 is expressed and displays a prevalent nuclear localization (Figure 3.9B). Subsequently, we performed a promoter-reporter assay in C2C12 cells to compare the ability of wt and Δ9-20 ZFP423 to regulate *Hes5* gene expression. Our results indicate that Δ9-20 ZFP423 fails to cooperate with NICD in *Hes5* gene activation (Figure 3.9C).

Since ZFP423 uses the same domain to interact functionally with both NICD and the SMAD complex, we asked whether and in which way ZFP423 might modulate the response of *Hes5* to BMP signaling activation (Takizawa et al., 2003). *In vivo*, *Zfp423* is expressed in the cerebellar primordium flanking the roof plate (Figure 3.1A), a territory

Figure 3.9: ZFP423 cooperates with NICD and BMP signaling activation to promote *Hes5* gene expression. Δ9-20 ZFP423 localizes partially in the cell nucleus but fails to activate *Hes5* gene expression in cooperation with NICD. **A)** scheme of the in frame deletion of exon 4 producing a protein devoid of Zn fingers 9-20, implicated in the interaction with SMAD proteins and the BMP-responsive element. **B)** Immunofluorescence analysis of the subcellular localization of a myc-tagged wt and Δ9-20 construct in COS7 cells. Note that the mutant protein has a nuclear localization, although in some cells it is also distributed in the cytoplasm. DAPI labels DNA. ovl, overlay. **C)** Histogram illustrating the results of a promoter-reporter assay revealing the lack of a co-operative interaction between Zfp423 Δ9-20 and NICD in *Hes5* gene activation. **D)** Cooperative activation of *Hes5* gene expression by BMP4 and Notch mediated by ZFP423. Real-time RT-qPCR analysis of RNA from C2C12 cells, mock transfected or transfected with either *Nicd, Zfp423*, or both. Cells were either left untreated or treated with BMP4 for 2 hours. Transfection with *Zfp423* strongly enhances the cooperative effect of NICD and BMP signaling on *Hes5* gene expression. **E)** RNAs from the C2C12 cell lysates analyzed in figure 3.9D were subjected to RT-qPCR using primers specific for flagNICD. FlagNICD expression levels are comparable in the presence and absence of Zfp423. This excludes the possibility that differences in *Hes5* expression depend on different levels of *flag-Nicd* in *Zfp423*-transfected vs. *Zfp423*-untransfected samples.*, $p<0.05$; **, $p<0.005$.


A
Zfp423Δ9-2 WT-F WT-R
Zfp423 wt WT-F MUT-R
B
Myc
DAPI
ovl
Zfp423 wt
Zfp423 Δ9-20
C
Hes5
Fold induction
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16
12
8
4
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Nicd - + - - + +
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ZfpΔ9-20 - - - + - +
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120
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Nicd - - + + - - + +
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Zfp423 - - - - + + + +

that expresses high levels of various BMP family molecules. To determine whether ZFP423 coordinates Notch- and BMP signaling recruiting both pathways in the activation of *Hes5* transcription, we performed an RT-qPCR experiment in C2C12 cells, measuring *Hes5* transcript levels in response to NICD and ZFP423, and in the presence or absence of purified BMP4 in the culture medium (Figure 3.9D). To exclude the possibility that *Hes5* activation could stem from unequal expression levels of NICD in cells co-transfected with NICD and ZFP423 versus NICD alone, the levels of NICD were analyzed by RT-qPCR and *Nicd* levels are virtually identical. (Figure 3.9E). Our results reproducibly indicate that, in the absence of ZFP423, a 2-hour BMP treatment does upregulate *Hes5* compared to the transcript levels achieved by transfecting the cells with *Nicd* alone The interaction between BMP4 and NICD observed in C2C12 cells confirms the results reported by other authors (Takizawa et al., 2003). However, cotransfecting C2C12 with ZFP423 significantly potentiates (over ten-fold) the co-activating effect of BMP4 on NICD-induced *Hes5* gene expression (Figure 3.9D), suggesting that this protein may play an important role integrating BMP and Notch signaling in dorsal territories of the neural tube.

Discussion

ZFP423 interacts functionally and molecularly with NICD to activate Hes5 gene expression

In this paper, we show that ZFP423 interacts functionally with the Notch intracellular domain to activate cooperatively and selectively the expression of one direct Notch target: *Hes5* (model in Figure 3.10). This effect occurs on a small stretch of *Hes5* proximal promoter, containing both a CBF1 binding site and a BMP responsive

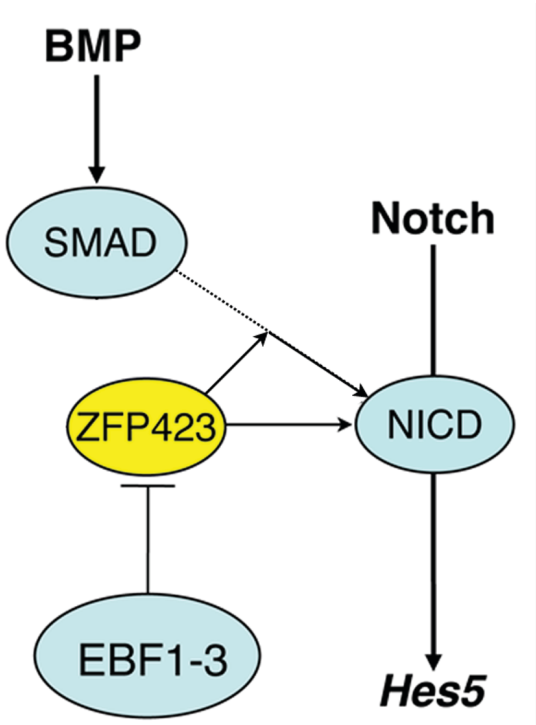

Figure 3.10: A working model of the interactions between ZFP423 and other networks in *Hes5* gene activation. *Hes5* expression is induced upon Notch signaling activation independently of ZFP423. ZFP423 boosts the effect of Notch signaling by forming a complex with NICD and by recruiting it onto the *Hes5* promoter. The interaction between the BMP4 transducer SMAD1-SMAD4 and ZFP423 further potentiates this effect (solid arrow), although BMP4 signaling might also cooperate with NICD in the absence of ZFP423 (stippled arrow). In overexpression experiments, EBF/COE transcription factors antagonize *Hes5* activation, likely by recruiting NICD and ZFP423 into a different transcriptional network.

element. This interaction occurs *in vivo*, as shown by the results of ChIP experiments. However, ZFP423 has no noticeable effect on the expression of other NICD targets, such as *Hes1* or *Nrarp*. This conclusion is supported by experiments conducted both in cell lines and *Xenopus* embryos. The results of both gain-of-function and loss-of-function experiments, conducted *in vivo* and *in vitro*, support the notion of a cooperation between ZFP423 and NICD in *Hes5* regulation. Strikingly, in C2C12 cells, that are negative for both the *Hes5* and *Zfp423* transcripts, the expression of *Hes5* is strictly dependent upon the addition of exogenous *Zfp423*, as *Nicd* overexpression alone is not sufficient to activate it significantly, while it activates other Notch targets. This result suggests that ZFP423 acts selectively to recruit NICD onto the *Hes5* promoter.

ZFP423 coordinates BMP4 and Notch signaling to activate Hes5 gene expression flanking the dorsal midline

Our results indicate that ZFP423, a known nuclear interactor of the BMP-dependent SMAD complex (Hata et al., 2000), acts to modulate the function of NICD. The corresponding transcript, *Zfp423*, is expressed in two symmetric stripes of cells flanking the roof plate in the cerebellar primordium (Figure 3.1, and Cheng et al., 2007). Thus, we wondered if ZFP423 might integrate Notch signaling and roof plate signals cell-autonomously to promote *Hes5* gene expression. Functional interactions between Notch and BMP signaling have been observed in urogenital and endothelial cells (Grishina et al., 2005; Itoh et al., 2004), and BMP has been known to activate *Hes5* transcription mildly in mouse neuroeptihelial cells (Takizawa et al., 2003), but the role of ZFP423 in this context was not examined. In C2C12 cells, we find that ZFP423 triggers a cooperative interaction between NICD and the SMAD complex, leading to a strong

activation of *Hes5* gene expression. While this result could not be replicated in *Xenopus* embryos, since BMP activation in early embryos interferes with neural induction, in vitro studies reproducibly reveal that the cooperative interaction existing between NICD and the BMP pathway is enhanced by ZFP423 and results in *Hes5* upregulation. For its cooperation with NICD (present paper) and SMAD1/4 (Hata et al., 2000), ZFP423 uses the same domain, containing Zn fingers 9-20. This is a very large domain, that likely accommodates both proteins permitting their cooperative rather than antagonistic interaction.

Previous reports have shown that *Zfp423* null mutants feature a disorganized cerebellar ventricular zone with disassembled radial glia. In neuralized P19 cells, ZFP423 regulates both *Hes5* and *Blbp* transcription in response to NICD. In vivo, *Hes5* transcription is strictly dependent upon Notch signaling activation and *Hes5* is expressed in asymmetrically dividing radial glia (Ohtsuka et al., 1999; Ohtsuka et al., 2001). In the cerebellar primordium, *Hes5* labels the cerebellar VZ and rhombic lip, whereas *Hes1* is highly expressed in the rhombic lip and isthmic organizer, but is downregulated in most of the VZ (Figure 3.1). We speculate that in dorsal territories of the cerebellar primordium the interaction occurring between Notch and BMP signaling, and enhanced by ZFP423, could maintain a pool of *Hes5* positive radial glial progenitors supporting several rounds of asymmetric, neurogenic cell division, and prevent the premature occurrence of terminal differentiation. In keeping with this interpretation, expression of the radial glia marker BLBP has been found reduced in *Zfp423* mutants (Alcaraz et al., 2006).

EBF TFs antagonize the ZFP423-NICD mediated activation of Hes5 gene expression

The cooperative interaction established by ZFP423 and NICD can be quenched by cotransfecting cells with cDNAs encoding TFs of the EBF ^{COE} family. EBF TFs also block the cooperative interaction between ZFP423 and N^{act} in *Xenopus* embryos. EBF TFs have been implicated in neuronal differentiation, and ZFP423 acts as an EBF antagonist both in promoter-reporter assays (Tsai and Reed, 1997) and, *in vivo*, in olfactory neurogenesis (Cheng and Reed, 2007). The reported ability of ZFP423 to block differentiation when expressed ectopically in postmitotic precursors, and to revert them to an undifferentiated state, could be explained partially by the ability, shown here, of ZFP423 to promote *Hes5* gene expression. Conversely, the ability of EBF TFs to couple cell cycle exit to the onset of differentiation may stem in part from recruiting ZFP423 into molecular processes other than its cooperation with Notch signaling. In other words, EBF TFs may compete with NICD for ZFP423 in differentiating radial progenitors, and recruit ZFP423 in neuronal differentiation and migration (Garcia-Dominguez et al., 2003). Our co-immuno-precipitation results indicate that EBF3 does not interfere significantly with the assembly of a ZFP423-NICD complex, thus suggesting that the presence of EBF TFs may recruit NICD and ZFP423 into a distinct regulatory network, sequestering it from the *Hes5* promoter.

In summary, ZFP423 synergizes with NICD and cell-autonomously integrates BMP signaling with the Notch pathway, leading to the selective upregulation of one direct target of Notch: *Hes5*. By doing so, *Zfp423* may promote the maintenance of an undifferentiated radial glial progenitor pool. *In vivo* gain-of-function studies are now required to fully elucidate the roles of Zfp423 in the context of cerebellar neurogenesis.

