Transcriptional control in the context of primary neurogenesis

in partial fulfillment of the requirements for the degree Doctor rerum naturalium (Dr. rer. nat.) in the Molecular Biology Program in the Faculty of Biology at the Georg-August University, Göttingen.

submitted by

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born in

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Affidavit

Herewith I declare, that I prepared the PhD thesis “Transcriptional control in the context of primary neurogenesis” on my own and with no other sources and aids than quoted.

07-31-2006 _____

Submission date Tiemo Klisch

List of Publications

Karabinos, A., Schulze, E., Klisch, T., Wang, J., and Weber, K. (2002). Expression profiles of the essential intermediate filament (IF) protein A2 and the IF protein C2 in the nematode Caenorhabditis elegans. *Mech Dev* 117, 311-4.

Klisch, T. J., Souopgui, J., Jürgens, K., Rust, B., Pieler, T., and Henningfeld, K. A. (2006). Mxi1 is essential for neurogenesis in Xenopus and acts by bridging the pan-neural and proneural genes. *Dev Biol* 292, 470-85.

Table of Contents

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Abstract

The Myc-Max-Mad network of bHLH-Zip transcription factors plays an essential role in a variety of cellular processes including proliferation, differentiation and apoptosis. Mxi1, a Mad family member, was previously isolated in our laboratory on the basis of its early expression pattern in the territories of primary neurogenesis. In the first part of this thesis work, a functional characterization of Xmxi during *Xenopus* embryogenesis was performed. Consistent with an early role in neurogenesis, Xmxi1 was found to be positively regulated by the panneural genes, and proneural genes, as well as negatively by the Notch pathway. Loss-of-function experiments demonstrated an essential role for Xmxi1 in the establishment of a mature neural state that can be activated by factors that induce neuronal differentiation, such as SoxD and X-ngnr-1. Overexpression of Xmxi1 resulted in a transient inhibition of neuronal differentiation, and at early tailbud stages both endogenous and ectopic neurogenesis were observed. While Xmxi1 enhances cell proliferation and apoptosis in the early *Xenopus* embryo, both activities appear not to be required for the function of Xmxi1 in primary neurogenesis.

During primary neurogenesis, cell to cell signaling mediated by the Notch pathway restricts the number of cells that undergo neuronal differentiation. In the second part of this thesis work, an unbiased screen to identify early target genes of the Notch effector Enhancer-of-split related 1 (ESR1) was performed. A library enriched in early ESR1 target genes was prepared by PCR subtractive amplification using *Xenopus* ectodermal explants and a hormone-inducible antimorphic form of ESR1 (ESR1-VP16-GR). Through microarray analysis, 2,304 clones from the library enriched in ESR1 target genes, together with an additional 25,138 cDNA clones from two unrelated libraries 9, were screened for regualtion by ESR1-VP16-GR. In total, 55 genes were identified of these, 12 are members of the Notch pathway. In whole embryos, 43 of the 55 genes were strongly induced by ESR1-VP16-GR.

List of Figures

List of Tables

1 Introduction

In anamniotes, such as fish and amphibians, neurogenesis occurs in two waves. The first wave is termed primary neurogenesis and generates an early nervous system required for the movements and responses of the larvae (Hartenstein, 1989). The primary neurons are born in the open neural plate shortly after gastrulation in three bilateral longitudinal domains and can be visualized by the expression of neuron-specific type II ß-tubulin (N-tubulin) (Figure 1A) (Hartenstein, 1989; Oschwald, 1991; Moody et al., 1996). *Xenopus* is an attractive model system to study primary neurogenesis due to the early expression, simplicity and accessibility of the primary neurons (Wullimann et al., 2005; Hartenstein, 1989).

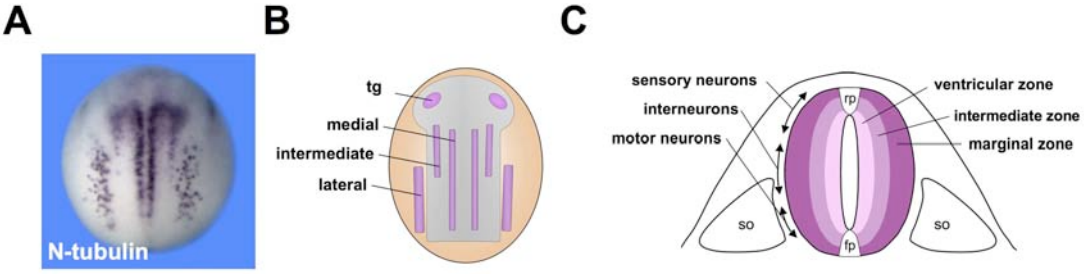

Figure 1 Proneural domains in *Xenopus* development. (**A**) Whole-mount in situ hybridization for N-tubulin at stage 15. Dorsal view, anterior up. (**B**) Schematic representation of the domains of primary neurogenesis in the open neural plate (grey). The characteristic pattern of primary neurogenesis is depicted as three longitudinal domains (purple) in the posterior neuroectoderm on both sides of the midline, as well as the trigeminal (tg) placodal areas in the anterior neural plate. (**C**) Cross-section of the posterior neural tube. Sensory neurons are derived from progenitors of the lateral stripes, interneurons from intermediate stripes and motor neurons from the medial stripes. The proliferating progenitors are located in the inner ventricular zone. As they become post-mitotic and initiate differentiation, they migrate into the intermediate zone. Terminally differentiated neurons are located in the outer marginal zone. tg, trigeminal placodes; rf, roof plate; fp, floor plate, so, somite.

After the neural folds rise to form the neural tube, the lateral, intermediate and medial stripes will give rise to sensory neurons, interneurons and motor neurons, respectively (Figure 1B) (Chitnis et al., 1995; Bally-Cuif and Hammerschmidt, 2003). The dividing neural progenitors are located in the inner ventricular zone of the neural tube. As these cells become post-mitotic and initiate differentiation, they migrate into the intermediate zone and ultimately to the outer marginal zone, where terminally differentiated neurons are located (Figure 1C) (Bellefroid et al., 1996). Secondary neurogenesis starts prior to metamorphosis at approximately stage 46, replacing the majority of the primary neurons with neurons required for the adult frog. While the latter phase of neurogenesis closer resembles that of amniotes, it is

thought to mimic the molecular events of primary neurogenesis, as most of the differentiation factors are present during both processes (Wullimann et al., 2005).

1.1 Neural Induction

The first step in the establishment of the vertebrate nervous system is the decision of the ectodermal cells to adopt a neural at the expense of an epidermal fate (De Robertis and Kuroda, 2004). One of the main driving forces of a neural fate is the absence of Bone Morphogenetic Protein (BMP) signaling, which is achieved through multiple mechanisms (Figure 2). During gastrulation, BMP activity is inhibited extracellularly by factors emanating from the dorsal-most region of the mesoderm, termed Spemann's Organizer. These secreted factors, such as Noggin, Chordin and Follistatin, associate with BMPs and inhibit binding to their cognate receptors, thereby lowering BMP activity (Wilson and Endlund, 2001; Smith et al., 1992; Sasai et al., 1994; Hemmati-Brivanlou et al., 1994). More recently it has been demonstrated that a blastula Chordin- and Noggin-expressing (BCNE) center is located in the dorsal animal cap of pre-gastrula embryos, which predisposes the prospective neuroectoderm to neural induction (Kuroda et al., 2004). Fibroblast growth factor (FGF) and also insulin growth factor (IGF) signaling further inhibit the BMP pathway by downregulating Smad1 activity, an intracellular transducer of BMP signaling (Pera et al., 2003; Richard-Parpaillon et al., 2002; Sasai et al., 1996; Launay et al., 1996). In addition, canonical Wnt signaling was shown to inhibit BMP at the transcriptional level in early cleavage stages (Baker et al., 1999).

However, BMP inhibition is not sufficient for neural induction in vertebrates (Launay et al., 1996; Sasai et al., 1996). In chick (Wilson et al., 2000), zebrafish (Kudoh et al., 2004) and more recently in *Xenopus,* it has been demonstrated that low levels of FGF signaling are required for the development of neural fates, independent of the role of FGF as an inhibitor of BMP signaling (Delaune et al., 2005). Active Wnt signaling may play a role in restricting the size of the neuroectoderm in *Xenopus* (Heeg-Truesdell et al., 2006).

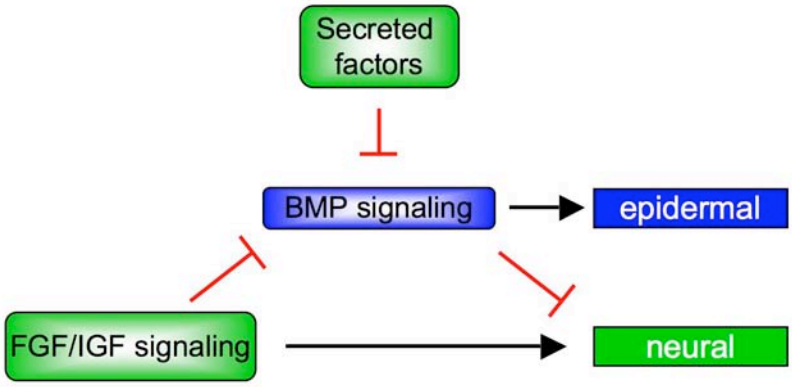

Figure 2 Neural induction in *Xenopus*. Scheme of the signaling events during neural induction. The attenuation of BMP activity establishes the neuroectoderm. The uncommitted ectoderm is characterized by high levels of BMP proteins. BMP signaling is inhibited intracellularly by FGF/IGF signaling and extracellularly by secreted factors from Spemann's Organizer during gastrulation. In addition, FGF signaling is required for neural induction independent of its role as an inhibitor of BMP signaling.

1.2 Panneural and prepattern genes

Several genes are induced within the neural plate by the events of neural induction. One of the earliest genes induced encodes for the high mobility group (HMG) box transcription factor, Sox3 (Penzel et al., 1997). While the medial and intermediate stripes of primary neurons are located within the Sox3 expression domain, the lateral stripe lies outside, suggesting the requirement of intermediate levels of BMP activity for their formation (Hardcastle and Papalopulu, 2000).

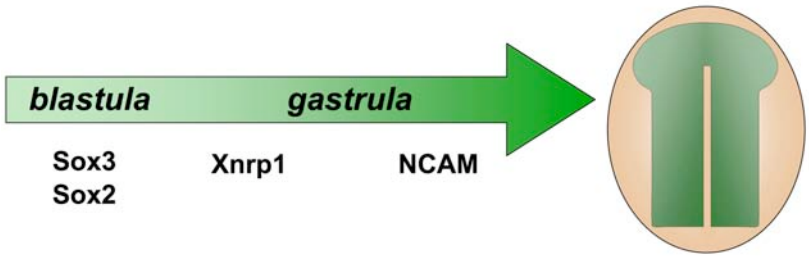

Figure 3 Schematic overview of neural plate maturation. The earliest panneural markers expressed in the prospective neuroectoderm are Sox3 and Sox2. By early gastrula stage Xnrp1 is expressed. By the end of gastrula stage the matured neural plate is demarcated by NCAM expression.

During maturation of the neuroectoderm, additional panneural markers, such as Sox2, nervous system-specific RNP protein-1 (Xnrp1) and neural cell adhesion molecule (NCAM) are expressed (Figure 3) (Mizuseki et al., 1998; Richter et al., 1990; Kintner and Melton 1987). SoxD, a more distantly related Sox family member, is also expressed in the open neural plate. In contrast to Sox2 and Sox3, SoxD is able to induce the neuronal determination factor

X-ngnr-1 at open neural plate stages (Mizuseki et al., 1998b). The combination of positively acting prepattern transcription factors, like Zic1, Zic3 and negatively acting ones, such as Zic2 and Xiro family members, contributes to the restriction of the proneural cascade to the domains of primary neurogenesis (Aruga, 2004; Moody and Je, 2002; Franco et al., 1999).

1.3 Proneural genes

Genetic and biochemical studies have revealed a remarkable conservation of principles and molecular mechanisms underlying neurogenesis in vertebrates and invertebrates (Bally-Cuif and Hammerschmidt, 2003). *Drosophila* achaete-scute complex and atonal are essential for the development of the fly nervous system and have been termed proneural genes and belong to the basic helix-loop-helix (bHLH) family of transcription factors. They heterodimerize with ubiquitously expressed bHLH proteins, called E-proteins, bind to E-box sequences (CACGTG) within the promoters of target genes and regulate their transcription (Jones, 2006). During vertebrate neurogenesis, several orthologs of these transcription factors have also been cloned and shown to be involved in neurogenesis (Jones, 2006; Lee et al., 1997; Ghysen and Dambly-Chaudiere, 1998).

One of the first genes that is exclusively expressed within the domains of primary neurogenesis in *Xenopus* is the bHLH transcription factor Neurogenin related 1 (X-ngnr-1), a *Drosophila* atonal ortholog (Figure 4). X-ngnr-1 expression prefigures the domains of primary neurogenesis during early gastrulation. Moreover, all known proneural genes identified in *Xenopus* can be induced by X-ngnr-1 overexpression, most in the neural ectoderm as well as in non-neural ectoderm (Ma et al., 1996; Bellefroid et al., 1996). Downstream differentiation genes include the zinc finger HLH protein XCoe2, a direct target of X-ngnr-1 (Dubois et al., 1998), the bHLH protein NeuroD (Lee et al., 1995) and the later acting zinc finger HLH transcription factor Xebf3 (Figure 4) (Pozzoli et al., 2001). Non-HLH transcription factors are also expressed within the territories of primary neurogenesis and have been shown to be essential for primary neurogenesis, including the zinc finger

proteins Neuronal Kruppel Like (NKL) and Myelin transcription factor 1 (X-MyT1) (Lamar et al., 2001; Bellefroid et al., 1996). These factors may operate in parallel, but can also act synergistically. For example, the bHLH protein XASH-3 synergizes with X-MyT1 to robustly induce N-tubulin (Figure 4) (Zimmerman et al., 1993; Bellefroid et al., 1996). In addition to proneural transcription factors, other proteins are required for primary neurogenesis including the RNA binding protein XSeb4R and the chromatin remodeling protein Brg1 (Boy et al., 2004; Seo et al., 2005). X-ngnr-1 not only induces downstream proneural transcription factors essential for neuronal differentiation, but also activates the Notch pathway, which inhibits neuronal differentiation

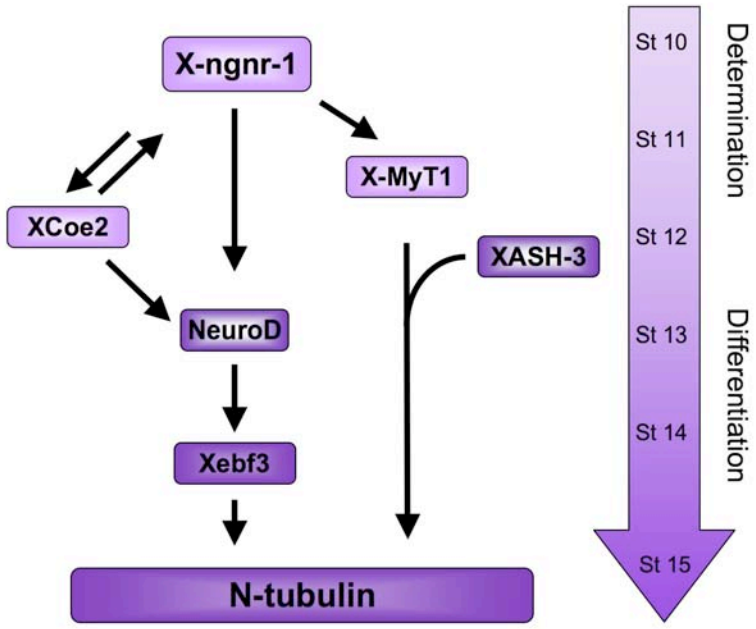

Figure 4 The proneural gene network. Scheme of the molecular events, which govern primary neurogenesis in *Xenopus*. X-ngnr-1 is the earliest gene expressed within the proneural domains and can activate other bHLH genes, like XCoe2, NeuroD, XASH-3, Xebf3 as well as non-bHLH transcription factors, such as X-MyT1. The approximate initiation of expression of each factor is roughly indicated by its position in the cascade. Ongoing differentiation is visualized by a purple gradient. The cascade ultimately leads to the activation of the post-mitotic neuronal expression marker N-tubulin.