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CHAPTER 4

THE EARLY B CELL FACTOR FAMILY OF PROTEINS PARTICIPATES IN TRANSCRIPTIONAL REGULATION OF XENOPUS MUSCLE CELL DEVELOPMENT

(Yangsook Song Green and Monica L. Vetter, in preparation for submission)

Abstract

EBF proteins have diverse functions in the development of multiple lineages, including neurons, B cells and adipocytes. During Drosophila muscle development EBF proteins are expressed in muscle progenitors and required for muscle cell differentiation, but there is no known function of EBF proteins in vertebrate muscle development. In this study, we examine expression of *ebf* genes in Xenopus muscle tissue and show that EBF activity is necessary for aspects of Xenopus skeletal muscle development, including somite organization, migration of hypaxial muscle anlagen toward ventral abdomen, and development of jaw muscle. From a microarray screen, we have identified candidate targets of EBF activity with known roles in muscle development. The candidate targets we have verified are MYOD, MYF5, M-Cadherin and SEB-4. *In vivo* overexpression of the *ebf2* and *ebf3* genes leads to ectopic expression of these candidate targets, and knockdown of EBF activity causes downregulation of the endogenous expression of the candidate targets. Finally we show that MYOD can upregulate the expression of *ebf* genes, indicating the presence of a positive feedback loop between EBF and MYOD that we find to be important for maintenance of MYOD expression in Xenopus. These results suggest that EBF activity is important for both stabilizing commitment and driving aspects of differentiation in Xenopus muscle cells.

Introduction

The processes of cell commitment and cell differentiation are important aspects of the development of muscle tissue. The group of transcription factors known as myogenic regulatory factors (MRFs) includes the proteins MYOD and MYF5, and these

transcription factors are critical in driving both commitment and differentiation in muscle tissue, as seen by the complete lack of muscle cells in Myod, Myf5 double knockout mice (Rudnicki et al., 1993). While much is known about regulation of the process of myogenesis, and the actions of the MRFs in particular, there is no complete picture of the regulatory networks of transcription factors that drive vertebrate muscle development. In *Drosophila*, the Early B cell factor (EBF, also known as COE (collier/olfactory/EBF)) family member Collier plays a role in muscle development, but the roles of EBF proteins in vertebrate muscle development have not been explored.

EBF family members are transcription factors involved in development in several different cell lineages, including neurons, B cells, adipocytes and muscle cells (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002; Lukin et al., 2008). These proteins contain a zinc finger DNA binding domain and an atypical helix-loop-helix dimerization domain (Hagman et al., 1993; Hagman et al., 1995; Wang and Reed, 1993; Wang et al., 1997). There are four family members in mammals (Ebf1, Ebf2, Ebf3 and O/E4), two known members in *Xenopus* (EBF2 and EBF3), and one in zebrafish (ZCOE2) (Bally-Cuif et al., 1998; Dubois et al., 1998; Garel et al., 1997; Hagman et al., 1993; Malgaretti et al., 1997; Pozzoli et al., 2001; Wang and Reed, 1993; Wang et al., 1997). Among invertebrates, the proteins Collier in *Drosophila* and UNC3 (*CeO/E*) in *C. elegans* belong to the EBF family (Crozatier et al., 1996; Prasad et al., 1998). EBF proteins have been shown to regulate many aspects of differentiation during neuronal, B cell and adipocyte development, however much less is known about their role during muscle development.

In *Drosophila*, the *collier* gene is expressed in progenitors for several muscles, and is required for myoblast fusion (Crozatier and Vincent, 1999). The expression of

collier in *Drosophila* is driven by both Collier itself and by the MYOD ortholog Nautilus, and this upregulation is synergistic when two genes are present together (Dubois et al., 2007). In mouse, Northern blot analysis shows that *Ebf3* is expressed in adult skeletal muscle (Garel et al., 1997). Furthermore, EBF proteins are known to bind to the negative regulatory element of the *glut4* gene in muscle (Dowell and Cooke, 2002), which allows for insulin-mediated glucose uptake in multiple tissue types (Kahn, 1998). However, the exact expression patterns, transcriptional targets, and functions of EBF genes in the development of vertebrate muscle are not understood.

The process of muscle development has been intensively investigated in multiple vertebrate models, including *Xenopus*. During vertebrate development, early mesoderm tissue forms somites, which contain myotome cells that will become myoblasts and give rise to muscle tissue. In *Xenopus*, cells in presomitic mesoderm undergo an early rotational event that gives rise to somites (reviewed in (Elinson, 2007).

Somites contain two separate muscle cell lineages. The region of the somite called the dermamyotome contains a dorsal lip, near the neural tube, with cells that will form epaxial muscles (muscles of the deep back), and a ventral lip, far from the neural tube, with cells that will form hypaxial muscles (muscles of the body wall and limbs) (Gros et al., 2005; Mariani et al., 2001). The hypaxial cells bud off from the somite and migrate ventrally along the body wall before completing the processes of muscle development (Martin and Harland, 2001, 2006). Next, myoblasts localize to their correct positions and exit the cell cycle. In most species, myoblasts then align with neighboring myoblasts, undergo fusion, and continue differentiation as multinucleated muscle fibers. However, in *Xenopus*, muscle cells generated before metamorphosis utilize amitotic rounds of nuclear

division to generate multi-nucleated muscle cells (Boudjelida and Muntz, 1987; Kielbowna, 1966).

Across many species, MYOD and MYF5 are expressed from the somite stage, and these bHLH proteins are well known for their critical role in myoblast commitment and differentiation. In *Xenopus*, MYOD and MYF5 are expressed even in presomitic mesoderm (Dosch et al., 1997; Hopwood et al., 1989, 1991), and when MYF5 function is blocked by morpholinos, normal development of both the presomitic mesoderm region and of somites is disrupted (Keren et al., 2005). MYOD and MYF5 are also expressed in *Xenopus* migrating hypaxial cells (Martin and Harland, 2001).

We performed a microarray screen of *Xenopus* animal cap tissue with active EBF3 protein, and unexpectedly found that several muscle-related genes were among the most strongly up-regulated targets (Green et al., in preparation), suggesting a role for EBF factors in regulating vertebrate muscle differentiation. We demonstrate the sufficiency and requirement of EBF2 and EBF3 for *in vivo* expression of the muscle-related genes *myod*, *myf5*, *seb-4 (*also called *rbm24)*, and *m-cadherin*, identified in our microarray screen. We also describe the expression patterns of *ebf2* and *ebf3* in the tissues that give rise to *Xenopus* skeletal muscle, and show a requirement for EBF2 and EBF3 activity in normal muscle development. Finally, we show that MYOD can drive expression of *ebf2* and *ebf3*, *in vivo*. Our results suggest several new functions of EBF proteins in vertebrate muscle development, and provide evidence in vertebrates of a reciprocal transcriptional relationship between EBF proteins and MYOD.

Materials and methods

Microinjection of RNA and morpholinos

The following constructs were used as DNA templates to make capped RNA: pCS2+Noggin (Richard Harland), pCS2+hGR-MT-Xebf2, pCS2+hGR-MT-Xebf3, p64T-MyoD-GR (Kolm and Sive, 1995), and pCS2+nβgal (Chitnis et al., 1995). Capped RNA was generated *in vitro* using the Message mMachine kit (Ambion). Antisense morpholino oligonucleotides (MOs) were designed by Gene Tools, and directed against a region at or near the translational start site of *ebf2* (5'-GCGCTTTGTCTCTCAAGGCAGTTCC-3') and *ebf3* (5'-GTATATTTTCCTGAATCCCAAACAT-3').

For microarray experiments, 1ng of hGR-XEBF3 mRNA and 0.2ng noggin mRNA were coinjected into *Xenopus* embryos at the one-cell stage. Alternatively, 0.4ng hGR mRNA and 0.2ng noggin mRNA were coinjected in control embryos. At stage 9, animal caps were dissected from the embryo, using either a Gastromaster or a syringe needle tip. Animal caps were treated with 30μM Dexamethasone (DEX) in 1x MMR for 4.5 hours before harvesting of total RNA.

For testing sufficiency of EBF and MYOD to drive target gene expression, a volume of 4nl containing RNA was injected into one blastomere of two cell stage embryos. The following amounts of capped RNA were used for injection: hGR-Xebf2 (0.5ng), hGR-Xebf3 (0.5ng), MyoD-hGR (0.5ng), and nβgal (30pg). For morpholino experiments, a volume of 3nl containing Xebf2 morpholinos (Gene Tools, 7.5μg or 10μg), Xebf3 morpholinos (Gene Tools, 7.5μg or 10μg), and nβgal (20pg) was injected into two vegetal blastomeres of 8-cell stage embryos. In all microinjections, nβgal capped RNA was co-injected with other capped RNA or morpholinos into embryos as a tracer.

Embryos were then grown and staged (Nieuwkoop and Faber, 1994). Embryos, which were injected with hGR-Xebf2, hGR-Xebf3 and MyoD-hGR, were treated with 30μM DEX from the gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). All embryos were then fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. After washing the embryos 3 times with PBS, X-gal staining was performed as described (Turner and Weintraub, 1994). Further fixation was done for one hour at room temperature or overnight at 4°C.

Real time PCR

The total RNAs generated for the microarray experiments were used for real time PCR (RT-PCR). To make cDNA from isolated total RNA from animal caps, the SuperScript II and oligodT12-18 primers (Invitrogen) were used according to the manufacture's instructions. MacVector Software was used to design the gene specific primers (Table 4.1). PCR was performed as previously described (Hutcheson and Vetter, 2001; Pozzoli et al., 2001).

Whole mount *in situ* hybridization

The following constructs were used to generate antisense RNA probes: pBS-Xebf2 (Pozzoli et al., 2001), pBS-Xebf3 (Pozzoli et al., 2001), pSP73-XmyoD (Hopwood et al., 1989), pBS-XMyf5 (Hopwood et al., 1991), M-cadherin (IMAGE ID 5440166, ATCC), XSEB-4 (IMAGE ID 4970239, ATCC), Actin alpha (IMAGE ID, 5542285 ATCC), and Tnnc1 (IMAGE ID 4407474, ATCC). Antisense RNA probe was generated *in vitro* using SP6, T7 or T3 RNA polymerase (Ambion) and labeled with digoxigeninin-

Table 4.1: Primer sequences used for RT-QPCR.

Name	Sequence
myod1 forward	5′- TCTGCCCCCTATTGGTCACTTG -3′
myod1 reverse	5′- CCCCATTGTCCGTATTCAACAC -3′
m-cadherin forward	5′- CCAAGATGGAACAAGGTCGCTC -3′
m-cadherin reverse	5′- GCTCATTTTTGACATTCAGTGCC -3′
actin alpha forward	5′- CCTGGACTTTGAAAATGAAATGGC -3′
actin alpha reverse	5′- CATACGGTCAGCAATACCTGGG -3′
seb-4 forward	5′- AGGCACCAAAGCAATCTTCTTG -3′
seb-4 reverse	5′- CCAGCGGTTCAAAGTTTCCC -3′
tnnc1 forward	5′- TGGGAGGGACTTATCACTAAATAGG -3′
tnnc1 reverse	5′- GGGCACAAAATGTCAAACGG -3′
myf5 forward	5′- AACCAGGCTTTTGAAACGCTC -3′
myf5 reverse	5′- TTCTTCCAGACCATTGAGGGC -3′
brachyury forward	5′- GGATCGTTATCACCTCTG -3′
brachyury reverse	5′- GTGTAGTCTGTAGCAGCA -3′
histone h4 forward	5′- TGCGGGATAACATTCAGGGC -3′
histone h4 reverse	5′- CGGTCTTCCTCTTGGCGTG -3′

11-UTP (Roche). Whole mount *in situ* hybridization was performed on the fixed and X-gal stained embryos as described (Harland, 1991; Kanekar et al., 1997).

Immunostaining

For whole mount immunostaining, pigmented embryos were bleached with 1% hydrogen peroxide and 5% formamide in 0.5X SSC solution under fluorescent light for about 1hour. The bleached embryos were fixed again with 4% PFA. 12/101 antibody hybridoma supernatant was used to stain differentiated skeletal muscle (Developmental Studies Hybridoma Bank, (Kintner and Brockes, 1984)). After washing embryos three times (1 hour for each wash) with PBS at 4°C, embryos were incubated with blocking solution containing 1% triton X-100 and 10% heat inactivated goat serum in PBS for 3 to 5 hours at RT. 12/101 antibody was diluted in the blocking solution (1:300) and incubated for 2 to 4 days at 4°C. The embryos were then washed 3 times (1 hour for each wash) with the blocking solution, and the Alexa 488 conjugated goat anti-mouse IgG secondary antibody (Invitrogen) was diluted in the blocking solution (1:1000) and incubated with embryos for 2 days at 4°C. The embryos were washed with PBS three times before being photographed.

Results

EBF3 drives expression of multiple muscle development genes in explanted *Xenopus* animal caps

To identify transcriptional targets of EBF3, we performed a microarray screen on *Xenopus* animal caps, comparing animal caps with and without active EBF3 protein (Green et al., in preparation; see GEO database). A hormone-inducible fusion protein

(hGR-XEBF3) was used to enable us to regulate EBF activity using the hormone dexamethasone (DEX). In the absence of DEX, EBF3 remains inactive. Adding DEX to the explants induces EBF3 activity (Kolm and Sive, 1995). We found that genes involved in muscle development were among the most strongly upregulated genes on the array, with *myod* being the second most strongly upregulated target of all genes. This study focuses on the muscle-related targets of EBF3. There were also significant candidate targets with expected neuronal functions, which are described elsewhere (Green et al., in preparation, and also see the GEO database).

We found a variety of candidate targets that have known functions or expression in muscle tissue, and we performed additional analysis on six of these: *myod* (80-fold upregulated, Genbank accession number BC073672), *muscle-cadherin* (*m-cadherin*, 39-fold, CF288050), *actin alpha* (34-fold, BC046739), *seb-4* (16-fold, BC072812), *tnnc1* (9-fold, BC082829), and *myf5* (6-fold, AJ009303). These results are the first to show *myod* transcriptionally downstream of an EBF family member, and suggest a potentially critical role of EBF proteins in *Xenopus* muscle development. To confirm our microarray results, we performed reverse transcriptase PCR (RT-PCR) and found that each candidate target gene listed above was upregulated in the presence of active hGR-XEBF3 (Figure 4.1).

EBF2 and EBF3 are sufficient and required for the expression of muscle targets *in vivo*

The function of EBF2 is known to be similar to that of EBF3, so EBF2 was also included in the remaining experiments of this study (Dubois et al., 1998; Pozzoli et al., 2001). In order to determine if EBF2 and EBF3 are sufficient for driving the expression of the candidate target genes *in vivo*, we examined the expression level of targets after

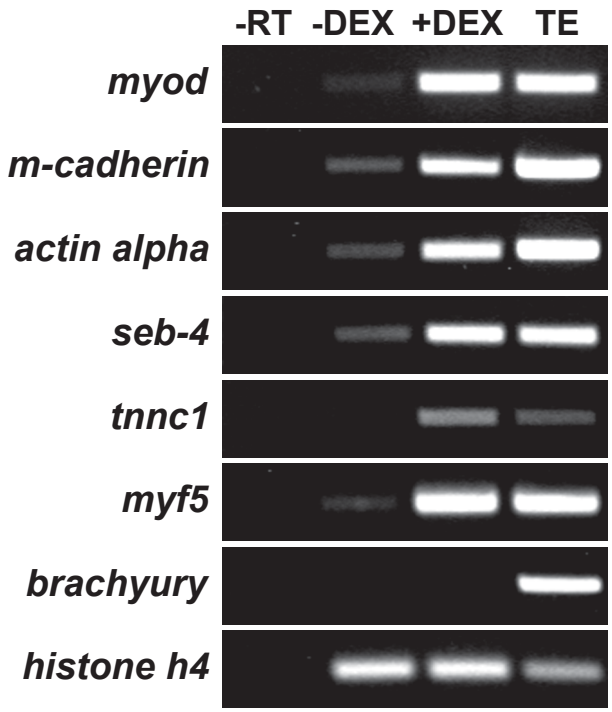


Figure 4.1: Confirmation of microarray by RT-PCR. hGR-XEBF3 mRNA and Noggin mRNA were injected into embryos at the single-cell stage. At the blastula stage, the animal caps were divided into two groups. One group was treated with DEX and the other, untreated, group was used as a control. Following a 4.5 hour incubation with or without DEX, total RNA was isolated from each group of animal caps. RT-PCR was conducted with the isolated total RNAs. The column labeled –RT is a negative control, in which reverse transcriptase was not added when cDNA was generated. cDNA from stage 12 (for *myf5*, *Xbra* and *histone h4*) or stage 27 (for the remaining genes) total embryos (TE) was used as a positive RT-PCR control. *brachyury* was used to ensure there was no mesoderm contamination in animal caps. *histone h4* was used as a loading control. All tested genes (except the loading and contamination controls) were upregulated in the presence of DEX.

overexpression of hGR-XEBF2 or hGR-XEBF3 in *Xenopus* embryos. Overexpression was achieved by injection of mRNA into one cell of two-cell stage embryos, followed by treatment of the embryos with DEX from the gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). The expression level of candidate target genes was then examined by whole mount *in situ* hybridization (WM-ISH) (Figure 4.2). We found that *myod* (16/16 embryos for hGR-XEBF2 and 16/16 embryos for hGR-XEBF3), *m-cadherin* (28/33 and 15/24), *seb-4* (18/18 and 24/24), and *myf5* (33/50 and 30/48) were upregulated by overexpression of EBF2 or EBF3 (Figure 4.2). However, the expression of *actin alpha* (17/24) and *tnnc1* (36/48) were downregulated by EBF3 (data not shown). We therefore believe that *actin alpha* and *tnnc1* are unlikely to be *in vivo* targets of EBF activity. The fact that the expression levels of *myod*, *m-cadherin*, *seb-4* and *myf5* are upregulated by overexpresion of EBF2 and EBF3 supports the microarray data, and suggests that EBF activity is sufficient to drive expression of these candidate genes *in vivo*.

To determine if EBF2 and EBF3 activity is required for the expression of our candidate target genes *in vivo*, we examined their expression level after knockdown of EBF2 and EBF3 using translation blocking antisense morpholinos (MO), targeting both factors together since they often act redundantly. To block EBF2 and EBF3 function, EBF2 MO and EBF3 MO were coinjected into two vegetal cells of eight-cell stage embryos, which make minimal contributions to neuronal tissue where EBF factors are also known to be required ((Dubois et al., 1998; Pozzoli et al., 2001) and Green et al., in preparation). The expression level of endogenous candidate target genes was examined at the early tail bud stage (stage 21/22), a stage in which the anterior somites are clearly formed, and the expression of each target is apparent (Figure 4.3). After knockdown of

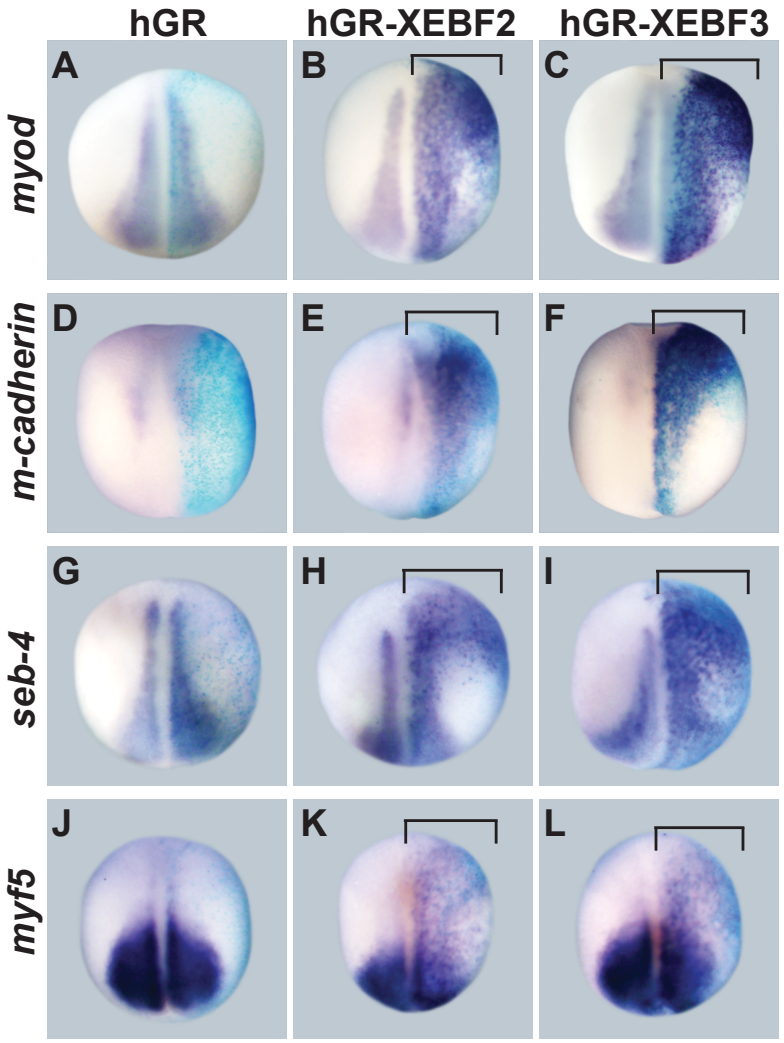


Figure 4.2: EBF2 and EBF3 are sufficient for muscle target gene expression. hGR-XEBF2 or hGR-XEBF3 mRNA were injected into one cell of two-cell stage embryos, followed by DEX treatment from the late gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). hGR mRNA was injected in control embryos. β-galactosidase mRNA was coinjected as a marker of the injected side. In all panels the right side is the injected side, showing the light blue color of X-gal staining. The (purple) expression of *myod* (B and C), *m-cadherin* (E and F), *seb-4* (H and I), and *myf5* (K and L) is strongly upregulated by EBF2 and EBF3 (brackets), while hGR does not change the expression level of the target genes (A, D, G, and J). All panels show dorsal views.