1.4 Neurogenic genes

Cell-to-cell signaling mediated by the single-pass transmembrane receptor Notch is fundamental to a variety of developmental processes including segmentation, myogenesis, and neurogenesis (Louvi and Artavanis-Tsakonas, 2006). Notch signaling is frequently used to single out one cell in a cluster of initially equal cells in a process termed lateral inhibition (Figure 5A)

(Lai, 2004). In *Drosophila,* one Notch receptor is present that can be activated by two ligands, Delta and Serrate (Radtke et al., 2005). In mammalians, 5 different ligands, Jagged1 and Jagged2 (orthologs of Serrate), Delta-like 1, Delta-like 3, and Delta-like 4 (orthologs of Delta), as well as 4 Notch receptors (Notch 1 to 4) have been identified (Beatus and Lendahl, 1998). In *Xenopus,* 4 ligands (X-Delta-1 and 2, Serrate 1 and 2) and one Notch receptor have been described (Chitnis et al., 1995; Jen et al., 1997; Kiyota et al., 2001; Coffman et al., 1990).

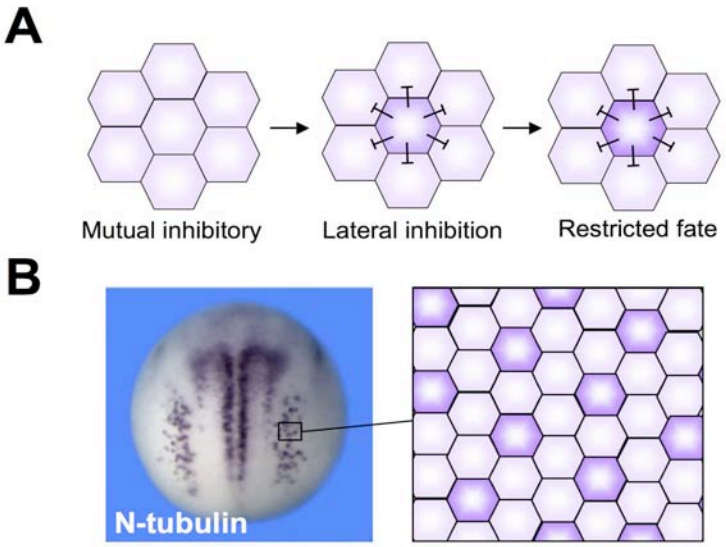

Figure 5 Lateral inhibition. (**A**) Schematic overview of negative feedback by Notch signaling. In a cell cluster, all cells initially share a specific cell fate potential. All send and receive Notch signals, known as mutual inhibitory Notch signaling. Later, one cell commits to a specialized fate (purple) and inhibits surrounding cells from adopting this fate. (**B**) Shown is the salt and pepper-like N-tubulin expression pattern, which is a result of Notch signaling restricting the number of neuronal precursors.

During primary neurogenesis in *Xenopus*, lateral inhibition restricts the number of neurons within the proneural domains to a salt and pepper-like pattern (Figure 5B). In the cell fated to become a neuron, X-ngnr-1 activates transcription of the cell surface ligand X-Delta-1, which binds to the Notch receptor on the neighboring cell (Figure 6) (Chitnis et al., 1995). The binding triggers a series of proteolytic cleavages in the Notch receptor leading to the release of the intracellular domain of Notch (NICD). NICD translocates to the nucleus, where it exchanges the corepressor complex bound to the *Xenopus* homologue of Suppressor of Hairless (XSu(H)) for a coactivator complex, thereby allowing transcription of target genes (Figure 6) (Wettstein et al., 1997). Some of the best-characterized Notch signaling targets are repressor bHLH transcription factors of the Enhancer of split related (ESR) gene family.

These proteins bind to E-box sequences and repress target genes, as well as heterodimerize with HLH proteins and inhibit their function (Davis and Turner, 2001). X-ngnr-1 is transcriptionally and functionally inhibited by Notch effectors, whereas the downstream transcription factor NeuroD is less sensitive to lateral inhibition (Ma et al., 1996; Chitnis and Kintner, 1996). The exact mechanism of how a cell in the proneural cell cluster escapes Notch signaling remains unclear, however, X-MyT1 activity is required (Chitnis and Kintner, 1996; Bellefroid et al., 1996).

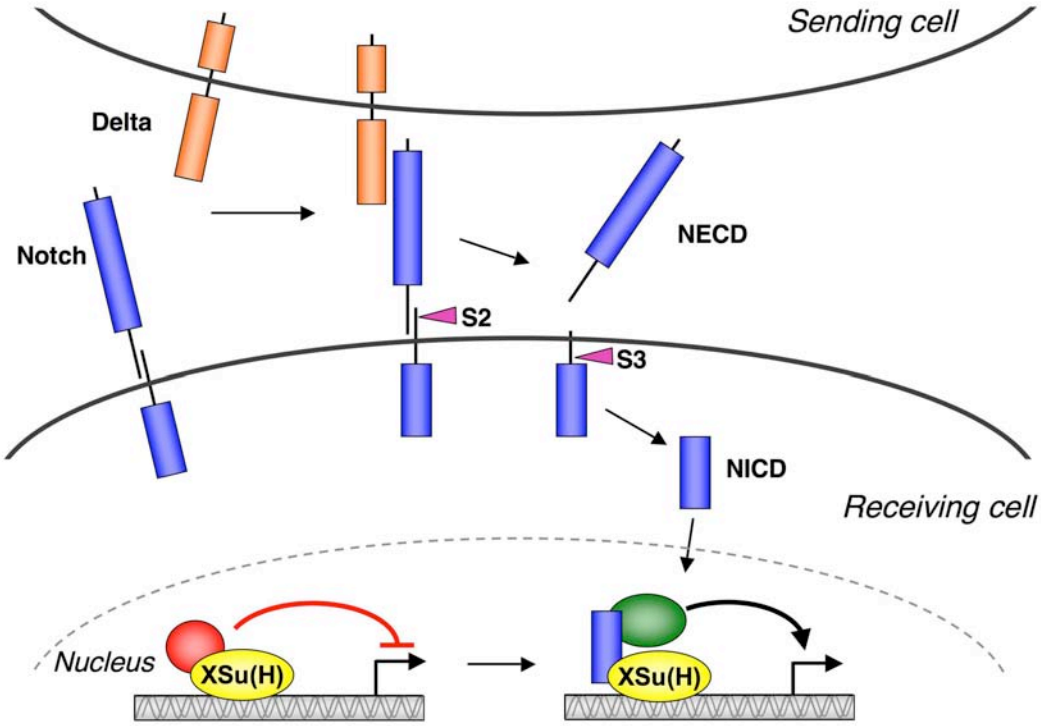

Figure 6 Schematic overview of Notch signaling during primary neurogenesis in *Xenopus*. X-Delta-1 transcription is induced by X-ngnr-1 in the sending cell, the ligand is localized to the cell membrane, where it binds to the Notch receptor on the neighbouring cell. After extracellular cleavage (S2), a membrane anchored multimeric complex with γ-secretase activity cleaves (S3) the intracellular domain of Notch (NICD). NICD translocates to the nucleus, where it binds to XSu(H) and activates transcription of Notch effectors.

1.5 Basic Helix-Loop-Helix Orange proteins

The ESR protein family belongs to the bHLH transcription factor superfamily and is related to the *Drosophila* Hairy and Enhancer-of-split proteins (E(spl)). This class of proteins is characterized by a conserved bHLH domain, which mediates DNA binding and dimerization. A highly conserved Orange domain, located C-terminal to the bHLH domain influences DNA binding preferences of individual members (Louvi and Artavanis-Tsakonas, 2006; Umbhauer et al.,

2001; Dawson et al., 1995). In addition, a C-terminal tetrapeptide affords transcriptional repression by recruitment of Groucho (Paroush et al., 1994; Fisher et al., 1996). Due to their structural features, members of the bHLH-Orange family can be divided into E(spl), Hairy, Hey and Stra13 subfamilies (Davis and Turner, 2001). With the exception of Stra13 proteins, all possess a repressor motif, WRPW in E(spl)/Hairy- and YRPW in Hey-type proteins. These proteins bind to E-Box (CANNTG) or the related consensus CACNAG sequence, termed N-Box (Nakagawa et al., 2000; Garriga-Canut et al., 2001). Although most bHLH-Orange proteins are directly activated by Notch signaling and therefore act as Notch mediators, some exceptions exist. For example, XHes6, a member of the E(spl) subfamily, is not induced by Notch signaling, but activated by proneural transcription factors. In contrast to other bHLH-Orange proteins, which inhibit primary neurogenesis upon overexpression, XHes6 overexpression promotes neuronal differentiation (Koyano-Nakagawa et al., 2000).

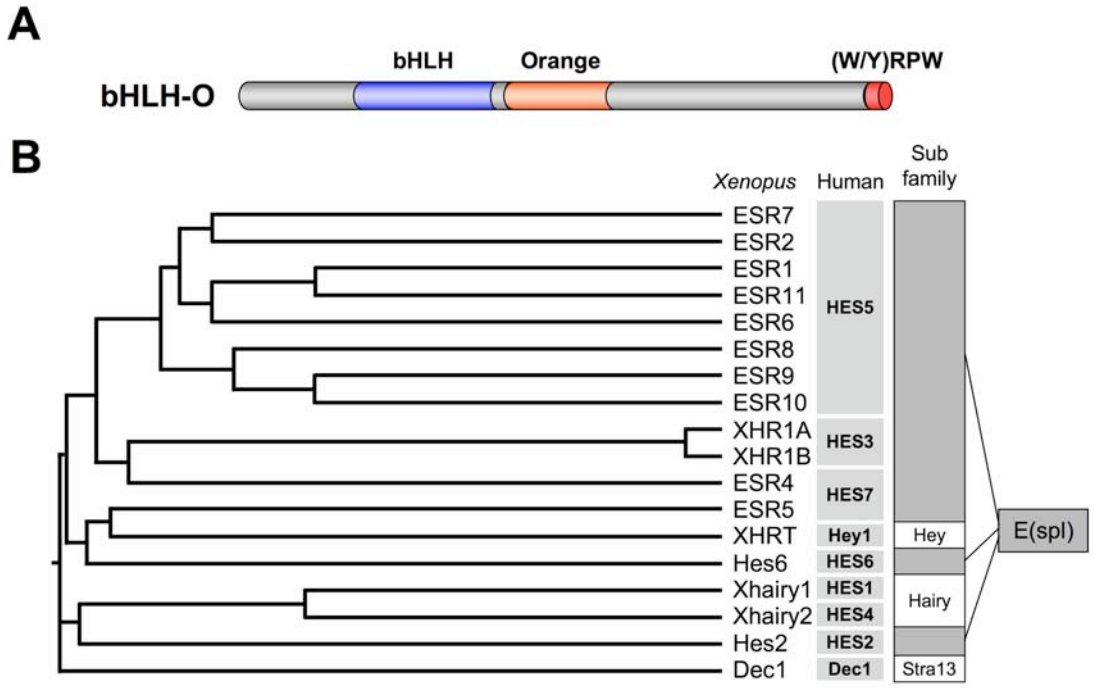

Figure 7 bHLH-O proteins. (**A**) Simplified representation of the domain structure of bHLH-Orange proteins. The basic helix-loop-helix (bHLH) domain is shown in blue, the Orange domain in orange and the repressor domain (WRPW in E(spl)/Hairy and YRPW in Hey) in red. (**B**) Phylogenetic tree of *Xenopus* bHLH-Orange genes. For simplicity only one pseudoallele is shown with the corresponding human ortholog. The subfamilies are indicated to the right. The E(spl) subclass is shaded.

In humans, seven HES (HES1 to HES7), three Hey (Hey1, Hey2 and HeyL) and two Stra13 genes have been identified. Numerous genes for each

human member of the bHLH-Orange family have been identified in *Xenopus*. Multiple members of the human HES5 are expressed within the domains of primary neurogenesis, such as ESR1, ESR3/7, ESR8, ESR9 and ESR10 (Figure 7) (Wettstein et al., 1997; Li et al., 2003;Shinga et al., 2001; Sölter, 2006). Therefore, they are potential mediators of lateral inhibition in the context of primary neurogenesis, although this is only reported for ESR1 and ESR10 (Lamar and Kintner, 2005).

While the Notch signaling pathway and its designated transcriptional targets have been under intensive investigation, studies of bona fide direct targets of bHLH-Orange proteins are limited. During myogenesis, XMyoD was shown to be a putative target of Xhairy-1 (Umbhauer et al., 2001). In the context of neurogenesis, MASH1 is a direct target of HES1 in mouse and *achaete* a direct target of Hairy in *Drosophila* (Castella et al., 1999; Ohsako et al., 1994). In *Xenopus*, X-ngnr-1 is a primary target of Notch effectors, but which bHLH-O protein is the direct repressor of X-ngnr-1 is not known. In a microarray-based study to identify targets of XHR-1, a bHLH-O protein expressed specifically in the midbrain-hindbrain region, several members of the ESR gene family have been found, arguing for a complex transcriptional cross-regulation among bHLH-O proteins (Takada et al., 2005).

1.6 Proneural expression groups

There is a strong correlation between gene expression and gene function in *Xenopus* primary neurogenesis. Many proneural genes, for example X-ngnr-1, X-MyT1 and Xebf3, are expressed in the same pattern as N-tubulin (Figure 8). These genes define the N-tubulin synexpression group. With the successive differentiation, their expression pattern becomes more refined in such a way, that an early acting proneural gene exhibits slightly broader domains in comparison to later acting downstream factors (Bellefroid et al., 1996).

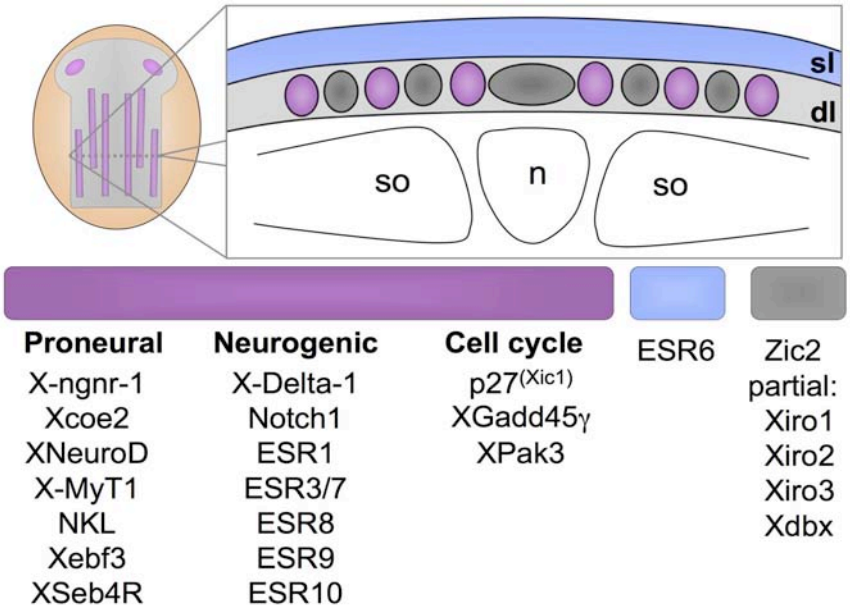

Figure 8 Summary of genes expressed within the N-tubulin synexpression group. The proneural domains (purple) are established in the deep layer (dl) of the neuroectoderm (light grey), while proneural activity is inhibited by ESR6 in the superficial layer (sl) (blue). Examples of genes, which are expressed in all three stripes of primary neurogenesis are listed. They are categoried into proneural, neurogenic and cell-cycle genes. In between the proneural domains, several negative regulatory genes are expressed, restricting the proneural domains (dark grey). While Zic2 is expressed in all intra-neuronal stripes, Xiro proteins and Xdbx are expressed only in a subset.

Members of the Notch pathway, such as X-Delta-1 and Notch-1, are also expressed in the longitudinal stripes of primary neurogenesis, although they have a slightly different overall expression pattern (Chitnis et al., 1995). In addition, several Notch effectors, ESR1, ESR3/7, ESR8, ESR9 and ESR10, are expressed within the proneural domains. Another set of genes, linked to the cell cycle, has been described to be essential for primary neurogenesis, including XPak3, XGadd45γ and $p27^{(Xic1)}$ (see below).

1.7 Terminal differentiation in *Xenopus*

One prerequisite for cellular differentiation is the withdrawal from the cell cycle. In *Xenopus*, mitotic cells are predominantly located within open neural plate at neurula stages (Saka and Smith, 2001). The superficial layer will only give rise to secondary neurons due to the inhibition of proneural transcription factors, most likely mediated by ESR6 (Chalmers et al., 2002). Neuronal progenitors in the deep layer, which will give rise to primary neurons, undergo one additional cell division, which is initiated at the neural plate border and extends medially (Hartenstein, 1989).

Several cell cycle regulators have been identified that are expressed within the regions of primary neurogenesis and can serve as mediators between proliferation and differentiation. These genes include the *Xenopus* ortholog of cyclin kinase inhibitors (CKI) of the Cip/Kip family $p27^{(Xic1)}$, growth-arrest-and-DNA-damage induced gene gamma (XGadd45γ) and the p21 activated serine/threonine kinase 3 (XPak3) (Ohnuma et al., 1999; Vernon et al., 2003; de la Calle-Mustienes et al., 2002; Souopgui et al., 2002). Although the epistatic relationship and the underlying molecular mechanisms of how they allow a neuronal precursor to exit the cell cycle are not known, all are required for *Xenopus* primary neurogenesis.

1.8 The Myc/Max/Mad Network

Myc proteins are members of the Myc/Max/Mad network of transcription factors, which has been shown to regulate a variety of cellular processes including proliferation, differentiation and apoptosis (Cole and Nikiforov, 2006).

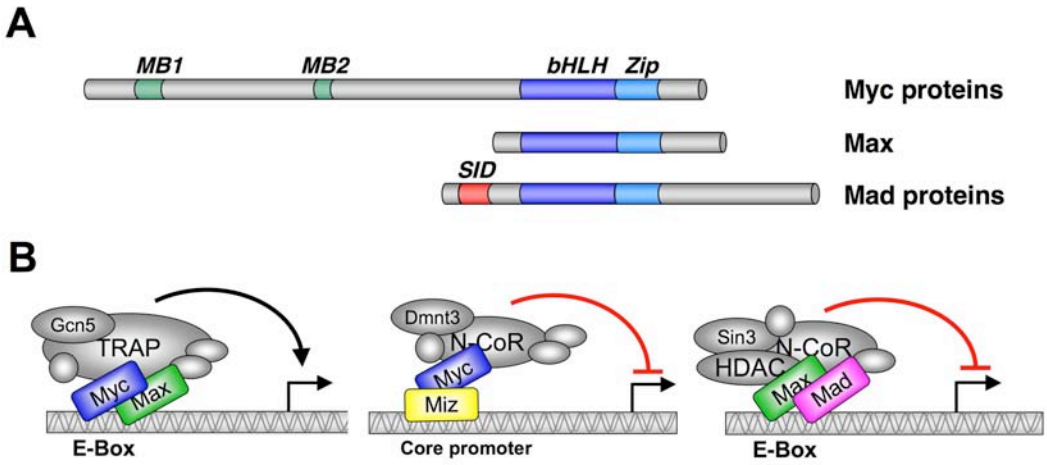

Figure 9 Schematic overview of Myc/Max/Mad protein domains and function. (**A**) All members of the Myc/Max/Mad Network posses a central bHLH leucine zipper domain (blue and light blue). While the Myc proteins harbor two conserved domains in the N-terminus, so called Myc Boxes (green), the Mad proteins contain a N-terminally Sin3 interaction domain (SID) (red), which mediates repression. (**B**) Myc:Max dimers can open the local chromatin state of the DNA by recruitment of multimeric coactivator complexes including TRAP and the histone acetylation (HAT) possessing protein Gcn5. This gene activation is E-Box dependent. Myc can also bind to Miz at core promoters, leading to an exchange of the Miz bound coactivator complex by a corepressor complex including N-CoR and the methylase activity possessing protein Dmnt3, which leads to a local condensation of DNA. Mad:Max dimers can bind to E-Box sequences and by recruitment of a multimeric corepressor complex via their SID domain, the local chromatin structure condenses.

Mycs, namely c-myc, N-myc and L-myc are bHLH proteins, which contain a leucine zipper adjacent to the bHLH domain, and in addition two

conserved domains in the N-terminus, called Myc boxes (MBI and MBII) (Figure 9A) (Colby et al., 1983; Hann et al., 1983; Nau et al., 1985; Schwab et al., 1983). Although the cellular mechanisms of Myc activity are highly complex, it has been shown that Myc proteins heterodimerize with their obligate dimerization partner Max and bind to E-box sequences (CACGTG) (Eisenman, 2006). The transcriptional activation is mediated primarily through recruitment of multimeric coactivator complexes, which lead to unwinding of the local chromatin structure (Figure 9B) (Nikiforov et al., 2002). Myc, together or independent of Max, can also associate with the Myc-interacting-zinc-finger protein 1 (Miz1) independent of E-Box binding, leading to repression of transcription (Figure 9B) (Kleine-Kohlbrecher et al., 2006; Brenner et al., 2005; Peukert et al., 1994). In general, the Mycs positively regulate cell proliferation and growth, and the deregulation of Mycs plays a significant role in the development of a variety of human tumors, including those of the nervous system (Nilsson and Cleveland, 2003; Grandory et al., 2000). A role in neurogenesis has also been reported for N-myc. A conditional knock out of N-myc in neuronal progenitor cells of mice argues for a role in proliferation and progenitor maintenance; and N-myc deficient mice show reduced brain size and massive increase of neuronal differentiation in the cerebral cortex (Knöpfler et al., 2002).

The Myc binding protein Max also binds to other bHLH-Zip proteins, including Mad1, Mxi1, Mad3 and Mad4, which form the Mad family of transcription factors (Ayer et al., 1993; Zervos et al., 1993; Hurlin et al., 1995). In addition to a bHLH-Zip domain, they possess a conserved repressor domain in the N-terminus, the Sin3 interaction domain (SID) (Figure 9A) (Baudino and Cleveland, 2001). Through recruitment of the corepressor complexes harboring Sin3 and N-CoR, Mad:Max heterodimers mediate transcriptional repression of E-Box containing target genes (Figure 9B) (Baudino and Cleveland, 2001). Mad:Max are thought to be cellular antagonists of Myc:Max heterodimers, as they can counteract the Myc:Max induced transformation activity (Rottmann and Luscher, 2006). More distantly related Mad-like proteins have also been identified, including Mnt, a much larger bHLH-Zip SID containing protein, and Mga, which possesses an additional protein-protein interaction domain (T-Domain) (Hurlin et al., 1997;

Hurlin et al., 1999). Mad proteins can also bind to Max-like (Mlx) and the RING finger protein Mip2 (Figure 10) (Billin et al., 1999; Yin et al., 1999).

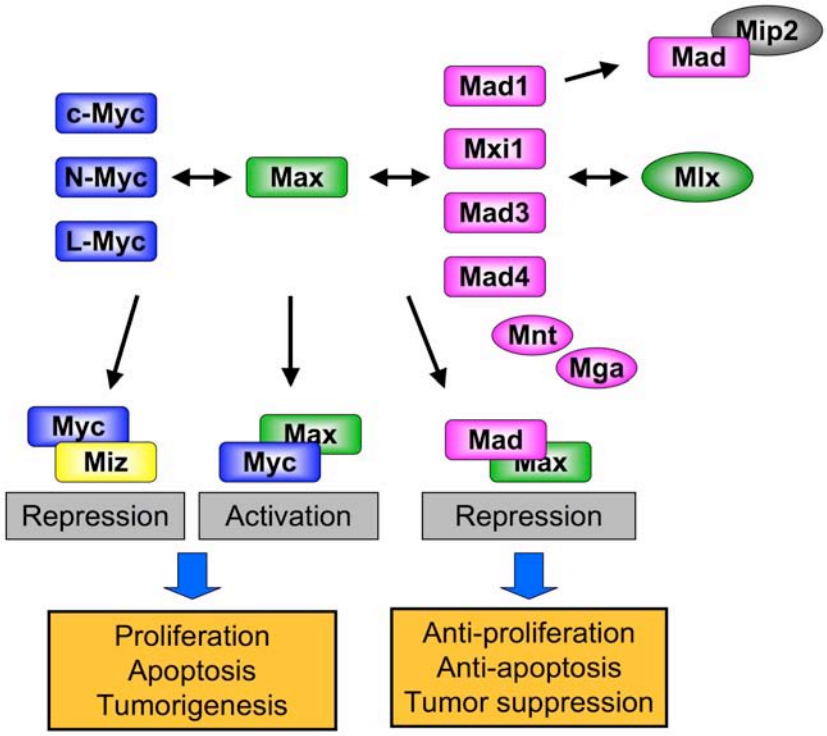

Figure 10 Interplay of the Myc/Max/Mad Network. The complex interplay between Mycs, Mads and Max, as well as other related proteins, like Mnt, Mga, Mlx and Mip2 is schematically illustrated. Myc:Max and Mad:Max dimers have unique but also overlapping target genes. Myc:Max interaction leads to proliferation, apoptosis and possibly also tumorigenesis, while differentiation is repressed. Mad:Max heterodimers inhibit proliferation and apoptosis, while allowing differentiation.

Extensive evidence demonstrating that Mxi1 and other members of the Mad family can induce growth arrest and inhibit Myc transformation has been obtained primarily through experiments performed in cell culture systems (Zervos et al., 1993; Hurlin et al., 1995; Chen et al., 1995; Eisenman, 2006). Studies elucidating the functional role of the Mads in normal differentiation processes and in embryonic development remain unclear, as does their contribution to Myc antagonism. Several recent reports have demonstrated that members of the Myc/Mad/Max network can directly control cell fate determination through a mechanism that is independent of proliferation and apoptosis. For example, c-Myc has been shown to be required for neural crest specification in *Xenopus*, and Mad1 has been identified as a direct activator of the PDX-1 gene during pancreatic development (Bellmeyer et al., 2003; Patane et al., 2003).

In *Xenopus*, Myc as well as Mad proteins exhibit complex and dynamic patterns of expression, arguing for distinct roles during development (King et al., 1986; Vize et al., 1990; Newman and Krieg, 1999; Bellmeyer et al., 2003; Jürgens et al., 2005). All Myc transcripts are maternally expressed, but are quickly degraded. While transcripts of L-Myc are not detected in later stages of embryogenesis, those of c-Myc are found at late gastrula stages and early neurula stages in the transversal and lateral neural folds that will later give rise to the neural crest (Bellmeyer et al., 2003; Newman and Krieg, 1999). N-Myc mRNA levels rise during neurula stages, with considerable expression in the anterior neural plate, and during tailbud stages in the proliferating cells of the neural tube (Vize et al., 1990). Xmad4 is transiently expressed in the cement and hatching gland and later in the developing liver and pronepheros (Newman and Krieg, 1999). Only recently, other members of the Mad family have been described in *Xenopus* (Jürgens et al., 2005). Xmad1 is expressed maternally and becomes localized to the dorsal midline during gastrulation. Later, it is found in the notochord and the overlying floor plate of the neural tube. At stage 27, Xmad1 transcripts are found in the hypochord and the postmitotic regions of the neural tube (Jürgens et al., 2005). Xmad3 transcripts are first detected in the eye vesicle and later in the midbrain and hindbrain areas. At stage 27, the transcripts are detected in the whole neural tube as well as the retina (Jürgens et al., 2005). In contrast to the other Mad family members, Xmxi1 is the only member exclusively expressed in the regions of primary neurogenesis, prefiguring the expression domains of X-ngnr-1 (Souopgui, 2002).

1.9 Aims

The Myc/Max/Mad network plays an essential role in a variety of cellular processes including proliferation, differentiation and apoptosis. This network has been under intensive investigation over the decade, however, its role during early embryogenesis still remains largely undefined. *Xenopus* Mxi1 is unique among other members of the *Xenopus* Myc/Max/Mad network in that it is strongly expressed in all territories of primary neurogenesis. Xmxi1 transcripts are present during gastrulation and early neurula stages, earlier and in broader domains as compared to the neuronal determination factor

X-ngnr-1. The early steps of neurogenesis prior to X-ngnr-1 expression are still largely unknown. Due to the temporal and spatial expression, Xmxi1 could participate in these early events leading to the restricted pattern of X-ngnr-1 expression. Therefore, a functional characterization of Xmxi1 is of interest and was conducted as described in the first part of this thesis work.

Primary neurons arise through an interplay between the proneural cascade, which drives differentiation of neuronal precursors, and Notch signaling, which restricts the number of cells undergoing neuronal differentiation. Direct target genes of Notch signaling include members of the Enhancer-of-Split Related (ESR) gene family of transcriptional repressors. Despite the fundamental role attributed to Notch signaling during embryogenesis, only a limited number of bona fide ESR target genes have been identified. The identification of ESR target genes will increase our knowledge of the underlying molecular mechanisms by which Notch influences development and unravel the interplay of positive and negative acting factors. Therefore, an unbiased screen to identify early target genes of ESR proteins was undertaken as described in the second part of this thesis work.

2 Material and Methods

2.1 Organisms

2.1.1 Xenopus laevis

The African clawed frog *Xenopus laevis* was used as experimental organism during this study. Pigmented or albino frogs were purchased from Nasco (Ft. Atkinson, USA). The embryonic staging was based on Nieuwkoop und Faber (1967).

2.1.2 Escherichia coli

XL1-Blue recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F´ proAB, lacIqZDM15, Tn10(Tetr)][c] (Bullock *et al*., 1987)

2.2 Oligonucleotides

2.2.1 RT-PCR oligonucleotides

Sox3	for	5´-GCGCACATGAACGGCTGGACTA-3´
Sox3	rev	5´-GTGTGGGAGGTGATGGCTGGAG-3´
5´UTR Nrp1	for	5´-AGTGCTTTGTCAGGAGAGATC-3´
5´UTR Nrp1	rev	5´-CTTCAACTGTTGTATTCACTG-3´
N-CAM	for	5´-CACAGTTCCACCAAATGC-3´
N-CAM	rev	5´-GGAATCAAGCGGTACAGA-3´
SoxD	for	5´-TCAGCAACAGGTCCAGTACC-3´
SoxD	rev	5´-TCTAACAAGATCCGACGCC-3´
Xmxi1	for	5´-CAAAGAGATGCAGTGGCCTC-3´
Xmxi 1	rev	5´-CCAATACTGTCTGAGCGCAC-3´
X-ngnr-1	for	5´-CAAGAGCGGAGAAACTGTGT-3´
X-ngnr-1	rev	5´-GAAGGAGCAACAAGAGGAAG-3´
Ebf2	for	5´-GGTATGGAGCGAGCTTATCA-3´
Ebf2	rev	5´-CTGTACAGAGCCTCCGCAAT-3´
X-MyT1	for	5´-GGCCATGTAAACAGCAACCGTAAC-3´
X-MyT1	rev	5´-AGAGGAGGAGGAAGAGGAAGTGCT-3´
NeuroD	for	5´-GTGAAATCCCAATAGACACC-3´

NeuroD	rev	5´-TTCCCCATATCTAAAGGCAG-3´
Xebf3	for	5´-CCTACAAGTCAAAGCAGTTCT-3´
Xebf3	rev	5´-CCTACTTGGTCATTAGCTTGA-3´
XPak3	for	5´-TGAATGCAAAGACTGCATCTGAGC-3´
XPak3	rev	5´-GCTCGTGCTTGAGTTTGAGTTTTC-3´
ESR9	for	5´-GCTTCCAAATATGCACAATCATCC-3´
ESR9	rev	5´-CCAGTCCCAGGAGTTGTGCATTT-3´
N-tub	for	5´-ACACGGCATTGATCCTACAG-3´
N-tub	rev	5´-AGCTCCTTCGGTGTAATGAC-3´
ODC	for	5´-GCCATTGTGAAGACTCTCTCCATTC-3´
ODC	rev	5´-TTCGGGTGATTCCTTGCCAC-3´

RT-PCR oligonucleotides used in the screen to identify ESR1 target genes are listed in the Appendix.

2.2.2 Morpholino oligonucleotides

Antisense morpholinos were obtained from Gene Tools. Morpholinos were dissolved to a stock concentration of 10 mg/ml in H_2O; mutated nucleotides are underlined.