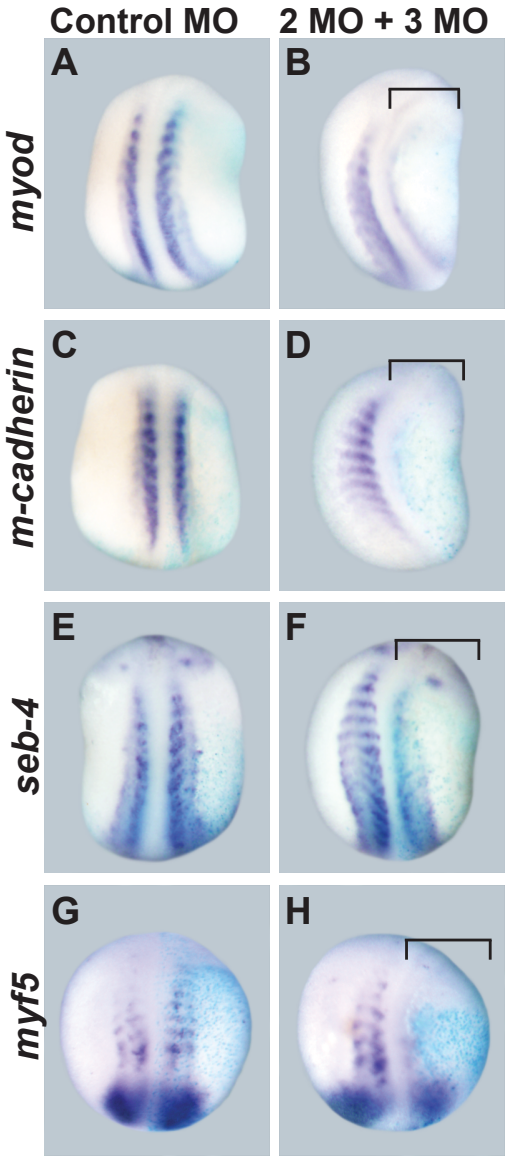


Figure 4.3: EBF2 and EBF3 are necessary for muscle target gene expression. Two vegetal cells of eight-cell stage embryos were injected with either XEBF2 MO and XEBF3 MO together, or control MO. β-gal mRNA was coinjected as a marker of the injected side. The expression level of target genes was examined at the stage 21/22. In all panels the right side is the injected side, showing the light blue color of X-gal staining. The (purple) expression of *myod* (B), *m-cadherin* (D), *seb-4* (F), and *myf5* (H) are downregulated by XEBF2 MO and XEBF3 MO (brackets), while control MO does not change their expression level (A, C, E, and G). All panels show dorsal views.

both EBF2 and EBF3, the expression of *myod* (16/18 embryos), *m-cadherin* (7/8), *seb-4* (6/10), and *myf5* (14/17) were downregulated. Control MO did not change the expression levels of these genes. These four genes were also downregulated by expression of RNA encoding truncated dominant negative EBF3 (DN-EBF), which blocks the function of endogenous EBF proteins by forming non-functional dimers that do not bind DNA (Green et al., in preparation, (Dubois et al., 1998; Hagman et al., 1993; Hagman et al., 1995) and data not shown). These knockdown experiments suggest that EBF factors are required for the expression of each of our candidate targets *in vivo*.

EBF2 and EBF3 are expressed in developing muscle tissue

Previous studies have focused mainly on neuronal expression of the *ebf2* and *ebf3* genes (Dubois et al., 1998; Pozzoli et al., 2001), but there is also apparent expression of *ebf3* in somites of stage 28 and stage 32 *Xenopus* embryos (Pozzoli et al., 2001). Since we have verified that EBF activity is critical for the expression of our muscle specific candidate target genes, we performed WM-ISH to obtain a more detailed picture of both *ebf2* and *ebf3* expression in somites and developing muscle tissue (Figure 4.4). At stage 22, there is expression of *ebf2* and *ebf3* in presomitic mesoderm tissue. Expression of *ebf2* and *ebf3* is detectable in somites as well, and this somite expression becomes very clear at stage 28 (Figure 4.4, arrows). The somites will give rise to structures including dorsal epaxial muscle and ventral hypaxial muscle. At stage 37, *ebf2* and *ebf3* maintain somitic expression, and maintain expression in the migrating hypaxial muscle tissue (Figure 4.4, arrowheads). In sections through the somites at stage 28, *ebf2* and *ebf3* appear to be expressed in the dermamyotome, which contains the cells that will form

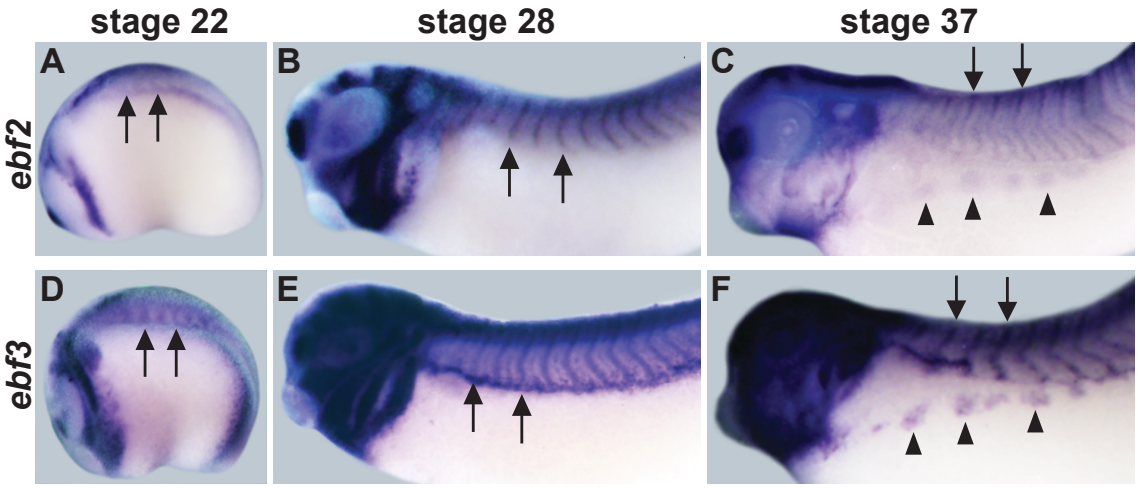


Figure 4.4: Expression patterns of ebf2 and ebf3 in Xenopus muscle. *ebf2* (A-C) and *ebf3* (D-F) are expressed in multiple developing nervous and muscle tissues. At stage 22, *ebf2* and *ebf3* are expressed in pre-somitic mesoderm. At all three stages, *ebf2* and *ebf3* are expressed in the developing somites (arrows). At stage 37, they are also expressed in the migrating hypaxial muscle anlagen (arrowheads).

epaxial and hypaxial muscle tissue (data not shown). These expression patterns provide a strong indication that *ebf2* and *ebf3* could regulate skeletal muscle development.

Classes and expression patterns of target genes

The EBF gene targets identified by microarray, and confirmed by EBF gain and loss of function experiments, were classified based on their known functions in *Xenopus* and other species. MYOD and MYF5 are basic helix-loop-helix transcription factors and also are myogenic regulatory factors (Braun et al., 1989; Davis et al., 1987; Hopwood et al., 1989, 1991). M-cadherin is a cell membrane protein (Donalies et al., 1991) and SEB-4 is an RNA binding protein (Fetka et al., 2000).

To determine if the functional relationships we identified above are meaningful relationships during *Xenopus* development, and to determine if the targets are expressed in a manner consistent with the expectation that they are involved in muscle development, we compared the expression domains of EBF2 and EBF3 target genes (Figure 4.5) with those of *ebf2 and ebf3* (Figure 4.4) by WM-ISH at stage 37. We chose this stage because most structures that will give rise to skeletal muscles, including somites, migrating hypaxial muscle anlagen and developing jaw muscle, are clearly detectable at this stage. The WM-ISH expression patterns of *myod*, *myf5* and *seb-4* in *Xenopus* have previously been published (Dosch et al., 1997; Fetka et al., 2000; Martin and Harland, 2001), but to our knowledge this is the first report of expression patterns of *m-cadherin* in *Xenopus*. All four genes are expressed in the tissues that will give rise to skeletal muscle. *myod* is expressed in a strong central band in the somites, with especially strong expression at the dorsal and ventral lips. It is also expressed in jaw muscle and

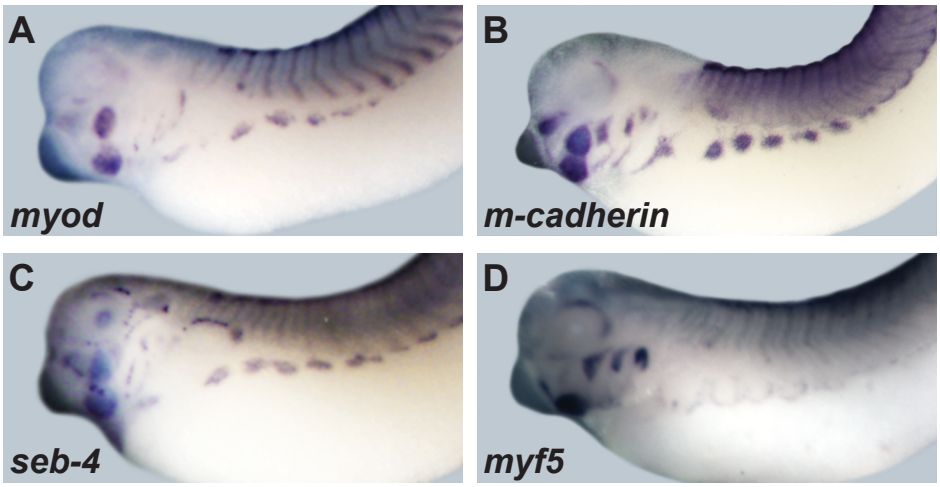


Figure 4.5: Expression patterns of muscle target genes in stage 37 embryos.
myod, *m-cadherin*, *seb-4*, and *myf5* are all expressed in the skeletal muscle. *myod* (A) and *seb-4* (C) are expressed in the somites, migrating hypaxial muscle anlagen and jaw muscle, and these expression patterns overlap with those of *ebf2* and *ebf3* (Figure 4.4). *m-cadherin* (B) is expressed in a weak central band in somites, with expression throughout the somite. It is also expressed in migrating hypaxial muscle anlagen and jaw muscle. *myf5* (D) expression in somites is weaker than other genes at this stage, and is expressed at the leading edge of migrating hypaxial muscle. This gene is also expressed in jaw muscle. All embryos show lateral views.

migrating hypaxial muscle anlagen (Dosch et al., 1997; Martin and Harland, 2001, and Figure 4.5). *m-cadherin* is expressed in a weaker central band in the somites, and with diffuse expression throughout the somites. It is also expressed in jaw muscle and migrating hypaxial muscle anlagen (Figure 4.5). The expression pattern of *seb-4* in muscle tissue is very similar to that of *myod*. It is expressed in the somites, jaw muscle and the migrating hypaxial muscle anlagen ((Fetka et al., 2000) and Figure 4.5). *myf5* is expressed in the somites, jaw muscle and migrating hypaxial muscle anlagen, but the expression pattern of *myf5* is different from other targets in the migrating hypaxial muscle anlagen, in that it appears to be at the leading edge, rather than within the bulk of the anlagen. Expression of *myf5* is also weaker than that of the other targets at this stage ((Dosch et al., 1997; Martin and Harland, 2001) and Figure 4.5). These expression patterns of *myod*, *m-cadherin*, *seb-4* and *myf5* are very similar to expression of *ebf2* and *ebf3* in somites and migrating hypaxial muscle anlagen. This strong correlation suggests that the transcriptional relationships we have identified could be very relevant for *Xenopus* muscle development.