Control-MO (Co-MO)	5´-CCTCTTACCTCAGTTACAATTTATA-3´
Xmxi1-MO	5´-ATCCACCAGCTCCACCGACTCTAAT-3´
MM-Xmxi1-MO	5´-AT<u>G</u>CAC<u>G</u>AGCT<u>G</u>CACC<u>C</u>ACT<u>G</u>TAAT-3´
Xmxi1-MO2	5´-TCCATCATCTCCTGCAGCTCCATCA-3´
X-ngnr-1-MO	5´-TGGTTAGCCCCAATGTTGCACTGAC-3´

2.2.3 General oligonucleotides

SP6	5´-TATTTAGGTGACACTATAG-3´
RR67	5´-GGAGAGCTTGGGCGACCTCACC-3´
EnR for	5´-CAGGAGTGCAGCCAATGC-3´
pCS2 rev	5´-CTCATCAATGTATCTTATCATGTCTG-3´
GR9	5´-ACCTCCAACAGTGACACCAGG-3´

2.3 Constructs

2.3.1 Overexpression constructs

MT-Xmxi1pCS2+ harbors the full open reading frame of Xmxi1 (DQ137875), including ATG and STOP codon. The fragment was generated by PCR amplification using Xmxi1pGEM-T as template (Jürgens, 2002), 5´Xmxi-1 (XhoI/ATG) 5´-GCCCTCGAGATGGAGCTGCAGGAGATGATG-3´ and 5´Xmxi1 (SalI/noATG) 5´-GCCGTCGACGAGCTGCAGGAGATG-3´ as primers and inserted into the XhoI site of MTpCS2+ (D. Turner and R. Rupp, http://sitemaker.umich.edu/dlturner.vectors). For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

MT-Xmxi1-DBMpCS2+ harbors the full open reading frame of Xmxi1 (DQ137875), including ATG and STOP codon with a point mutation in the basic region, which abolishes DNA binding (Fischer et al., 1993; Prochownik et al., 1998). The fragment was generated with the QuikChange® XL Site-Directed Mutagenesis Kit for site-directed mutagenesis, (Stratagene) using MT-Xmxi1pCS2+ as template and Xmxi1 dnamutup 5´-CCCACAATGAACTGGCGAAGAACCGGAGAGCC-3' and Xmxi1 dnamutdown 5´-GGCTCTCCGGTTCTTCGCCAGTTCATTGTGGG-3' as primers. The mutation is underlined, for sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

Xmxi1-GRpCS2+ harbors the full open reading frame of Xmxi1 (DQ137875), incuding ATG but without STOP codon to obtain a hormone inducible GR fusion protein. The fragment was generated by PCR amplficication using Xmxi1pGEM-T, 5´Xmxi-1 (XhoI/ATG) 5´-GCCCTCGAGATGGAGCTGCAGG AGATGATG-3´ and 3´Xmxi1 (SalI/noSTOP) 5´-GCCGTCGACCGAGCTGAA GGACAAC-3´ as primers and inserted into the XhoI site of GRpCS2+ (D. Turner and R. Rupp). For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

MT-ΔNXmxi1pCS2+ harbors the C-terminal part of the coding sequence of Xmxi1 (aa63-238) (DQ137875), including ATG and STOP codon. The fragment was generated by PCR amplification using Xmxi1pGEM-T as template (Jürgens, 2002), ΔNXmxi1 (XhoI/ATG) 5´-CCGCTCGAGATGGAGA

GTTCCGACCCA ATG-3´ and 5´Xmxi1 (SalI/noATG) 5´-GCCGTCGACGAGC TGCAGGAGATG-3´ as primers and inserted into the XhoI site of MTpCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

EnRpCS2+ harbors the N-terminal part of the coding seuquence of the drosophila repressor engrailed (aa1-298) (M10017), including ATG but without STOP codon. The fragment was PCR amplified using EnR for (MfeI/ATG) 5´-GCAATTGGATGGCCCTGGAGGATCG-3´ and EnR rev (EcoRI/noSTOP) 5´-CGAATTCCGTCCCAGAGCAGATTTCTCTGG-3´ as primers and inserted into the EcoRI site of pCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

EnR-Xmxi1pCS2+: ΔNXmxi1 was subcloned from MT-ΔNXmxi1pCS2+ into the XhoI site of EnRpCS2+. For sense RNA, the construct was linearized with SacII and RNA transcribed with SP6 RNA polymerase.

MT-Mad1pCS2+ harbors the full open reading frame of Xmad1 (AY964104), without ATG but STOP codon. The fragment was generated by PCR amplification using Xmad1pBKCMV as template (Jürgens et al., 2005), 5´Xmad1 (SalI/noATG) 5´-GTTGTCGACGCGGCCCCGG-3´ and 3´Xmad1 (SalI/STOP) 5´-GCCGTCGACCTGATTGGCTGTTTAAGGAA-3´ as primers and inserted into the XhoI site of MTpCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

MaxpCS2+ harbors the full open reading frame of Xmax1 (L04923), including ATG and STOP codon. The fragment was generated by PCR amplification using *Xenopus* stage 25 cDNA, 5´Xmax (BamHI/ATG) 5´-CACGGATCCAT GAGCGATAACGATGACATCG-3´ and 3´Xmax (EcoRI/STOP) 5´-CGGGAAT TCTTAGCTTGCGTCCATCCGTAG-3´ as primers and inserted into the BamHI and EcoRI sites of pCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

5´UTR-Xmxi1-MT-GFPpCS2 harbors part of the 5'UTR of Xmxi1 (-73 to -7) (DQ137875). The fragment was PCR amplified using Xmxi1pGEM-T as

template (Jürgens, 2002), 5´Xmxi1-UTR (BamHI) 5´-CACGGATCCGA ATTCCGGCACGAGGTCGCAC-3´ and 3´Xmxi1-UTR (BamHI) 5´-CACGGA TCCCCCCAGGACTCACAATATCCACCAG-3´ as primers and inserted into the BamHI site of MT-GFPpCS2+ (Klymkowsky, 1999). For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

5´UTR-Xmxi1-MT-Xmxi1pCS2 harbors part of the 5'UTR of Xmxi1 (-73 to -7) (DQ137875). The fragment was PCR amplified using Xmxi1pGEM-T as template (Jürgens, 2002), 5´Xmxi1-UTR (BamHI) 5´-CACGGATCCGAATTC CGGCACGAGGTCGCAC-3´ and 3´Xmxi1-UTR (BamHI) 5´-CACGGATCC CCCAGGACTCACAATATCCACCAG-3´ as primers and inserted into BamHI site of MT-Xmxi1pCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

ESR1-GRpCS2+ harbors the full open reading frame of XESR1 (AF383157) without STOP codon. The fragment was PCR amplified using the RZPD clone IMAGp998N1310807Q template, ESR1for (EcoRI) 5´-TCAGAATTCTACCAT GGCTCCTACCAGCATTTC-3´ and ESR1rev (XhoI) 5´-ATCACTCGAGCC AGGGGCGCCATATTTTGTTGG-3´ as primers and inserted into the EcoRI-XhoI sites of GRpCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

ESR1-VP16-GRpCS2+ harbors the full open reading frame of XESR1 (AF383157) without the C-terminal WRPW repressor peptide and STOP codon to obtain an antimorphic hormone inducible GR fusion protein. The fragment was PCR amplified using the RZPD clone IMAGp998N1310807Q template, ESR1for (EcoRI) 5´-TCAGAATTCTACCATGGCTCCTACCAG CATTTC-3´ and ESR1VP16rev (XhoI) 5´-ATCACTCGAGTATTTTGTTGGTGT TGCTTGCCA-3´ as primers and inserted into the EcoRI and XhoI sites of VP16-GRpCS2+. For sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

ESR1-VP16-GR-DBMpCS2+ harbors the full open reading frame of XESR1 (AF383157) without the C-terminal WRPW repressor peptide with three point mutations in the basic region, which abolishes DNA binding (Ström et al.,

1997). The fragment was generated with the QuikChange® XL Site-Directed Mutagenesis Kit for site-directed mutagenesis, (Stratagene) using ESR1-VP16-GRpCS2+ as template and ESR1 dnamutup 5´-GAAAGCCCATTGTGGCAGCGATGCGCGCAGACAGGATTAACAAC-3' and ESR1 dnamutdown 5´-GTTGTTAATCCTGTCTGCGCGCATCGCTGCCA CAATGGGCTTTC-3' as primers. The mutation is underlined, for sense RNA, the construct was linearized with NotI and RNA transcribed with SP6 RNA polymerase.

hBcl2pBluescript RN3 harbors 68 nts 5´UTR, hBcl2 open reading frame and 1109 nts of 3´UTR. The constuct was a kind gift of Dr. Kristine A. Henningfeld. For sense RNA, the construct linearize with SfiI and RNA transcribed with T3 RNA polymerase.

Noggin (Smith et al., 1993); **Notch-ICD** (Coffman et al., 1993); **MT-X-ngnr-1** (Ma et al., 1996); **MT-NeuroD** (Lee et al., 1995); **SoxD** (Mizuseki et al., 1998); **NLS LacZ** (Chitnis et al., 1995); and **MT-GFP** (Klymkowsky, 1999)

2.3.2 Constructs for whole-mount in situ hybridization

Xmxi1pGEM-T (Jürgens, 2002); **Sox3pBS** (Rust, 2005); **EpiKpGEM-T** (XK81) (Souopgui, 2002); **XPak3** (Souopgui et al., 2002); **X-ngnr-1** (Ma et al., 1996); **X-MyT1** (Bellefroid et al., 1996); **N-tubulin** (Chitnis et al., 1995); **NeuroD** (Lee et al., 1995); **X-Delta1** (Chitnis et al., 1995); **ESR9** (Li et al., 2003); and **p27(Xic1)** (Ohnuma et al., 1999).

Whole-mount in situ constructs used in the screen to identify ESR1 target genes are listed in the Appendix.

2.3.3 Constructs for real-time RT-PCR standard curves

The amplified RT-PCR products were cloned into the pGEM-T vector. The accession number and region in nucleotides are given with respect to the ATG. The constructs were used for generation of a standard curve for real-time RT-PCR analysis.

Xmxi1 QRTpGEM-T (DQ13787, 298 to 607); **X-ngnr-1 QRTpGEM-T** (U67778, 206 to 539); **XMyT1 QRTpGEM-T** (U67078, 1365 to 1808); **Xebf3 QRTpGEM-T** (AF040994, 986 to 1376); **NCAM QRTpGEM-T** (M25696, 2792 to 3134); **NeuroD QRTpGEM-T** (U28067, 964 to 1203); **5´UTR-Nrp1 QRTpGEM-T** (BC084959, -933 to -758); **N-tubulin QRTpGEM-T** (X15798, 80 to 329); **ODC QRTpGEM-T** (X56316, 222 to 441); **XPak3 QRTpGEM-T** (AF485330, 511 to 751); **Sox3 QRTpGEM-T** (BC072222, 433 to 737); **SoxD QRTpGEM-T** (BC093551, 490 to 805); **Ebf2 QRTpGEM-T** (AF040993, 872 to 1213).

2.4 Total RNA extraction and cDNA synthesis

Total RNA was isolated with the RNAqueous®-Micro Kit (Ambion). To lyse the cells, 20 to 50 animal caps or three whole embryos were macerated with a 29-Gauge syringe in 100 µl lysis buffer and centrifuged at maximum speed for 2 min. The lysis buffer containing the total RNA was removed without debris and the manufacturer's protocol was followed. The RNA was eluted in 30 µl, 75°C pre-heated elution buffer and subject to DNAse treatment for at least 2 hours at 37°C to digest genomic DNA. DNAseI was inactivated with DNAse inactivation solution (Ambion). For cDNA synthesis, 50 µg total RNA was used in a 10 µl cDNA reaction containing 5 mM $MgCl_2$, 2.5 ng random hexamer, 5 mM dNTP mix, 0.8 U RNAse OUT (Invitrogene), 20 U reverse transcriptase (Applied Biosystems) in 1X Taq incubation buffer without $MgCl_2$ buffer (Qbiogene). After an initial incubation at 20°C for 20 min to anneal the random hexamer primers, cDNA synthesis was carried out for 50 min at 72°C and terminated by heating to 95°C for 5 min.

2.5 RT-PCR analysis

For semi-quantative RT-PCR, 5 µl cDNA was used in a total volume of 25 µl containing 0.2 mM RT primers each, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase in 1X Taq incubation buffer without $MgCl_2$ buffer (Qbiogene). A Histone H4 RT-PCR was carried out to control equal cDNA concentrations and test for DNA contamination using cDNA and total RNA, respectively. For real-time RT-PCR analysis, 5 µL cDNA was used in a total volume of 50 µl containing

0.2 mM RT primers each in 1X iQ™SYBR® Green Supermix (Biorad). Analysis was done in the iCycler system (Biorad). All samples were normalized to levels of ornithine decarboxylase (ODC), which was used as loading control. The copy numbers were calculated based on a standard curve for each analyzed gene. All measurements were done in duplicates.

2.6 *In vitro* synthesis of RNA

2.6.1 Capped sense RNA

For capped mRNA used for microinjection, the mMessage-mMachine™ Kit was used according to the manufacturer's protocol (Ambion). For a 20 µl reaction, 1 µg linearized plasmid was used. Transcription was carried out at 37°C for at least 2 hours. The DNA template was removed by addition of 5 U DNAseI to the reaction mix and incubation at 37°C for at least 30 min. The reaction was purified with the RNeasy Mini Kit (Qiagen), eluted in 20 to 30 µl RNAse-free H_2O and 2 µl aliquots were stored at -80°C.

2.6.2 Antisense RNA

For antisense RNA used as probes for whole mount in situ hybridization 1 µg linearized template was used in a total reaction volume of 25 µl containing 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.64 mM UTP, 0.36 mM digoxigenin-UTP, 0.03 µM DTT, 1.6 U RNAse OUT (Invitrogene), 0.05 U Pyrophosphatase, 0.8 U RNA polymerase in 1X transcription buffer. For ESR screen probes, inserts were PCR amplified using the appropriate vector primers, purified (Qiagen), eluted in 30 µl elution buffer and 8 µl used as RNA template. After 3 hours at 37°C, the DNA template was digested by addition of 5 U DNAseI. The mix was incubated at 37°C for 30 min and purified using the RNeasy Mini Kit (Qiagen). The RNA was eluted twice with 50 µl RNAse-free H_2O and stored in Hyb Mix at -20°C.

2.7 Embryo culture and injections

HCG: 2000 U/mL human chrorionic gonadotropin (HCG) (Sigma)

10X MBS Salts: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.8

1X MBS: 1X MBS Salts, 0.7 mM $CaCl_2$

Dejelly solution: 2% (w/v) L-cysteine hydrochloride in 0.1X MBS, pH 8.0

Injection buffer: 1% (w/v) FICOLL in 1X MBS

Nile blue staining: 0.01% (w/v) Nile Blue chloride, 89.6 mM Na_2HPO_4, 10.4 mM NaH_2PO_4, pH~7.8

5X MBS AC: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.05mM $CaCl_2$, 1.65 mM $Ca(NO_3)_2$, pH 7.8

Agar dishes: 60 mm petri dishes, coated with 0.7% agar made with 0.8X MBS AC.

Embryos were obtained from *Xenopus laevis* by HCG induced egg-laying using 800 U HCG. Spawn was *in vitro* fertilized and embryos staged according to Nieuwkoop and Faber, 1994. Embryos were injected in one blastomere of the two-cell stage or one dorsal of the four-cell stage as described. As lineage tracer, 50 pg nuclear lacZ mRNA was coinjected. For ectodermal explants and western blotting experiments, both blastomeres were injected omitting nuclear lacZ mRNA. In coinjection experiments, GFP mRNA was used to normalize the total mRNA concentrations.

Animal caps were dissected from stage 8.5-9 embryos in agar dishes in 0.8X MBS AC. Animal caps were cultured in 0.8X MBS AC in agar dishes until sibling controls reached the desired stage, then shock frozen and stored at -80°C.

2.8 Chemical treatments

2.8.1 Dexamethasone treatment

500X DEX: 20 mM dexamethasone in ethanol, stored in the dark, stable for up to 3 month.

Embryos were injected with inducible mRNA constructs and treated with fresh 1X DEX at various stages. The embryos were continuously kept in solution until fixation.