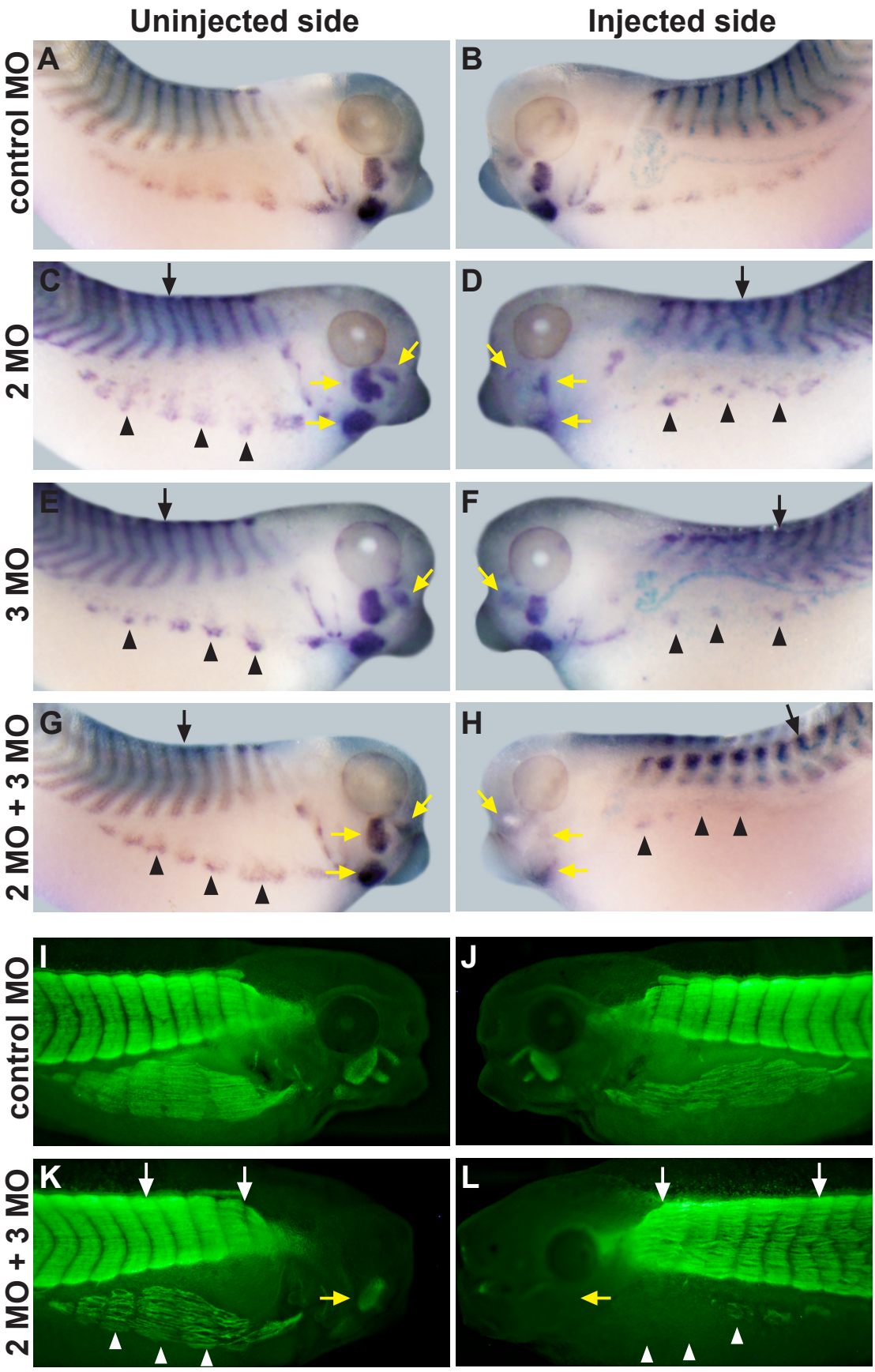
EBF2 and EBF3 are involved in *Xenopus* muscle development

In order to determine if EBF2 and EBF3 have a functional requirement in the morphology of *Xenopus* muscle development, we assessed this after knockdown of EBF2 and EBF3 with morpholinos. These morpholinos were injected either independently or together into two vegetal cells of eight-cell stage embryos. We then examined the expression pattern of the skeletal muscle marker *myod* (Dosch et al., 1997; Hopwood et al., 1989; Martin and Harland, 2001) after knockdown of EBF2 and EBF3 at stage 39/40

(Figure 4.6, A-H) by using WM-ISH. After knockdown of EBF2 and EBF3, the amount of migrating hypaxial muscle anlagen became smaller, and the anlagen migrated a shorter distance (black arrowheads) than on the injected side. The chevron shape of somites was more irregular (black arrows) than the uninjected side, and *myod* expression levels in jaw muscle were downregulated (yellow arrows). These defects of muscle development were present following single knockdown of either EBF2 or EBF3, and were more severe after double knockdown of both. We also found similar muscle defects after injection of DN-EBF (data not shown). There were no visible defects after injection of control MO (Figure 4.6, A and B). Since *myod* is one of our candidate targets of EBF activity, we verified our findings by labeling embryos with an antibody against the differentiated skeletal muscle marker 12/101 (Kintner and Brockes, 1984) after knockdown of EBF2 and EBF3 (Figure 4.6, I-L). At stage 39/40, skeletal muscle tissue staining positively with 12/101 antibody can be seen in somites, jaw, and abdomen. When EBF2 MO and EBF3 MO were coinjected, the region of skeletal muscle tissue was reduced in jaw (yellow arrows) and abdomen (white arrowheads) compared to the uninjected side. In the somites, the segmentation between somites was not clear, and the chevron shape was abnormal (white arrows). Control MO does not affect muscle differentiation (Figure 4.6, I and K). These defects of muscle development after knockdown of EBF2 and EBF3 give us good evidence that EBF proteins are required for normal *Xenopus* skeletal muscle development.

Figure 4.6: Defective skeletal muscle development after knockdown of EBF2 and EBF3

Two vegetal cells of eight-cell stage embryos were injected with control MO or EBF2 MO (2 MO) and EBF3 MO (3 MO), either alone or together. β-gal mRNA was coinjected as a marker of the injected side. At stage 39/40, *myod* expression was examined (A-H), and 12/101 antibody was used as a marker of skeletal muscle tissue (I-L). The left column (panels A, C, E, G, I, and K) shows the uninjected control side of the embryos, and the right column (panels B, D, F, H, J, and L) shows the injected side. All panels show lateral views. After injection of 2 MO or 3 MO, *myod* expression patterns show that the chevron shape of somites is abnormal (black arrows), the amount of hypaxial muscle anlagen is smaller, and the migration distance is reduced (black arrowheads) compared to the uninjected side. The expression of *myod* in jaw muscle is also reduced (yellow arrows). When 2 MO and 3 MO were coinjected (H), these defects were more severe than 2 MO or 3 MO alone (D and F). Control MO does not affect these phenotypes (B). 12/101 antibody staining shows that when 2 MO and 3 MO were coinjected, somite segmentation is not complete, and the chevron shape of somites is abnormal (white arrows). Also jaw muscle differentiation is reduced (yellow arrow) and abdominal hypaxial muscle differentiation is strongly reduced (white arrowheads), while control MO shows a mild defect of only hypaxial muscle differentiation (J). To visualize the injected side after immunostaining, beta-galactosidase antibody (not shown) was coimmunostained with 12/101 antibody.


Uninjected side
Injected side
control MO
2 MO
3 MO
2 MO + 3 MO
control MO
2 MO + 3 MO
A
B
C
D
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I
J
K
L

MYOD can upregulate the expression of *ebf2* and *ebf3* *in vivo* in a positive feedback loop

In *Drosophila*, the MYOD ortholog Nautilus can drive expression of the *ebf* ortholog *collier* (Dubois et al., 2007). We therefore asked whether MYOD can also regulate *ebf* gene transcription in *Xenopus*. MYOD-hGR (Kolm and Sive, 1995) mRNA was injected into one cell of two-cell stage embryos, followed by treatment of embryos with DEX from the gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). The expression levels of *ebf2* and *ebf3* were then examined by WM-ISH. The expression levels of both *ebf2* and *ebf3* were upregulated by activated MYOD (31/31 for *ebf2* and 19/19 for *ebf3*, Figure 4.7). Combined with our result that EBF activity drives *myod* expression, this suggests that MYOD and EBF may have a reciprocal transcriptional interaction in vertebrates.

Discussion

While many functions of EBF proteins are proposed for regulation of neural and B cell development, nothing has been reported about their role in muscle development in vertebrates. Our finding that the *ebf2* and *ebf3* genes are expressed in developing muscle tissue in *Xenopus*, and that EBF activity is required for the normal development of muscle tissue reveals an unexpected role for EBF factors in vertebrate muscle development. We have identified multiple genes, with known function in muscle tissue, to be downstream of EBF transcriptional activity. These genes represent potential routes whereby EBF activity can help regulate commitment, differentiation, and migration of muscle cells.

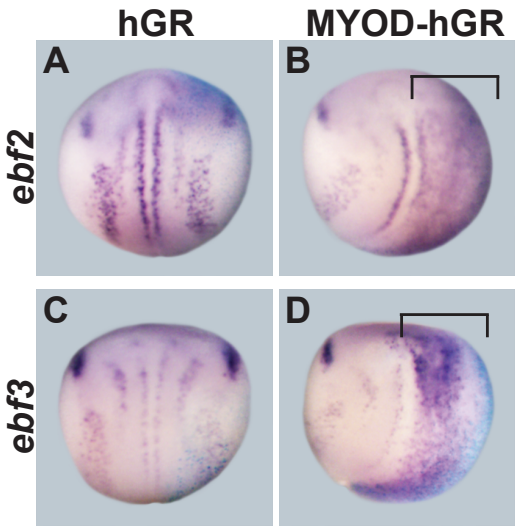

Figure 4.7: MYOD drives expression of *ebf2* and *ebf3*. MYOD-hGR mRNA or control, hGR mRNA was injected into one cell of two-cell stage embryos, followed by DEX treatment from the late gastrula stage (stage 11/11.5) to the neurula stage (stage 14/15). β-gal mRNA was coinjected as a marker of the injected side. In all panels the right side is the injected side, showing the light blue color of X-gal staining. The expression of *ebf2* (B) and *ebf3* (D) is strongly upregulated by activated MYOD-hGR (brackets), while control, hGR injection does not change the expression level of *ebf2* (A) or *ebf3* (C). All panels show dorsal views.

Ebf functions in muscle cell determination to drive *myod* and *myf5* expression

Our discovery that MYOD and MYF5 are potential transcriptional targets of EBF3 demonstrates a potentially important role for EBF proteins in muscle cell determination. Myogenic regulatory factors (MRFs) including MYOD, MYF5, Myogenin and MRF4 are bHLH transcription factors and form heterodimers with other bHLH proteins, such as the ubiquitously expressed E proteins. These heterodimers are critical for driving transcription of muscle-related genes (Biressi et al., 2007; Buckingham, 2001; Chanoine and Hardy, 2003; Pownall et al., 2002; Shih et al., 2008). In particular, *MyoD*/*Myf5* double knockout mice display a complete absence of muscle cells (Rudnicki et al., 1993).