2.8.2 Hydroxyurea and aphidicolin (HUA) treatment

10X MBS Salts: 880 mM NaCl, 10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.8

1X MBS: 1X MBS Salts, 0.7 mM $CaCl_2$

Proteinase K: 5 µg/ml Proteinase K in 0.1X MBS

HUA: 20 mM hydroxyurea, 150 mM aphidicolin in 0.1X MBS

Mitosis can be blocked by incubation of the embryos in a cocktail of S-Phase inhibitors (Harris and Hartenstein, 1991). Embryos subject to HUA treatment were treated at stage 10 with Proteinase K for 5 min. After washing extensively with 0.1X MBS, embryos were incubated in HUA as described in Harris and Hartenstein (1991). If an inducible construct was injected, dexamethasone was also added at the desired stage to the buffer. Embryos were kept in this solution continuously until fixation. In the following WMISH, the Proteinase K treatment was shortened by 5 min.

2.9 X-gal staining

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

10X MEM: 1 M Mops, 20 mM EGTA, 10 mM $MgSO_4$, pH 7.4, sterile filtered and stored in the dark

X-gal: 40 mg/mL 5-Bromo-4-chloro-3-indolyl-b-D-galactosidase (X-gal) in formamide, stored in the dark at -20°C

$K_3Fe(CN)_6$: 0.5 M in H_2O, stored in the dark

$K_4Fe(CN)_6$: 0.5 M in H_2O, stored in the dark

MEMFA: 4% (v/v) formaldehyde in 1X MEM

X-gal staining solution: 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in 1X PBS

Dent's solution: 20% (v/v) DMSO in methanol

Embryos were grown to the desired stage and fixed for 20 min in MEMFA. After washing three times for 10 min in PBS, embryos were transferred to X-gal staining solution until staining was sufficient, typically 10 to 20 min. The reaction was stopped by washing the embryos three times in PBS and afterwards fixed in MEMFA for 25 min. For whole mount in situ hybridization and BrdU incorporation assay, embryos were dehydrated with absolute ethanol and stored at -20°C. For TUNEL staining, embryos were dehydrated with methanol and stored at -20°C. For pH3 staining, embryos were dehydrated with methanol, transferred to Dent's solution and stored at least 24 hours at -20°C.

2.10 Whole-mount in situ hybridization

20X SSC: 3 M NaCl, 0.3 M NaCitrat, pH 7.2 - 7.4

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

5X MAB: 500 mM maleic acid, 750 mM NaCl, pH 7.5

Hybridization Mix (Hyb Mix): 50% Formamid (deionisiert), 1 mg/ml Torula-RNA, 10 µg/ml Heparin, 1X Denhardt's, 0.1% Tween-20, 0.1% CHAPS, 10 mM EDTA in 5X SSC

NBT: 100 mg/mL in 70% Dimethylformamide; stored at -20°C

BCIP: 50 mg/mL in 100% Dimethylformamide; stored at -20°C

EtOH series: 100%, 75%, 50% ethanol in H_2O, respectively, 25% ethanol in PTw

MeOH series: 100%, 75%, 50%, 25% methanol in H_2O, respectively

PTw: 0.1% Tween-20 in 1X PBS

Proteinase K: 5 µg/ml Proteinase K in 0.1X PBS

PTw/MEMFA: 4% (v/v) formaldehyde in PTw.

RNAse Solution: 10 µg/ml RNAse A, 0.01 U/ml RNAse T1 in 2X SSC

MAB/BMB: 2% BMB in 1X MAB

MAB/BMB/HS: 2% BMB, 20% heat treated horse serum in 1X MAB

Antibody solution: 2% BMB, 20% heat treated horse serum, 1:2000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase (Roche) in 1X MAB

APB: 100 mM Tris-HCl, pH 9.0, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% TWEEN-20

Color reaction solution: 80 µg/ml NBT, 175 µg/ml BCIP in APB

Whole-mount in situ hybridization (WMISH) was performed essentially as described (Harland, 1991; Hollemann et al., 1999) using antisense RNA labeled with digoxigenin-11-UTP. Double in situ hybridization was performed according to Knecht et al. (1995). All steps were performed at ambient temperature with mild shaking. Embryos were rehydrated with the EtOH series to PTw, washed three times in PTw for 10 min and subjected to Proteinase K treatment to allow better penetration of the RNA probe. Stage 15 embryos were incubated for 6 min, later stage embryos were incubated for no longer than 15 min in Proteinase K. Embryos were washed twice in 0.1M triethanolamine, pH 7.5, to stop Proteinase K digestion and acetylated by adding 25 µl acetic anhydrite to fresh triethanolamine. After 5 min, another 25 µl acetic anhydrite was added. Then embryos were fixed in PTw/MEMFA

for 25 min, washed five times in PTw, transferred to Hyb Mix and incubated for 5 hr at 65°C in a water bath. Hyb Mix was exchanged for the antisense RNA probe and incubated overnight at 65°C in a water bath.

The next day, the RNA probe was collected and stored -20°C for reuse. After washes in Hyb Mix for 10 min at 65°C, three times in 2X SSC for 15 min at 65°C, non hybridized RNA probe was removed by RNAse digestion for 1 hour at 37°C in RNAse solution. The digested probe removed by washing once in 2X SSC at 37°C and twice 0.2X SSC at 65°C. After exchanging the buffer to MAB, embryos were blocked in MAB/BMB for 20 min and MAB/BMB/HS for 40 min to minimize unspecific binding of the antibody. The solution was replaced with antibody solution and incubated for 5 hours. The embryos were washed three times for 10 min with MAB and then overnight in MAB.

After three rinses with MAB for 5 min, the caps were exchanged and the embryos transferred to APB. After three washes in APB for 5 min each, alkaline phosphatase was detected in color reaction solution. Embryos were kept at 4°C in the dark until staining was sufficient. The embryos were transferred to 100% Methanol to stop the reaction and to minimize background staining. Then embryos were rehydrated with the MeOH series to MEMFA and fixed overnight in MEMFA.

2.11 Sections

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

gelatin/albumin: 4.88 mg/ml gelatin, 0.3 g/ml bovines serum albumin, 0.2 mg/ml sucrose in PBS. The gelatin was dissolved by heating the solution to 60°C. Albumin and sucrose was added, filtered with a 0.45 μm filter (Satorius) and stored at -20°C.

Mowiol: 5 g Mowiol was stirred overnight in 20 ml PBS. After addition of 10 ml glycerol, the solution was stirred again overnight. Not dissolved Mowiol was collected by centrifugation for 30 min at 20,000 g. The supernatant was pH adjusted to pH~7.0 (using pH strips) and stored at -20°C.

Specimens were transferred to PBS and after equilibration in gelatin/albumin for 20 min, mounted by addition of glutaraldehyde. Sections (30 µm) were cut on a Leica VT1000M vibratome (Hollemann et al., 1999) and mounted in Mowiol.

2.12 BrdU incorporation assay

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

EtOH series: 100%, 75%, 50% ethanol in H_2O, respectively, 25% ethanol in PBS

Proteinase K: 5 µg/ml Proteinase K in 0.1X PBS

PBS/Triton: 0.3% Triton X-100 in 1X PBS

PTw: 0.1% Tween 20 in 1X PBS

Incubation buffer: 66 mM Tris, 0.66 mM $MgCl_2$, 1 mM ß-mercaptoethanol

1° AB solution: 1:10 dilution of mouse monoclonal anti-BrdU in incubation buffer

2° AB solution: 20% (v/v) heat-treated hourse serum, 1:50 dilution of horseradish peroxidase-coupled anti-mouse IgG antibody in PTw

The BrdU experiment was performed as described by Hardcastle and Papalopulu (2000) using the BrdU labeling and detection Kit II (Boehringer Mannheim) with the following exceptions. Three times, 10 nl of undiluted 5-bromo-deoxyuridine (BrdU) solution were injected into stage 15 embryos: to both sides of dorsal midline and once in the ventral hemisphere. After 1.5 hours, embryos were fixed and subject to X-gal staining. BrdU was detected with an anti-BrdU antibody, as described below. All steps were performed at ambient temperature with mild shaking. Embryos were rehydrated with the EtOH series to PBS, washed five times in PBS and Proteinase K treated for 5 min. After two additional washings (5 min) in PBS, embryos were treated with 2 N HCl for 1 hour. Embryos were washed in PBS three times for 5 min and rinsed in PBS/Triton for 5 min. After blocked in 3% heat-treated horse serum for 3 hours, embryos were washed 15 min in incubation buffer. Incubation buffer was replaced for 1° AB solution, embryos incubated at 37°C for 3 hours and washed overnight in PBS. The next day, embryos were washed three times in PTw for 10 min, then for 60 min in 20% heat-treated horse serum in PTw. Subsequently, the embryos were incubated for 5 hours in 2° AB solution, after which embryos were washed overnight in PTw. Horseradish peroxidase activity was detected as described for WMISH.

2.13 phosphorylated Histone 3 (pH3) assay

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

MeOH series: 100%, 75%, 50% methanol in H_2O, respectively and 25% methanol in 1X PBS

PBS-TB: 0.05% Tween-20, 0.2 % BSA in 1X PBS

PBS-TBN: 0.3M NaCl in PBS-TB

1° AB solution: 20% heat-treated horse serum, 5% DMSO, 1:200 dilution rabbit anti-pH3 antibody (Biomol)

2° AB solution: 20% heat-treated horse serum, 5% DMSO, 1:5,000 dilution goat anti-rabbit IgG horseradish peroxidase-coupled secondary antibody (Sigma)

The pH3 assay was performed essentially as described (Dent et al., 1989). All steps were performed at ambient temperature with mild shaking. After rehydration with MeOH series to PBS, embryos were pre-incubated in 20% heat-treated horse serum in PBS for 2 hours. The solution was exchanged to the 1° AB solution and incubated for 3 hours. After two washes in PBS-TB for 2 hours, the embryos were washed overnight in fresh PBS-TB. Embryos were incubated in PBS-TBN for 3 hours. After a brief wash in PBS-TB, the embryos were transferred to the 2° AB Solution for 5 hours. The embryos were washed twice in PBS-TB for 2 hours, once in PBS TBN for 2 hours, again in PBS-TB for 2 hours before they were washed overnight in PBS-TB. Horseradish peroxidase activity was detected as for WMISH.

2.14 TUNEL assay

10X PBS: 1.75 M NaCl, 1 M KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

MeOH series: 100%, 75%, 50% methanol in H_2O, respectively and 25% methanol in 1X PBS

PTw: 0.1% Tween 20 in 1X PBS

TdT Buffer: 1X TdT (Invitrogene) in 1X PBS

PBS/EDTA: 1 mM EDTA in 1X PBS

PBT: 2 mg/ml BMB, 0.1% Triton-X-100 in PBS

Antibody solution: 20% heat-treated horse serum, 1:2,000 dilution of anti-digoxigenin alkaline phosphatase coupled antibody (Roche)

The TdT-mediated dUTP digoxygenin nick end-labeling (TUNEL) staining technique was modified from Hensey and Gautier (1997). Embryos were rehydrated with the MeOH series to PBS. After one wash in PBS for 10 min, the solution was exchanged twice with PTw, 15 min each. Embryos were washed twice in PBS for 20 min before they were incubated for 1 hour in TdT Buffer. During this pre-incubation step and the following overnight incubation,

the vials were standing upright. End-labeling was carried out overnight at room temperature in TdT buffer containing 0.5 mM digoxygenin-dUTP and 150 U/ml terminal deoxynucleotidyl transferase (Invitrogene). The embryos were washed twice for 2 hours with PBS/EDTA at 65°C in a water bath. After washing the embryos four times for 1 hour in PBS, PBS was exchanged with PBT and incubated for 20 min. The embryos were blocked for 1 hour by incubation in PBT containing 20% heat-treated horse serum. The embryos were placed in Antibody solution and incubated overnight at 4°C. To remove unbound antibody, the embryos were washed 8 times in PBT and then washed overnight in PBT at 4°C. The chromogenic reaction was performed as described for WMISH.

2.15 Protein isolation

Homogenizing Buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 15% Glycerol. Before use, 1 tablet complete protease inhibitors (Roche) was added per 50 ml Homogenizing buffer.

15 to 20 embryos were used for stabilization experiments. For each embryo 10 µl homogenizing buffer was added and homogenized. After a 20 min centrifugation at maximum speed at 4°C, the supernatant was collected and centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant was removed and protein concentration was measured using the Bradford method (Bradford, 1976). Equal amounts of total protein were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001 and subject to western blotting.

2.16 T_NT *in vitro* translation

In vitro translation was performed in a 12.5 µl reaction with the TNT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's user manual. Proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001. The dried gel was exposed to an autoradiography sensitive phosphoimager screen and scanned the following day (Amersham).

2.17 Western blotting

Transfer Buffer: 39 mM glycine, 48 mM Tris-HCl, pH 7.5, 0.04% SDS, 20% Methanol

PTw: 0.1% Tween 20 in PBS

Blocking buffer: 5% (w/v) milk powder in PTw

1° AB solution: 5% (w/v) milk powder, 1:2,000 to 1:10,000 dilution of primary antibody in PTw

2° AB solution: 5% (w/v) milk powder, 1:10,000 to 1:20,000 dilution of secondary antibody coupled to horseradish peroxidase (HRP) in PTw

Proteins were separated by denaturing SDS-polyacrylamide gel electrophoresis according to Sambrook and Russell, 2001 and transferred for 1 hour to a nitrocellulose membrane (0.45 μm, Schleicher & Schuell) for 1 hour using the semi-dry blotting method (Sambrook and Russell, 2001). The membrane was blocked overnight in blocking buffer. After two brief washes in PTw, the membrane was incubated with 1° AB solution for 2 hours. After two brief washes in PTw, one 20 min in PTw and two brief washes in PTw, the membrane was incubated in 2° AB solution for 1 hour. After two brief washes and one for 20 min in PTw, the ECL Direct™ nucleic acid labeling and detection system was used to visualize the proteins (Amersham).

2.18 cDNA library generation

To identify early ESR1 target genes in *Xenopus*, a hormone inducible antimorphic construct, ESR1-VP16-GR was generated, which activates X-ngnr-1 expression in whole embryos as well as in the animal cap system. Animal caps of ESR1-VP16-GR injected embryos were prepared, divided into two populations, of which one was induced at stage 10 with dexamethasone. After three hours at 14°C, 80 animal caps of each population were collected and total RNA was prepared. The dexamethasone treated RNA population (ESR1-VP16-GR, +Dex) should be enriched with early ESR1-VP16-GR targets and served as “Tester” during the cDNA substraction, while the uninduced RNA population (ESR1-VP16-GR, -Dex) served as “Driver”. Differentially expressed cDNAs were identified using the PCR cDNA Substraction Kit (Clontech) according to the manufacturer's protocol. The SMART™ PCR cDNA Synthesis Kit (Clontech) was used to pre-amplify 1 µg total RNA of each population according to the manufacturer's protocol. During

the subtractive hybridization procedure, cDNAs represented in both populations are removed and only cDNAs, which are present in the tester (ESR1-VP16-GR, +Dex) are left for PCR amplification. After two rounds of PCR amplification, the PCR products were purified using the PCR purification Kit (Qiagen) and eluted in 30 µl elution buffer. The fragments were ligated into the pGEM-T T/A cloning vector (Promega). 2304 clones were picked and used for microarray-based validation.

2.19 Microarray

A microarray based approach was used to identify strongly upregulated genes of ESR1-VP16-GR within the enriched cDNA library. In addition to 2304 clones of the cDNA library enriched with ESR1-VP16-GR target genes, 9,216 clones of an oocyte cDNA library enriched with vegetally localized RNAs (Claussen and Pieler, 2004) as well as 15,912 clones of the RZPD clone set RZPDp988 were spotted on each slide to improve normalization of individual signal intensities and identify additional ESR1-VP16-GR targets. To increase the statistical significance of the result, a total of ten arrays (dye swap + five technical replicates) were used during the hybridization, which followed the swap design (Kerr and Churchill, 2001). The total RNA from dexamethasone treated ESR1-VP16-GR animal caps and dexamethasone treated control caps were used as hybridization targets to eliminate genes, which are upregulated by the dexamethasone treatment alone. Each slide was hybridized with a mixture of 2 µg of Cy3-labeled and 2 µg Cy5-labeled probe.

2.19.1 Slide generation

cDNA clones were amplified in a 100 µl PCR reaction, purified using the Beckman-Coulter Biomek 2000 pipette robot, eluted in 45 µl spotting solution I (Schott Nexterion Spot I) and spotted on Schott Nexterion Slide E epoxy slides with the VersArray Chipwriter Pro robot (Bio-Rad). After incubation once in 0.1X Triton X-100 for 5 min, twice in 3.7% HCl for 2 min and once in 10 mM KCl for 10 min, the slides were boiled for 3 min in H_2O and blocked in 1X Nexterion Block E blocking solution at 50°C for 50 min (Schott).

2.19.2 Target generation

Total RNA was isolated using an optimized Trizol (Invitrogen) method. The RNA quality and quantity was determined with an Agilent 2100 BioAnalyzer and a NanoDrop ND-1000 spectrometer. The SMART cDNA Fluorescent Probe Amplification Kit (Clontech) was used according to the manufacturer's protocol with the following modification: progress of the amplification was monitored by removing aliquots of the ongoing PCR reaction. The DNA amount was quantified using PicoGreen (Invitrogen). All PCR reactions were subsequently stopped before the reached the plateau phase. This kit provides for indirect, "two-step" labeling of the target cDNA. Two-step labeling typically incorporates higher levels of label than direct, single-step procedures that directly incorporate fluorescently tagged nucleotides during cDNA synthesis. After single strand (ss) cDNA synthesis using 2.25 µg total RNA (split into 3 reactions, 750 ng total RNA each) for each RNA pool, the reactions were purified using the PCR Purification Kit (Qiagen). Ten amplification reactions were set up for each condition, using 100 ng ss cDNA as template, yielding a total of around 25 µg ds cDNA. This ds cDNA was used as template to produce aminoallyl-dUTP modified fragments with random primers, to which the N-hydroxysuccinimide activated Cy-Dyes (GE Healthcare) were coupled to in a second step. After removal of unbound dye, the dye incorporation rate was determined with Nanodrop ND-1000..

2.19.3 Hybridization and analysis

Hybridization buffer: 50% Formamide, 5X SSC, 0.1 % SDS, 5X Denhardt`s, 0.5 mg/ml mouse Cot-1 DNA, 0.125 mg/ml poly(d)A

The two Cy-labeled samples were combined (2 µg of each pool for one slide), concentrated using a speedvac and dissolved in 40 µl hybridization buffer. After a 5 min denaturation step at 95°C, the hybridization mix was uniformly distributed on the slides and incubated at 42°C overnight overnight in a sealed chamber. The following steps were performed in an ozone free environment: After an initial wash in 2X SSC including 0.2 % SDS for 10 min and another wash in 2X SSC (10 min) and 0.2X SSC (10 min), the slides were dried in a nitrogen stream and scanned using a microarray scanner (Agilent,

G2625BA). Computational analysis was done as described in (Landgrebe et al., 2004).

3 Results

3.1 Characterization of Xmxi1

3.1.1 Expression of Xmxi1

In a large-scale expression screen aimed at identifying novel *Xenopus* genes in the context of neurogenesis, one clone exhibited broad expression throughout the territories of primary neurogenesis (Souopgui, 2002). The isolated clone was a fragment of the *Xenopus* homologue of Mxi1 (Xmxi1). Xmxi1 mRNA is first detected by whole-mount in situ hybridization analysis broadly throughout the dorsal ectoderm during gastrulation. The expression of Xmxi1 is very similar to one of the earliest proneural genes, X-ngnr-1 (Ma et al., 1996). As shown in Figure 11A, a direct comparison of their expression patterns during gastrulation reveals that Xmxi1 expression domains are much broader and prefigure the domains of primary neurogenesis demarcated by X-ngnr-1. At stage 13 in the posterior neural plate, transcripts are localized in three broad stripes on both sides of the dorsal midline, encompassing the medial, intermediate and lateral proneural domains that will later give rise to the motor-, inter- and sensory neurons, respectively (Figure 11E).

Xmxi1 transcripts are also found in additional cell populations of the anterior neural plate that express other proneural genes including the trigeminal and olfactory placodes, as well as cells of the future ventral midbrain and hindbrain. Double labeling of stage 14 embryos with Xmxi1 and Sox3, a panneural marker expressed in proliferating cells, showed that their expression domains are partially overlapping, including the different layers of the posterior neuroectoderm (Figure 11B-D). In contrast to Sox3, Xmxi1 is excluded from the anterior neural plate, a territory in which neuronal differentiation is delayed until the tadpole stage (Papalopulu and Kintner, 1996). Moreover, the lateral stripe of Xmxi1, similar to that of the neuronal differentiation marker N-tubulin, is outside of the territory stained by Sox3 (Figure 11C, arrowhead) (Hardcastle and Papalopulu, 2000). During later neurula stages, Xmxi1 continues to be broadly expressed throughout the neural plate but is excluded from the anterior neural folds (Figure 11F). A

transversal section of a stage 16 embryo revealed that Xmxi1 transcripts are now predominately restricted in the deep layer of the neuroectoderm where primary neurogenesis occurs (Figure 11G) (Hartenstein, 1989).

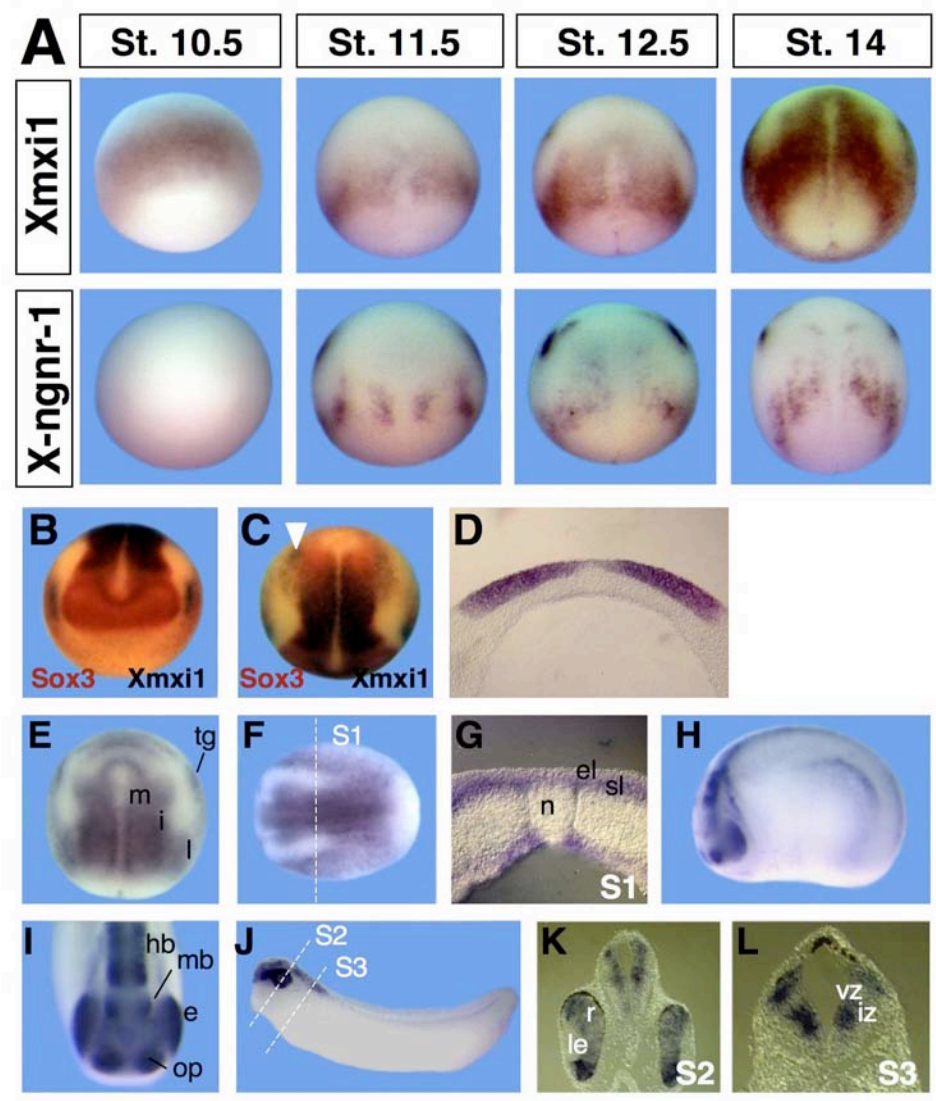

Figure 11 Expression of Xmxi1. (**A**) Whole-mount in situ hybridization expression analysis of Xmxi1 in comparison with X-ngnr-1. (**B-D**) Double labeling of Xmxi1 and Sox3 expression. Sox3, red; Xmxi1, dark blue. (**B**) Stage 14, anterior view. (**C**) Stage 14, dorsal view. White arrowhead marks the lateral stripe of Xmxi1. (**D**) Transversal section of B. (**E-L**) Spatial expression of Xmxi1 during later stages of *Xenopus* development. (**E**) Stage 13, dorsal, anterior up. (**F**) Stage 16, dorsal view, anterior left. (**G**) S1 transversal section. (**H**) Stage 22, lateral. (**I**) Stage 27, anterior (**J**) Stage 37, lateral. (**K**) S2 transversal section. (**L**) S3 transversal section, dark brown staining at the roof of the spinal chord is pigmentation not Xmxi1 staining. el, epithelial layer; hb, hindbrain; i, intermediate; iz, intermediate zone; l, lateral; le, lens; m, medial; mb, midbrain; n, notochord; op, olfactory placode, r, retina; sl, sensory layer; st, stage; tg, trigeminal placodes; vz, ventricular zone.

At stage 27, Xmxi1 expression was detected throughout the CNS including the eye, the olfactory placodes, midbrain, hindbrain and spinal cord (Figure 11I). In a transversal section of a stage 37 embryo, expression of Xmxi1 in the eye is restricted to the retina (Figure 11K), while the expression

in the neural tube is located in the proliferating neural progenitors of the ventricular zone and the intermediate zone, where cells start to differentiate (Figure 11L).

3.1.2 Regulation of Xmxi1

An early role for Xmxi1 in the regulation of primary neurogenesis is strongly suggested by both the temporal and spatial expression of Xmxi1. To study the regulation of Xmxi1, the influence of known positive and negative regulators of neurogenesis on Xmxi1 expression in whole embryos was evaluated (Figure 12). Synthetic mRNAs were injected into one blastomere of two-cell stage embryos together with LacZ mRNA as a lineage tracer. The embryos were subjected to whole-mount in situ hybridization after X-gal staining, to visualize the side of injection and marker gene expression. The injected half of the embryo is visualized by light blue staining. The panneural HMG-box transcription factor Sox3 ectopically activates Xmxi1 expression in the whole embryo (96%, n=23), while it inhibits the neuronal differentiation marker N-tubulin at open neural plate stage (92%, n=60) (Figure 12A, B). X-ngnr-1 also ectopically activates Xmxi1 expression (100%, n=37), which is similar to its effect on N-tubulin (Figure 12C, D). The downstream proneural transcription factor NeuroD also strongly induces Xmxi1 and N-tubulin within the neural plate, as well as in the nonneural ectoderm (87%, n=45) (Figure 12E, F).

In addition to its proneural activity, X-ngnr-1 activates the Notch pathway, which restricts the number of cells that undergo neuronal differentiation. To determine the influence of Notch signaling on the expression of Xmxi1, Notch signaling was increased or decreased, by overexpression of the intracellular domain of XNotch1 (Notch-ICD) or a dominant-negative version of X-Su(H) mutated in the DNA binding domain (DN-SuH) (Chitnis et al., 1995; Wettstein et al., 1997). Injection of mRNA encoding Notch-ICD negatively regulated Xmxi1 (96%, n=24) expression, as well as X-ngnr-1 (Figure 12G, H). Conversely, DN-SuH increased the density of Xmxi1 (92%, n=27) and X-ngnr-1 in the domains of primary neurogenesis (Figure 12I, J). These results demonstrate that Xmxi1 is negatively regulated by lateral inhibition.

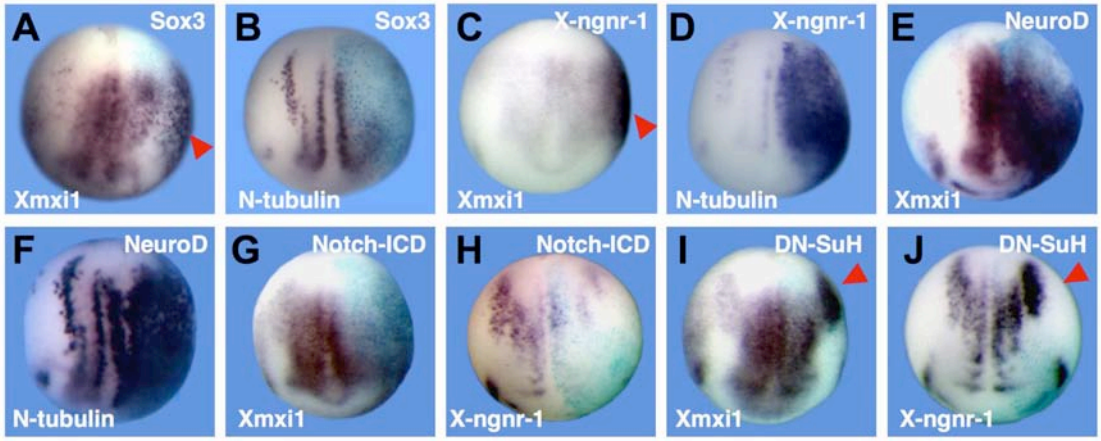

Figure 12 Regulation of Xmxi1 expression during primary neurogenesis. (**A-J**) Whole-mount in situ hybridization of stage 14 embryos injected with the corresponding RNAs as indicated in the upper right hand corner. The antisense probes used are indicated in the lower left hand corner. The injected side (ß-gal, light blue) is on the right and embryos are shown as a dorsal view, anterior down. Overexpression of Sox3 (50 pg) induces ectopic expression of Xmxi1 (**A**), while inhibiting N-tubulin expression (**B**). X-ngnr-1 (50 pg) and NeuroD (500 pg) induces ectopic expression of Xmxi1 (**C** and **E**) and N-tubulin (**D** and **F**). Activation of the Notch pathway by overexpression of Notch-ICD (50 pg) represses Xmxi1 (**G**) and X-ngnr-1 (**H**). In contrast, inhibition of the Notch pathway by the DN-SuH (300 pg) increased the density of Xmxi1 (**I**, arrowhead) and X-ngnr-1 (**J**, arrowhead).

The regulation of Xmxi1 was also investigated in ectodermal explants (animal caps) from blastula stage embryos; these explants are normally fated to become epidermal tissue, but can be converted to derivatives of all three germ layers. The animal blastomeres of two-cell stage *Xenopus* embryos were injected bilaterally with mRNA and animal caps were dissected at blastula stage. Total RNA was isolated at stage 14 and analyzed by RT-PCR. As shown in Figure 13, animal caps neuralized with the BMP inhibitor noggin afford a strong induction of the panneural markers Sox3 and NCAM, but Xmxi1 transcript levels do not significantly increase. While Sox3 overexpression in whole embryos afforded ectopic activation of Xmxi1 (Figure 12A), the Sox3 levels induced by noggin may be insufficient for activation in animal caps. Consistent with its ability to activate Xmxi1 in the nonneural ectoderm of whole embryos, X-ngnr-1 robustly activated not only N-tubulin and NCAM, but also Xmxi1 in animal caps, as well as in whole embryos (Figure 12C, D, Fig.13). Interestingly, Sox3 was not induced by X-ngnr-1 in the animal cap assay as well as in whole embryos (Fig.13). This may reflect the incompatibility with neuronal differentiation as previously suggested by their mutual exclusive expression patterns (Bourguignon et al., 1998; Bellefroid et al., 1998). We also investigated the regulation of Xmxi1 by SoxD, an early panneural transcription factor that can induce both neural and

neuronal differentiation in ectodermal explants (Mizuseki et al., 1998). As shown in Fig.13, SoxD activates Xmxi1.