In this study we show that in *Xenopus myod* and *myf5* are candidate targets of EBF proteins, and that *ebf2* and *ebf3* can in turn be regulated by MYOD. MYOD is expressed in early presomitic mesoderm. *ebf2* and *ebf3* are detected by WM-ISH in pre-somitic mesoderm as well, but not at the early stages when *myod* is present. This suggests that EBF proteins are likely involved in maintaining and reinforcing the expression of *myod* rather than initiation of *myod* expression. Maintenance of *myod* expression by EBF proteins appears to be important in *Xenopus*, since we find that knockdown of EBF activity strongly reduces the expression of *myod*, and disrupts normal skeletal muscle development, including that of hypaxial muscle and jaw muscle. Our study therefore suggests that EBF proteins are involved in *Xenopus* myogenic determination by maintaining and reinforcing the expression of *myod* and *myf5*. It is known that MYOD also can drive its own expression (Thayer et al., 1989; Weintraub et al., 1989), but in *Xenopus* perhaps this auto-regulation of MYOD is not fast enough, or strong enough, on

its own, to give proper myogenic specification.

Our finding, conversely, that MyoD drives expression of *ebf* genes is analogous to the finding in *Drosophila* that the MyoD ortholog Nautilus drives *collier* expression (Dubois et al., 2007). However, in two types of microarray screens for MyoD targets in mouse cultured cell lines, *Ebf* was found to either be a weak target of MyoD, or to even be downregulated (Bergstrom et al., 2002). Also, *Ebf* genes were not found to be a MyoD target by ChIP analysis in a mouse cultured cell line (Cao et al., 2006). There is no *Ebf1/Ebf2/Ebf3* triple knockout mouse though, and the *Ebf2* knockout mouse shows reduced sizes of skeletal muscle (Giacomo Consalez, unpublished observation), so the full contribution of EBF activity to mouse muscle development remains unknown. The fact that our findings are somewhat at odds with the microarray and ChIP experiments could be due to the species difference or differences in timing from our experiment.

EBF functions in muscle cell differentiation to drive *m-cadherin* and *seb-4* expression

The known functions of the candidate targets M-cadherin and SEB-4 suggest that EBF proteins may control aspects of myoblast migration and differentiation. The cadherin family of transmembrane proteins is generally involved in adhesion between cells, and is therefore important in a wide array of developmental processes. M-cadherin is present in developing and adult skeletal muscle, and at the adult neuromuscular junction. During development, it is known to be involved in the differentiation of skeletal muscle, with special importance for myoblast fusion (Charrasse et al., 2007; Cifuentes-Diaz et al., 1996; Donalies et al., 1991; Moore and Walsh, 1993; Pouliot et al., 1994; Zeschnigk et al., 1995). There are also reports of its involvement in muscle cell migration

in zebrafish somites (Cortes et al., 2003), and of its association with microtubules in a myoblast cell line (Kaufmann et al., 1999).

Since we find that *m-cadherin* is expressed in *Xenopus* from as early as the pre-somitic mesoderm period and continuing through the events of muscle differentiation, it is possible that it is involved in steps including somite formation, hypaxial muscle migration, and maintenance of proper cell relationships during late myoblast differentiation. The fact that EBF drives *m-cadherin* expression, and that expression of *ebf2* and *ebf3* overlaps with that of *m-cadherin* through most of muscle development, suggest a new avenue of importance for the EBF family of transcription factors in muscle development.

It has been shown that SEB-4 is necessary for myogenesis (Li et al., 2010; Miyamoto et al., 2009). SEB-4 is likely involved in regulation of cytoskeletal events in muscle development, since it is a *Xenopus* homolog of the *C. elegans* protein SUP-12, which regulates splicing of *unc-60* mRNA (Anyanful et al., 2004). UNC-60 is the ortholog of actin depolymerizing factor (ADF)/cofilin which controls actin filament dynamics (Bamburg, 1999; Bamburg et al., 1999; Maciver and Hussey, 2002). The *seb-4* gene is expressed in the presomitic mesoderm from gastrulation, and its expression is restricted to somites, jaw muscle and myocardium at the tailbud stage ((Fetka et al., 2000) and Figure 4.5). Since it is expressed at the somite stage, and since somite rotation in *Xenopus* involves actin rearrangement (Kragtorp and Miller, 2006), *seb-4* may be necessary for proper somite rotation. Our study shows that *seb-4* expression is heavily dependant on the presence of EBF activity.

We show that knockdown of EBF activity leads to delayed migration of hypaxial

muscle anlagen, defective somite organization, and reduced differentiation of skeletal muscle. It seems likely that EBF proteins could control these muscle cell migration and differentiation events in *Xenopus* by controlling the expression of *m-cadherin* and *seb-4*. In addition, MYOD and other MRFs can drive both *m-cadherin* expression and *seb-4* expression (Hsiao and Chen, 2010; Li et al., 2010). Since *myod* is downstream of EBF2 and EBF3, there may be multiple possible pathways for EBF proteins to drive *m-cadherin* and *seb-4* expression, including indirectly, through MYOD.

The transcriptional relationship between EBF proteins and bHLH proteins

We believe that our systematic study of transcriptional targets of EBF proteins, together with evidence from other reports and other species, is expanding the scope of evidence for reciprocal transcriptional relationships between EBF proteins and bHLH proteins involved in cell commitment and differentiation in multiple cell lineages. First, during neuronal development EBF proteins have been shown to act upstream of bHLH genes in multiple contexts. For example, EBF2 can drive expression of the proneural bHLH genes *ngnr-1* and *neurod* in *Xenopus* (Dubois et al., 1998; Pozzoli et al., 2001). Additionally, misexpressed mouse *Ebf1* drives expression of *ngn1* and *ngn2* in chick spinal cord (Garcia-Dominguez et al., 2003). We also recently found that the bHLH gene *nscl-1* is transcriptionally regulated by EBF activity (Green et al., in preparation). These findings show striking similarity to what we report here, namely that the bHLH genes *myod* and *myf5* are regulated by EBF2 and EBF3.

Conversely, there is also evidence that bHLH proteins can drive expression of *ebf* genes in multiple contexts. For example, in *Xenopus*, the bHLH transcription factors

NGNR-1, NeuroD and ATH5 can upregulate *ebf2* and *ebf3* (Dubois et al., 1998; Logan et al., 2005; Pozzoli et al., 2001; Seo et al., 2007), and Nautilus drives *collier* expression in Drosophila (Dubois et al., 2007). Also, misexpressed *ngn2* drives *ebf1* and *ebf3* expression in chick spinal cord (Garcia-Dominguez et al., 2003). In our current study we further show that MYOD can drive expression of *ebf2* and *ebf3* in *Xenopus* embryos.

These studies support the idea that EBF factors and bHLH proteins have reciprocal transcriptional relationships in multiple lineages. Because EBF proteins and bHLH proteins appear to control the expression of each other in positive feedback loops in both neuron and muscle tissues, and possibly in multiple species, we suggest that there may be an ancient transcriptional relationship between these two gene families. Evidence also exists of reciprocal relationships between EBF and bHLH proteins in B cell development (Greenbaum and Zhuang, 2002; Kee and Murre, 1998; Kwon et al., 2008; Seet et al., 2004; Smith et al., 2002; Zhuang et al., 2004). All of these relationships appear to be primarily centered on stabilizing commitment of cells to a particular lineage. Interestingly, the potential spectrum of activities in muscle tissue suggested by our experiments, including stabilizing commitment, directing migration, and directing cytoskeletal organization, is very analogous to the range of activities driven by EBF proteins in neural development.

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CHAPTER 5

CONCLUSION

In the production of multiple cell lineages, complex networks of transcription factors drive the developmental processes of cell commitment and differentiation. Our understanding of these networks is limited by a lack of knowledge of the range of transcriptional targets of the different families of transcription factors. EBF proteins are involved in the processes of cell commitment and differentiation in several lineages, and while there are some known targets of EBF transcriptional activity, there has previously been no systematic approach to identify their targets *in vivo*. We have screened for targets of EBF proteins by using microarray analysis and verified some of the candidates to be *in vivo* targets important for nervous system development in *Xenopus* embryos (Chapter 2). We have discovered that the protein ZFP423 potentiates Notch signaling, and that EBF proteins can reduce Notch signaling by blocking the function of ZFP423 (Chapter 3). We have also shown that EBF proteins are necessary for normal *Xenopus* muscle development and control the expression of several muscle specific genes (Chapter 4). Taken together, these experiments expand our understanding of how EBF proteins function in the complex regulatory networks of the development of multiple animal cell lineages.

EBF functions during neuronal cell commitment and differentiation

Our results indicate that EBF proteins have at least two types of functions in neuronal development. Following the sequence of developmental events, the first relates to EBF protein interactions with ZFP423 (Chapter 3), and the second relates to EBF transcriptional targets (Chapter 2).

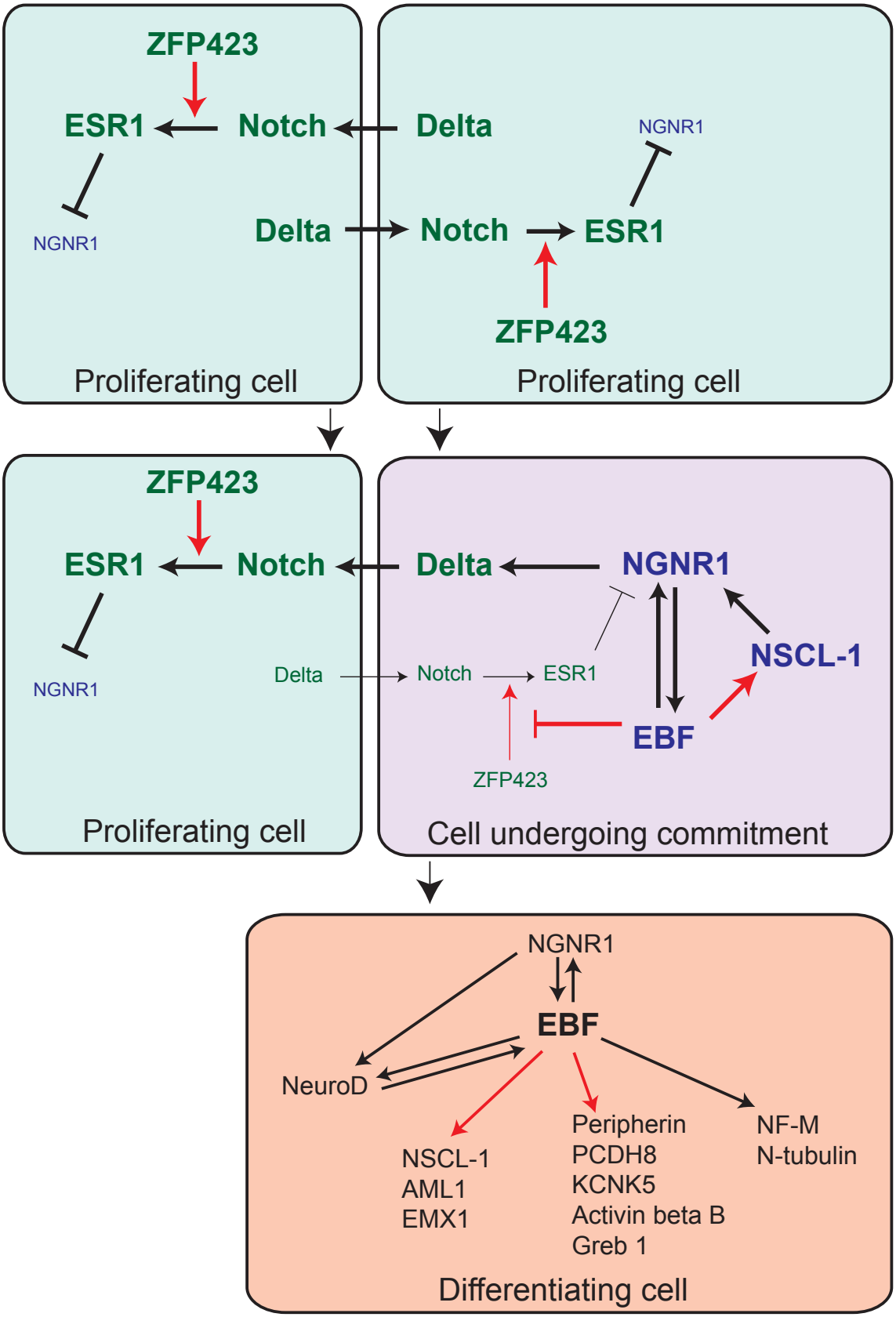
EBF can participate in neuronal cell commitment by inhibiting Notch signaling