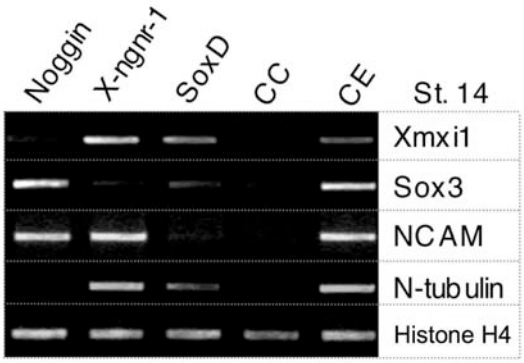

Figure 13 Regulation of Xmxi1 in animal caps. Inhibition of BMP signaling by noggin (50 pg) is not sufficient to activate Xmxi1 or N-tubulin although it neuralized the animal caps as seen by expression of Sox3 and NCAM. X-ngnr-1 (25 pg) strongly activates Xmxi1 and N-tubulin, but cannot activate the early panneural marker Sox3. SoxD (200 pg) moderately activates Xmxi1 and N-tubulin. Histone H4 was used as loading control. Injected RNAs are listed at the top; CC, control caps; CE, control embryos.

3.1.3 SoxD activates Xmxi1 in the absence of X-ngnr-1

Since overexpression of SoxD also increases the levels of X-ngnr-1, activation of Xmxi1 may be indirect (Yeo and Gautier, 2005). Therefore, we asked if the induction of Xmxi1 transcripts by SoxD was maintained in the absence of X-ngnr-1. Towards this end, an antisense morpholino oligonucleotide (MO) was designed targeting X-ngnr-1 mRNA (Figure 14A). The MO binds to the mRNA and blocks the translation of the endogenous target mRNA, therefore lowering the concentration of the corresponding protein, while the mRNA levels remain constant (Heasman et al., 2002). In an *in vitro* coupled transcription/translation assay the X-ngnr-1-MO, but not a control MO, inhibited translation of X-ngnr-1 (Figure 14B). Correspondingly, in whole embryos, the X-ngnr-1-MO inhibited neuronal differentiation on the injected side (loss of N-tubulin, 85%, n=39) (Figure 14C). The control MO did not alter the expression of N-tubulin (Figure 14D). Moreover, the loss of N-tubulin expression upon injection of the X-ngnr-1-MO could be rescued by coinjection of X-ngnr-1 mRNA (100%, n=110, no loss of N-tubulin plus ectopic N-tubulin) (Figure 14E).

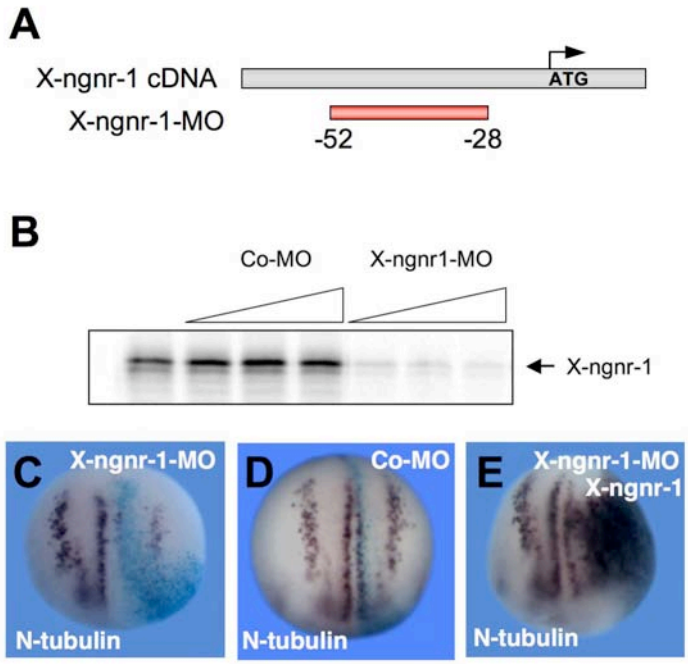

Figure 14 X-ngnr-1-MO inhibits primary neurogenesis. (**A**) Sequence and location of X-ngnr-1-MO with respect to the transcriptional start site of X-ngnr-1. (**B**) Morpholino specificity test using an *in vitro* transcription/translation assay (TNT, Promega), demonstrating the effectiveness of X-ngnr-1-MO. As template, a construct containing a partial 5´-UTR and the coding region of X-ngnr-1 was used. The protein was visualized by the incorporation of ^{35}S-Met. The concentrations of the MO were 0.02 µg, 0.2 µg, 2 µg in 12.5 µl total reaction volume. (**C**-**E**) Whole-mount in situ hybridization of N-tubulin expression of stage 14 embryos injected with X-ngnr-1-MO (15 ng), standard control MO (Co-MO) (15ng) and X-ngnr-1 (25 pg) as indicated in the upper right hand corner. The injected side (ß-gal, light blue) is on the right and embryos are shown as a dorsal view, anterior down.

As shown by real time PCR analysis, overexpression of SoxD alone or in combination with the X-ngnr-1-MO increased Xmxi1 transcript levels in animal caps to the same extent. However, as anticipated, the X-ngnr-1-MO but not the standard control MO inhibited SoxD induction of N-tubulin. (Figure 15). This loss of N-tubulin expression was not due to inhibition of X-ngnr-1 transcript levels and could be rescued by coinjection of X-ngnr-1 mRNA further demonstrating specificity of the MO. The above results demonstrate that Xmxi1 is regulated by proneural and panneural genes, further supporting an early role for Xmxi1 in the context of primary neurogenesis.

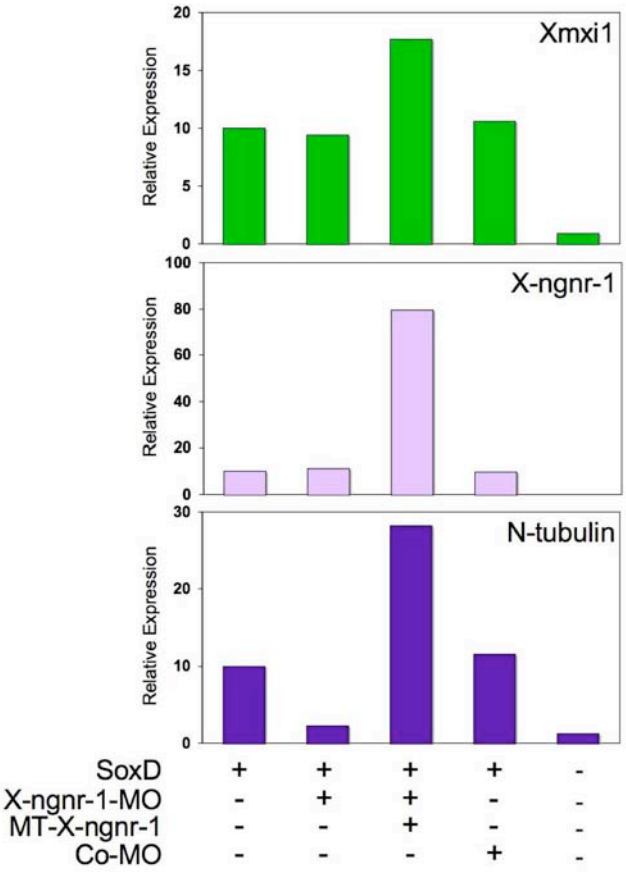

Figure 15 SoxD activation of Xmxi1 does not require X-ngnr-1. Real-time RT-PCR analysis of animal caps isolated from embryos injected with SoxD (200 pg), X-ngnr-1-MO (15 ng), X-ngnr-1 (20 pg) and standard control MO (Co-MO) (15 ng) as indicated and cultured until sibiling embryos reached stage 14. Expression levels were normalized to ornithine decarboxylase (ODC) and compared to the induction capacity of SoxD, which was set to 10. Note X-ngnr-1 RT primers detect both endogenous and injected X-ngnr-1 RNA.

3.1.4 Xmxi1 is essential for primary neurogenesis

The requirement of Xmxi1 during primary neurogenesis was evaluated using a MO to inhibit translation of the endogenous Xmxi1 (Figure 16A). To test the MO for specificity, an *in vitro* coupled transcription/translation reaction was performed. Increasing amounts of Xmxi1-MO blocked translation of Xmxi1. A five nucleotide mismatch MO (MM-Xmxi1-MO), which should not bind to the 5´UTR of Xmxi1, does not inhibit translation (Figure 16A, B). A second MO, which binds to a different site in the 5´UTR of Xmxi1 (Xmxi1-MO2), also blocks the translation of MT-Xmxi1 *in vitro*.

To test the specificity of the MO *in vivo*, the 5´UTR of Xmxi1 was fused to the open reading frame of GFP (5´UTR-Xmxi1-GFP). The RNA was injected in both blastomers of the 2-cell stage and GFP expression was visualized at gastrula stages (Figure 16C). Coinjection of Xmxi1-MO only

blocked the translation of 5´UTR-Xmxi1-GFP mRNA and not mRNA encoding GFP alone. The MM-Xmxi1-MO as well as the standard control MO did not inhibit translation of the corresponding mRNAs (Figure 16C).

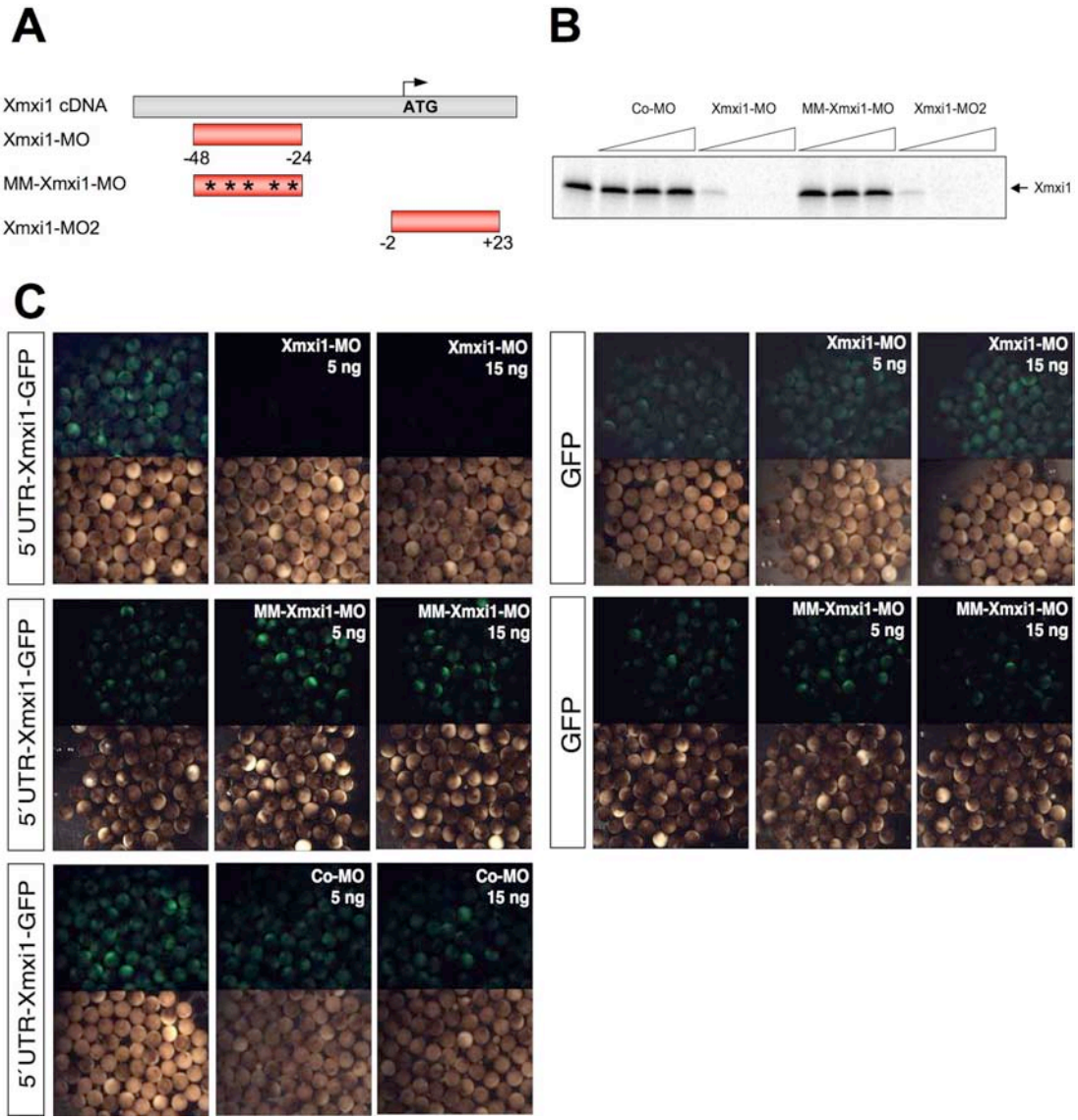

Figure 16 Xmxi1 *in vitro* and *in vivo* morpholino specificity test. (**A**) Sequence and location of Xmxi1-MO, Xmxi1-MO2 and MM-Xmxi1-MO with respect to the transcriptional start site of Xmxi1 mRNA. Asterisk indicate locations of mismatches. (**B**) *In vitro* transcription/translation assays (TNT, Promega), demonstrating the effectiveness of the indicated morpholino oligonucleotides (Xmxi1-MO and Xmxi1-MO2, MM-Xmxi1-MO and Co-MO). As template, a construct containing a partial 5´-UTR and the coding region of Xmxi1 was used. The protein was visualized by the incorporation of ^{35}S-Met. The concentrations of the MO were 0.02 µg, 0.2 µg, 2 µg in 12.5 µl total reaction volume. (**C**) Xmxi1-MO specifically blocks translation *in vivo*. The 5´-UTR of Xmxi1 was fused in front of the GFP coding sequence (5´-UTR-GFPpCS2). The 5´-UTR-Xmxi1-GFP (200 pg) or GFP (200 pg) mRNA was injected alone or together with Xmxi1-MO, MM-Xmxi1-MO or Co-MO as indicated in the upper right hand corner into both blastomeres of 2-cell stage *Xenopus* embryos. Embryos were analyzed for GFP fluorescence at stage 10.5 to 11.

Injection of the Xmxi-MO (12.5 ng) into one dorsal blastomere of four-cell stage embryos, led to an inhibition of neuronal differentiation, as marked by N-tubulin (86%, n=67) (Figure 17A). The MM-Xmxi1-MO did not

alter N-tubulin expression (100%, n=48) (Figure 17H). The suppression of N-tubulin by the Xmxi1-MO was not due to the loss of the neural precursor cell population, as Sox3 expression was not significantly altered (93%, n=23) (Figure 17D). However, the early proneural genes X-ngnr-1 (72%, n=79) and X-MyT1 (80%, n=15) were both inhibited by the Xmxi1-MO (Figure 17B, C). To further demonstrate specificity of the loss-of-function phenotype, Xmxi1-MO2 was injected. Xmxi1-MO2 also inhibited expression levels of X-ngnr-1 (71%, n=76), X-MyT1 (73%, n=63) and N-tubulin (85%, n=79) (Figure 17E-G).