The Notch signaling pathway is important for keeping neuronal progenitors in the cell cycle to provide a sufficient number of cells during development. With our collaborators, we show in Chapter 3 that ZFP423, which is a protein known to interact with EBF (Hata et al., 2000; Tsai and Reed, 1997, 1998), can induce the expression of *hes5/esr1 in vitro* and in *Xenopus* embryos. HES5/ESR1 is a well-known Notch target that blocks the activity of proneural bHLH proteins and prevents neuronal progenitors from progressing toward commitment and differentiation (reviewed in Artavanis-Tsakonas et al., 1999; Kageyama et al., 2009). However, the other Notch targets *hairy1* and *nrarp* are not upregulated by overexpressed ZFP423. The lack of effect on *nrarp* expression in particular is interesting, since *nrarp* is thought to function as a negative-feedback regulator of Notch signaling, and is therefore acting in an opposite direction from canonical Notch targets (Lamar et al., 2001). These results suggest that ZFP423 strengthens Notch signaling and helps prevent neuronal cell commitment (Figure 5.1).

Bringing an end to the activity of ZFP423 and the Notch signaling pathway in a cell are important steps for initiating neuronal cell commitment. It is known that ZFP423 can associate with EBF proteins, and block their ability to function as transcriptional activators of several genes involved in neuronal differentiation (Tsai and Reed, 1997, 1998). Our finding that coinjection of *zfp423* and *ebf2* or *ebf3* diminishes the upregulation of *esr1* by ZFP423 suggests that EBF proteins function as inhibitors of ZFP423, and block the ability of ZFP423 to potentiate Notch signaling. Interestingly, EBF2 has been shown to upregulate the expression of the Notch ligand *delta-1* in *Xenopus* embryos (Dubois et al., 1998). This previous study and our findings suggest that

Figure 5.1: Model of EBF functions during neuronal cell commitment and differentiation

(A) During proliferation of progenitors, ZFP423 helps maintain the proliferating state by potentiating the ability of Notch to drive expression of *esr1*. (B) *ngnr-1* is an important transcription factor in cells that have begun to commit to a neuronal fate, and NGNR-1 drives the expression of both *delta1* and *ebf* genes. EBF proteins may participate in strengthening neuronal cell commitment in two ways. First, they reinforce expression of ngnr-1, either directly or through NSCL-1. Second, they block the function of ZFP423, and thereby reduce the proliferative effects of Notch signaling. (C) EBF induces the expression of many genes involved in neuronal cell differentiation, directly or indirectly, as part of a complex transcriptional regulatory network. Black arrows indicate previously reported pathways, and red arrows indicate novel pathways described in this thesis.


ZFP423
ESR1
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Notch
ESR1
NSCL-1
EBF
ZFP423
Proliferating cell
Cell undergoing commitment
NGNR1
EBF
NeuroD
NSCL-1
AML1
EMX1
Peripherin
PCDH8
KCNK5
Activin beta B
Greb 1
NF-M
N-tubulin
Differentiating cell

EBF proteins promote neuronal cell commitment cell-intrinsically by suppressing Notch signaling, and promote lateral inhibition cell-extrinsically by increasing the expression of the Notch ligand Delta (Figure 5.1).

EBF functions during neuronal cell commitment and differentiation by controlling the expression of diverse targets

EBF proteins have known roles in promoting neuronal cell commitment and differentiation. For differentiation, EBF proteins are known to be involved in cell type specification, neurite growth and neuronal cell migration. With our microarray screen, we have generated an extensive list of candidate targets of EBF proteins. We selected several genes that either have known functions for neuronal development or the potential to be involved in neuronal development, and verified that their expression depends on EBF activity *in vivo* (Chapter 2). These targets include transcription factors that can participate in neuronal cell commitment and differentiation, and non-transcription factors that can participate in neuronal cell differentiation (Figure 5.1). The functions of these targets help us to expand the understanding of EBF activity during neuronal development (Figure 5.2).

EBF functions during neuronal cell commitment by driving expression of other transcription factors. During neuronal cell commitment, previous reports have shown that EBF proteins function to stabilize cell commitment rather than initiate commitment. Another interpretation of this is that EBF proteins likely participate in the irreversible determination stage of commitment, but not the reversible specification stage of commitment. EBF proteins stabilize commitment by driving the expression of the proneural bHLH transcription factor *ngnr-1*, which is a critical intrinsic signal that

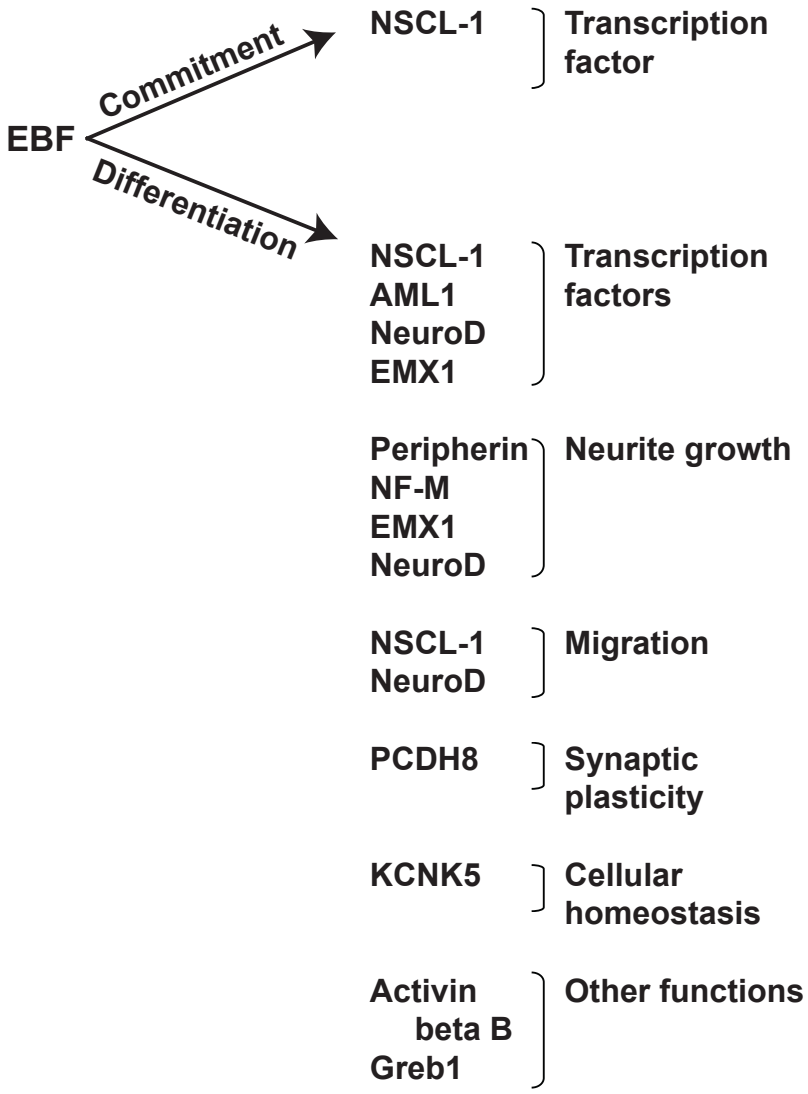

Figure 5.2. Functions of the neuronal targets of EBF. EBF proteins may participate in neuronal cell commitment through NSCL-1, and may participate in neuronal cell differentiation through a variety of classes of target genes.

promotes neuronal cell commitment (Dubois et al., 1998; Garcia-Dominguez et al., 2003; Ma et al., 1996).

Among our newly identified targets of EBF proteins, the presence of the bHLH transcription factor NSCL-1 strengthens the role of EBF in neuronal cell commitment. NSCL-1 is known to be involved in neuronal cell differentiation. But in *Xenopus* embryos, NSCL-1 can drive expression of the proneural bHLH transcription factor NGNR-1 (Bao et al., 2000), which suggests that NSCL-1 is also an important transcription factor for neuronal cell commitment. The fact that EBF activity drives expression of *nscl-1* provides more information about the route by which EBF proteins affect neuronal cell commitment. This does not alter the interpretation that EBF proteins are participating in the stabilization of commitment, since *ebf* genes are expressed after *ngnr-1* expression begins. Overall, our results suggest two new potential routes for EBF proteins to drive neuronal cell commitment: first, through inhibition of ZFP423 activity, and second, by driving expression of *nscl-1*.

EBF functions during neuronal cell differentiation by driving expression of other transcription factors. *ebf* genes are expressed broadly in the CNS and PNS in many different species. Gain and loss of function experiments show that EBF proteins are important for many aspects of neuronal cell differentiation, and some known targets of EBF activity are transcription factors while others are not. For example, when overexpressed in *Xenopus* embryos, EBF2 and EBF3 lead to ectopic expression of neuronal specific markers like N-tubulin and NF-M (Dubois et al., 1998; Pozzoli et al., 2001).

In this study, five of the ten targets of EBF3 that we analyzed in detail are indirect targets, supporting the conclusion that driving transcription factor targets is an important part of EBF function. NSCL-1, NeuroD, AML-1 and EMX1 are all transcription factors that are targets of EBF activity. They are known to be involved in neuronal differentiation in many different neurons in several species and are strongly upregulated by EBF. For example, NSCL-1 can affect differentiation of GnRH neurons in mouse and cooperates with ATH5 to promote retinal ganglion cell differentiation (Kruger et al., 2004; Schmid et al., 2007; Xie et al., 2004), while AML1 promotes differentiation of several types of sensory and motor neurons, including DRG neurons (Chen et al., 2006; Kramer et al., 2006; Theriault et al., 2004; Yoshikawa et al., 2007). We therefore conclude that these transcription factor targets are a key element of the role of EBF proteins in promoting neuronal differentiation.

EBF induces the expression of genes involved in neurite growth. Thalamocortical axons in Ebf1 null mice, olfactory axons in Ebf2 and Ebf3 null mice, and axons of motor neurons in UNC-3 *C. elegans* mutants show pathfinding defects (Garel et al., 1999; Prasad et al., 1998; Wang et al., 2004). In *Drosophila collier* mutants, type IV neurons show problems with dendritic arborization (Crozatier and Vincent, 2008; Hattori et al., 2007; Jinushi-Nakao et al., 2007). These results suggest that EBF proteins have critical roles for neurite growth. Among the targets we verified, Peripherin, NF-M, EMX1 and NeuroD have known functions for neurite growth. In particular, Peripherin and NF-M are neuronal intermediate filament proteins found in the cell body and neurites (Belecky-Adams et al., 2003; Fiumelli et al., 2008; Garcia et al., 2003; Gervasi et al., 2000; Helfand et al., 2003; Lin and Szaro, 1995; Smith et al., 2006). *Emx1* and *Emx2*

double mutant mice show defects of thalamocortical pathfinding (Bishop et al., 2003), similar to phenotypes of *Ebf1* knockout mice (Garel et al., 1999). There is also evidence that NeuroD participates in neurite outgrowth, as it has been shown to promote neurite growth in a neuroblastoma cell line and in cerebellar slice cultures (Cho et al., 2001; Gaudilliere et al., 2004). Our finding that Peripherin, NF-M, EMX1 and NeuroD are candidate targets of EBF activity suggest multiple possible mechanisms by which EBF may help regulate neurite growth.

EBF drives the expression of genes involved in cell migration. Evidence that EBF proteins are required for some neuronal cell migration comes from experiments showing that fbm neurons migrate to the wrong location in *Ebf1* null mice (Garel et al., 2000) and that the migration of GnRH neurons and Purkinje neurons is delayed in *Ebf2* null mice (Corradi et al., 2003; Croci et al., 2006). Interestingly, *Nscl*-1 and *Nscl*-2 double knockout mice have a phenotype of delayed migration of GnRH neurons similar to that found in *Ebf2* null mice (Kruger et al., 2004). Migration of dentate precursor cells and newly born granule cells from the neuroepithelium to the dentate gyrus is defective in *NeuroD* knockout mice, and misexpressed *NeuroD* leads to faster neuronal cell migration in mouse cortical stem cells (Ge et al., 2006; Liu et al., 2000). These results suggest that EBF proteins may participate in neuronal cell migration by controlling the expression of NSCL-1 and NeuroD.

Possible new functions for EBF proteins in neuronal differentiation. Several newly discovered EBF targets suggest that EBF may be involved in previously unknown functions. EBF may participate in regulation of synaptic plasticity through PCDH8, since the rat homolog Arcadlin affects the number of dendritic spines in cultured hippocampal

neurons (Yasuda et al., 2007) and is required for activity-induced long term potentiation (Yamagata et al., 1999). EBF also may participate in cellular homeostasis of mature neurons through KCNK5, which control several aspects of homeostasis in kidney cells (Barriere et al., 2003; Reyes et al., 1998; Warth et al., 2004). In the mouse brainstem, KCNK5 appears to be involved in maintaining the membrane potential of chemoreceptor cells (Gestreau et al., 2010). The newly discovered EBF targets Activin beta B and GREB1 do not yet have defined functions in the nervous system, but they are expressed in neuronal tissue, suggesting the possibility of more potential roles of EBF genes in neurons (our data, and Belecky-Adams et al., 1999; Dohrmann et al., 1993; Roberts et al., 1996).

EBF functions during *Xenopus* muscle development

While many functions of EBF proteins have been proposed for the regulation of neuronal and B cell development, nothing has been reported about their role in promoting muscle development in vertebrates. Our finding that the *ebf2* and *ebf3* genes are expressed in developing muscle tissue in *Xenopus*, and that EBF activity is required for the normal development of *Xenopus* muscle tissue, is the first to show EBF family involvement in vertebrate muscle development. We have identified multiple genes with known function in muscle tissue to be downstream of EBF transcriptional activity (Chapter 4). These genes represent potential routes whereby EBF activity may help regulate commitment, differentiation, and migration of muscle cells (Figure 5.3).

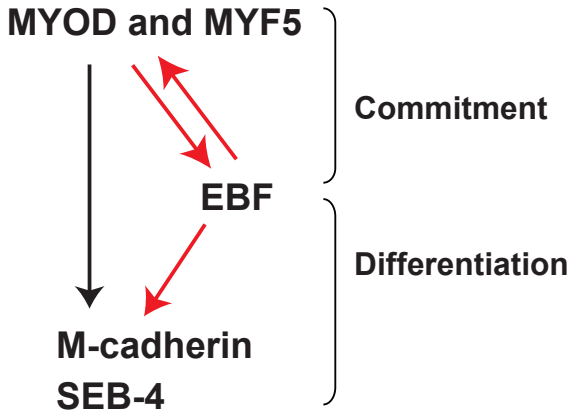

Figure 5.3: EBF functions during Xenopus muscle cell commitment and differentiation in a transcriptional regulatory network. EBF proteins may participate in muscle cell commitment and differentiation in a manner similar to the way EBF proteins participate in neuronal cell development. EBF proteins are downstream of the MRF MYOD, but can also reinforce the expression of *myod* and *myf5*. This positive feedback loop may help to stabilize muscle cell commitment. EBF proteins also can induce the expression of *m-cadherin* and *seb-4,* which will likely help the committed muscle cell to differentiate. Black arrows indicate previously reported pathways, and red arrows indicate novel pathways described in this thesis.

Ebf functions in muscle cell commitment by reinforcing the expression of *myod* and *myf5*

Our discovery that MYOD and MYF5 are candidate transcriptional targets of EBF3 demonstrates a potentially important role for EBF proteins in muscle cell commitment. The muscle cell determinants MYOD and MYF5 are myogenic regulatory factors that lead muscle progenitors to commit to become myoblasts (Biressi et al., 2007; Buckingham, 2001; Chanoine and Hardy, 2003; Pownall et al., 2002; Shih et al., 2008). Their importance is demonstrated by the fact that *MyoD*, *Myf5* double knockout mice show a complete absence of muscle cells (Rudnicki et al., 1993).

In this study we show that *myod* and *myf5* are transcriptional targets of EBF proteins and that *ebf2* and *ebf3* are targets of MYOD. MYOD is expressed in presomitic mesoderm earlier than *ebf2* and *ebf3*. This suggests that EBF proteins are likely involved in maintaining and reinforcing the expression of *myod* rather than initiation of *myod* expression. Maintenance of *myod* expression by EBF proteins appears to be important in *Xenopus*, since we find that knockdown of EBF activity strongly reduces the expression of *myod*, and disrupts normal skeletal muscle development, including that of hypaxial muscle and jaw muscle. We therefore suggest that EBF proteins may be involved in *Xenopus* myogenic commitment by maintaining and reinforcing the expression of *myod* and *myf5*.

EBF functions in muscle cell differentiation by inducing the expression of *m-cadherin* and *seb-4*

During development, M-cadherin participates in muscle cell adhesion and migration (Charrasse et al., 2007; Cifuentes-Diaz et al., 1996; Cortes et al., 2003;

Donalies et al., 1991; Moore and Walsh, 1993; Pouliot et al., 1994; Zeschnigk et al., 1995). There is evidence that M-cadherin can associate with cytoskeletal structural proteins. During myoblast fusion M-cadherin controls the activity of the Rac1 GTPase (Charrasse et al., 2007), which controls actin polymerization. M-cadherin also can form a complex with the cytoplasmic proteins catenin and plakoglobin (Kuch et al., 1997), and this M-cadherin–catenin complex can associate with microtubules in a myoblast cell line (Kaufmann et al., 1999).

It has been shown that SEB-4 is necessary for myogenesis (Li et al., 2010; Miyamoto et al., 2009). SEB-4 is potentially involved in regulation of cytoskeletal events in muscle development, given that it is a *Xenopus* homolog of the *C. elegans* protein SUP-12 that regulates splicing of *unc-60* mRNA (Anyanful et al., 2004). UNC-60 is the ortholog of actin depolymerizing factor (ADF)/cofilin, which controls actin filament dynamics (Bamburg, 1999; Bamburg et al., 1999; Maciver and Hussey, 2002).

We show that knockdown of EBF activity leads to delayed migration of the hypaxial muscle anlagen, defective somite organization, and reduced differentiation of skeletal muscle. It seems likely that EBF proteins could control these muscle cell migration and differentiation events in *Xenopus* by regulating the expression of genes involved in forming cytoskeletal structure including *m-cadherin* and *seb-4*. In addition, MYOD and other MRFs can drive both *m-cadherin* expression and *seb-4* expression (Hsiao and Chen, 2010; Li et al., 2010). Because *myod* is downstream of EBF2 and EBF3, there are multiple possible pathways for EBF proteins to drive *m-cadherin* and *seb-4* expression, including indirectly through MYOD (Figure 5.3)

Overview of EBF functions in development of cells from multiple lineages

We have expanded our knowledge of how EBF proteins can promote neuronal cell commitment and differentiation, by controlling genes involved in these processes and by inhibiting Notch signaling through ZFP423. In addition, we have discovered that EBF proteins can participate in muscle cell commitment and differentiation by inducing the expression of muscle specific genes involved in these processes.

The involvement of EBF proteins in muscle, neuron, B-cell, and adipocyte lineages raises the question of how they perform different tasks in these different tissues. There is evidence that the identity of a cell is determined by a combinatorial code of transcription factors that cooperate to drive expression of the correct subset of genes (reviewed in Barrera and Ren, 2006; Tumpel et al., 2009). It is easy to see how transcription factors with expression in only a single tissue could contribute to such a combinatorial code. EBF proteins, though, number only a few family members, and these are expressed in a variety of different tissues. It is less clear how they could drive expression of very different genes in these different tissues to contribute to such a code. A possible explanation is that earlier, tissue specific factors drive the processes of chromatin remodeling necessary to allow EBF proteins access to the correct target genes for that tissue (for a review of the role of chromatin remodeling in cell differentiation, see Barrera and Ren, 2006; Hager et al., 2009; Istrail and Davidson, 2005). This is an example of epigenetic regulation of expression, given that the same enhancer elements could be present in the regulatory regions of a neuron-specific target gene and a muscle-specific target gene, but EBF proteins would only have access to the neuron-specific target gene in a neuroblast and the muscle-specific target gene in a myoblast. This

explanation is supported by the fact that in neuron, muscle, and B cell development, *ebf* gene expression appears later than expression of factors that specify tissue type.

Interestingly, in both neuron and muscle tissues, *ebf* genes are targets of bHLH transcription factors that drive commitment, including NGNR-1 and MYOD. Although the expression of *ebf* genes is later than that of the bHLH genes (Dubois et al., 1998, and Chapters 2 and 4), EBF can reinforce the expression of bHLH genes in positive feedback loops. These positive feedback loops between bHLH and EBF suggest that during both neuronal and muscle development, EBF proteins lead progenitors to stabilize their committed state. There are also positive feedback loops between bHLH and EBF proteins in B-cell development (Zhuang et al., 2004), and positive feedback loops between EBF proteins and transcription factors that drive differentiation of pre-adipocytes (Jimenez et al., 2007).

Taken together, the evidence points to a striking similarity of EBF protein functions in different tissue types. They function both up- and downstream of cell-type commitment genes in muscle, neuron, and B-cell commitment, and appear to be important for stabilizing commitment. Then, they function in muscle, neuron, B-cell, and adipocyte differentiation to drive expression of both transcription factors and non-transcription factors, and therefore act as part of the respective combinatorial codes of transcription factors for development of these different lineages.

Summary

Commitment and differentiation are critical processes during the development of an animal cell, and combinatorial networks of transcription factors are important for these

processes. This thesis shows that the EBF transcription factor proteins have potentially important roles for cell commitment and differentiation during the development of *Xenopus* muscle cells and expands our knowledge of how EBF proteins participate in neuronal cell development. In addition to driving transcription of key sets of target genes, EBF also functions in neuroblasts by preventing the activity of its protein partner ZFP423. Placing EBF activity in the regulatory networks of transcription involved in development opens new insight into how this family of transcription factors can function in different lineages during animal development.

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