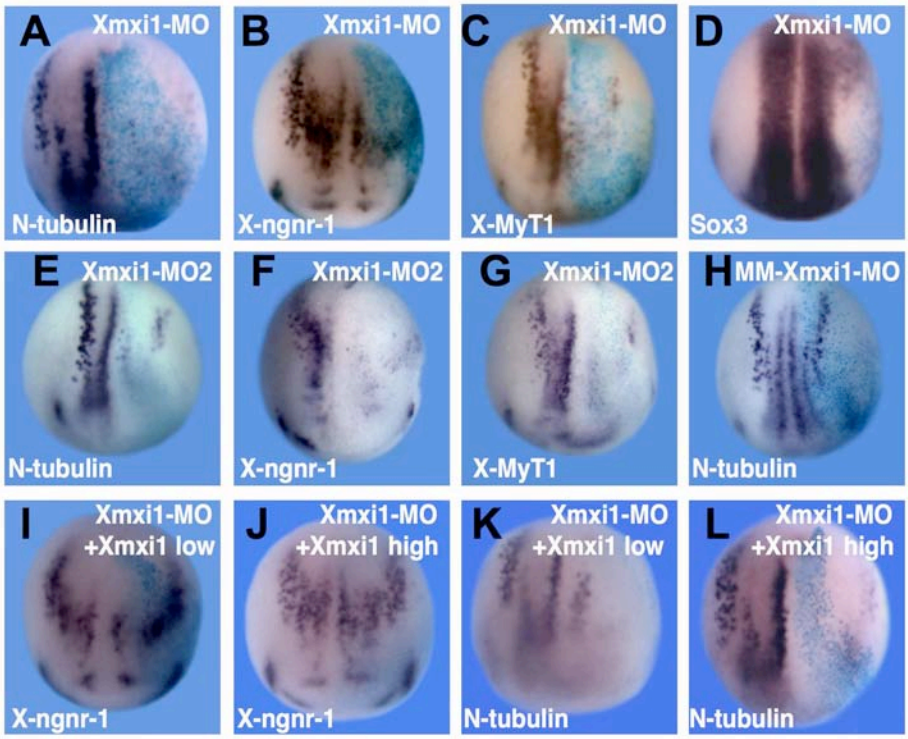

Figure 17 Xmxi1 is required for primary neurogenesis. (**A**-**L**) Whole-mount in situ hybridization of stage 14 embryos injected with Xmxi1-MO (12.5 ng), Xmxi1-MO2 (15 ng), mismatch morpholino MM-Xmxi1-MO (12.5 ng), MT-Xmxi1 (low (5 pg) or high (500 pg)) as indicated in the upper right hand corner. The antisense probes used are indicated in the lower left hand corner. The injected side (ß-gal, light blue) is on the right and embryos are shown as a dorsal view, anterior down.

The loss of X-ngnr-1 expression could be rescued by coinjection of a myc-tagged version of Xmxi1 (MT-Xmxi1) (5 or 500 pg) together with the morpholino, further demonstrating specificity of the MO (65% of the embryos, n=110, exhibited no reduction of X-ngnr-1 on the injected side) (Figure 17I, J). However, it should be noted that attempts to rescue the morpholino-induced inhibition of N-tubulin by titrating the concentration of Xmxi1 was not possible, even with doses as low as 5 pg (Figure 17K, L). The failure to rescue the loss of N-tubulin expression may partially be attributed to the finding that

MT-Xmxi1 overexpression results in a transient inhibition of neuronal differentiation downstream of X-ngnr-1 (see below).

3.1.5 Xmxi1 is required for X-ngnr-1 activation by SoxD

As SoxD is expressed earlier than Xmxi1, and SoxD activates transcription of both Xmxi1 and X-ngnr-1 (Figure 13 and Mizuseki et al., 1998), we asked if Xmxi1 is required for the function of SoxD. In whole embryos, overexpression of SoxD afforded a strong ectopic activation of X-ngnr-1 (ectopic X-ngnr-1, 90% n=20) that was effectively inhibited by the presence of the Xmxi1-MO (ectopic X-ngnr-1 with much weaker staining than SoxD alone, 30% n= 23) (compare Figure 18A, B). Moreover, this inhibition could be rescued by coinjection of MT-Xmxi1 (ectopic X-ngnr-1, 84% n=19) (Figure 18C).

To further provide evidence that Xmxi1 is required for SoxD function we used the animal cap assay coupled with real-time RT-PCR analysis. SoxD induced both the panneural markers Nrp-1 and NCAM, as well as X-ngnr-1 and N-tubulin (Figure 18D). However, in the presence of the Xmxi1-MO, but not the unspecific standard control MO (Co-MO), the activation of X-ngnr-1 by SoxD was significantly inhibited. Consistent with the results obtained in the whole embryo, the inhibitory effect of the Xmxi1-MO could be rescued by coinjection of MT-Xmxi1. In addition, the Xmxi1-MO suppressed the induction by SoxD of the late panneural markers Nrp-1 and NCAM.

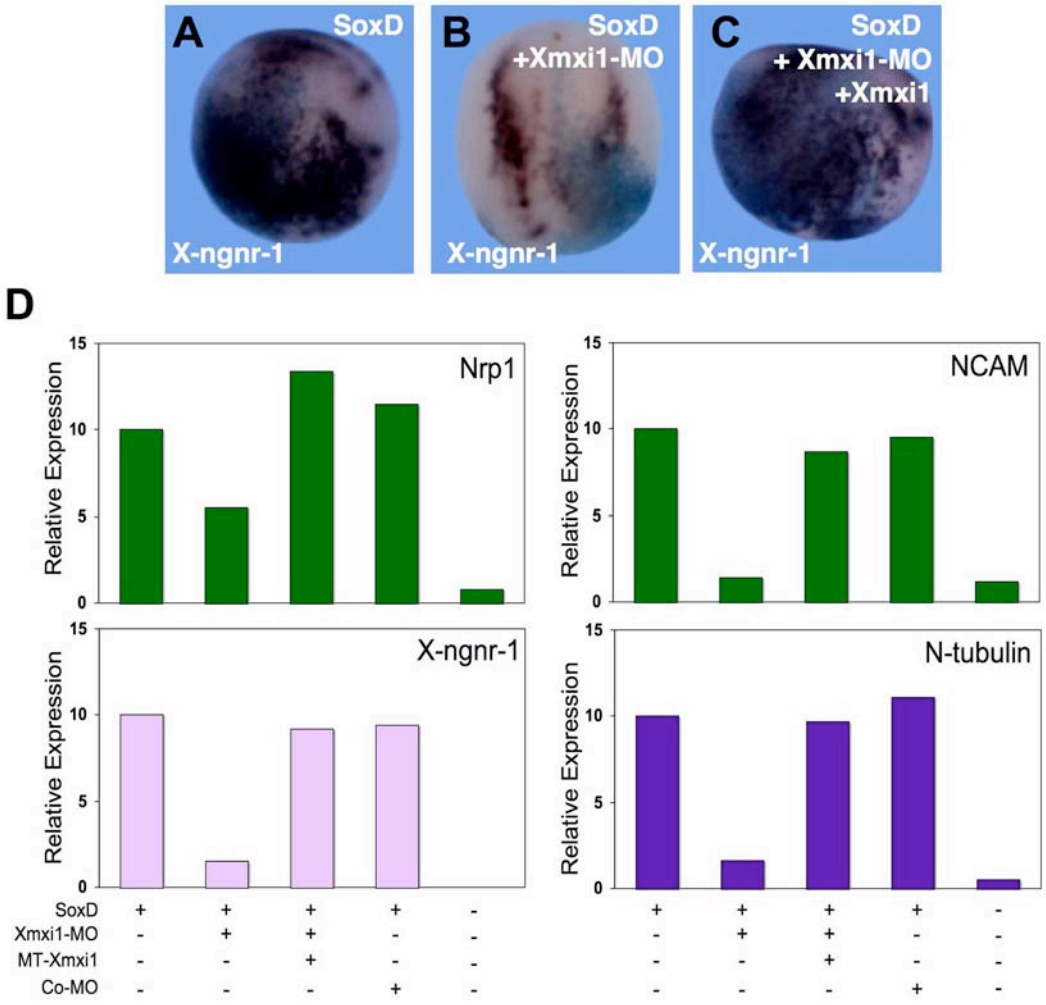

Figure 18 Xmxi1 is required for SoxD-induced neuronal differentiation. (**A-C**) Whole-mount in situ hybridization of stage 14 embryos injected with SoxD (200 pg), Xmxi1-MO (12.5 ng) or MT-Xmxi1 (500 pg) as indicated in the upper right hand corner. The antisense probes used are indicated in the lower left hand corner. The injected side (ß-gal, light blue) is on the right and embryos are shown as lateral view (A, C) or dorsal view, anterior down (B). (**D**) Real-time RT-PCR analysis of animal caps isolated from embryos injected with SoxD (200 pg), Xmxi1-MO (12.5 ng), Co-MO (12.5 ng) and MT-Xmxi1 (500 pg) as indicated and cultured until sibiling embryos reached stage 14. Expression levels were normalized to ODC and were compared to the induction capacity of SoxD injected animal caps, which was set to 10.

3.1.6 Xmxi1 is required for X-ngnr-1 induced neuronal differentiation.

The results obtained thus far argue for a role of Xmxi1 upstream of the proneural genes. However, as shown in Figure 12C, X-ngnr-1 ectopically activates Xmxi1 suggesting that Xmxi1 may also have a function downstream of the proneural genes. To investigate this possibility, the animal cap assay and real-time RT-PCR analysis was performed. In the presence of the Xmxi1-MO but not the Co-MO, the X-ngnr-1-induction of the downstream proneural genes (X-MyT1 and NeuroD) and N-tubulin were inhibited

(Figure 19). However, X-ngnr-1 not only induces neuronal differentiation mediated by the proneural genes, but also induces neuralization, as illustrated by the activation of general panneural markers such as Nrp-1 and NCAM. Interestingly, the Xmxi1-MO slightly reduced activation of Nrp-1 by X-ngnr-1, but NCAM, which marks the mature neural cells, was strongly inhibited (Figure 19) (Sasai et al., 2001). These results are consistent with the notion that Xmxi1 is an essential component of neurogenesis required to obtain a mature neural state that can respond to factors that induce neuronal differentiation.

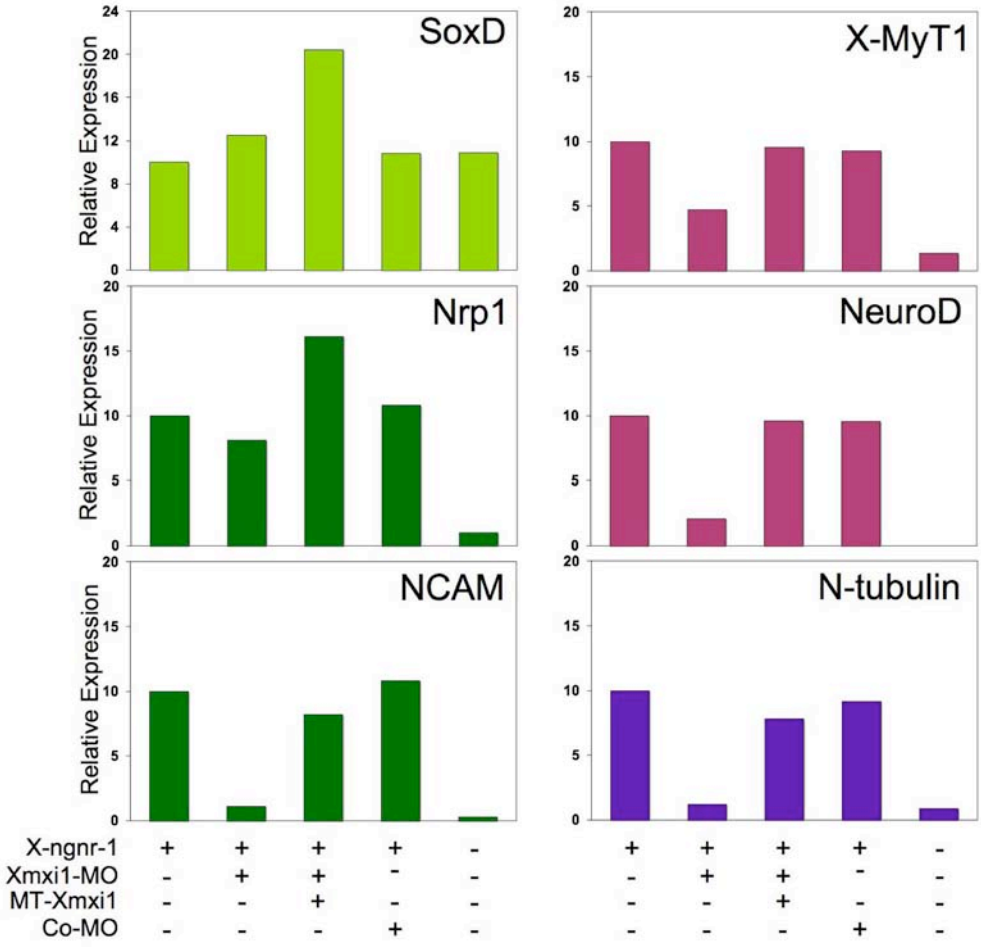

Figure 19 Xmxi1 is required for X-ngnr-1-induced neuronal differentiation. Animal caps were isolated from embryos injected with X-ngnr-1 (25 pg), Xmxi1-MO (12.5 ng), Co-MO (12.5 ng) and MT-Xmxi1 (500 pg) as indicated, cultured until sibiling embryos reached stage 14 and analyzed by real-time RT-PCR. Expression levels were normalized to ODC and were compared to the induction capacity of X-ngnr-1 injected animal caps, which was set to 10.

3.1.7 Xmxi1 overexpression inhibits differentiation

To further study the role of Xmxi1 in the context of primary neurogenesis, gain-of-function experiments in *Xenopus* embryos were performed. As

mammalian members of the Myc and Mad families have been found to exert a short half-life in cell culture (Hann and Eisenman, 1984; Ayer et al., 1993), we checked the stability of Xmxi1 in *Xenopus* embryos by injecting mRNA encoding for Xmxi1 bearing a Myc epitope (MT) at the N-terminus. During open neural plate stages, the Myc-tagged Xmxi1 protein is present as judged by western blot analysis (see Figure 27).

Injection of MT-Xmxi1 mRNA (500 pg) into the animal pole of one blastomere at the two-cell stage negatively influenced neuronal differentiation, as shown by the loss of N-tubulin expression on the injected side of the embryo (70%, n=134) (Figure 21A). The inhibition of primary neurogenesis was not the result of loss of the neural precursor pool as Sox3 expression was not reduced but dramatically expanded and found in the nonneural ectoderm of the injected side (77%, n=128) (Figure 21B). The increase in Sox3 expression occured at the expense of epidermis, as shown by the loss of epidermal keratin expression (72%, n=22) (Figure 21C). Thus, Xmxi1 and Sox3 exhibit positive reciprocal regulation (Figure 12B, 21B).

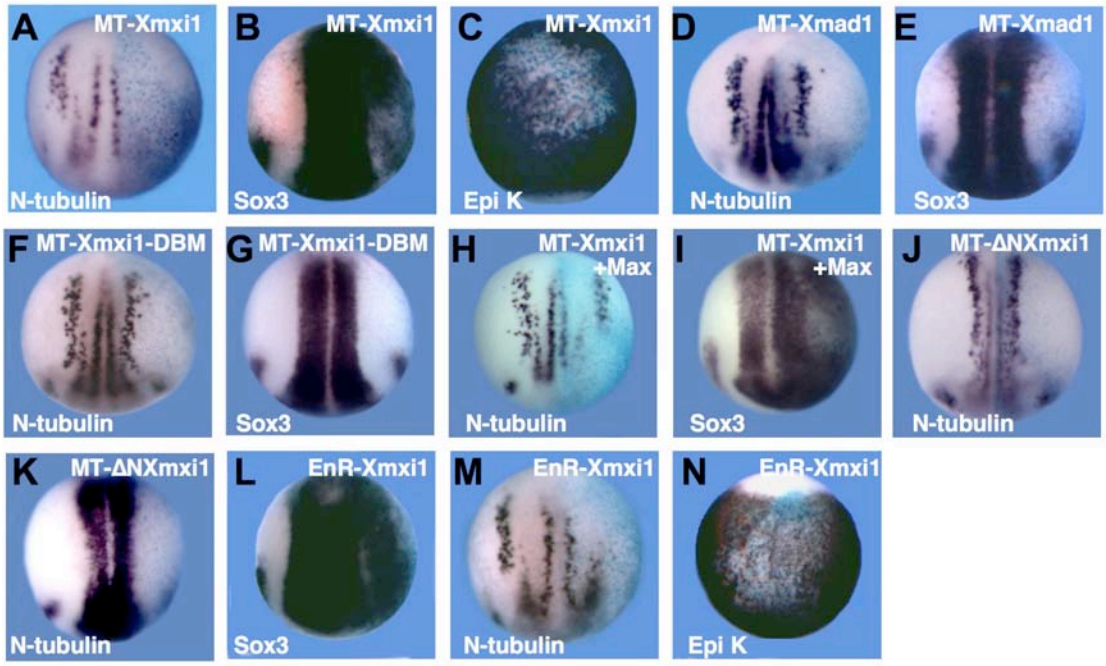

Figure 20 Xmxi1 activates ectopic Sox3 and inhibits neuronal differentiation. (**A-N**) Whole-mount in situ hybridization of stage 14 embryos injected with 500 pg of MT-Xmxi1, MT-Xmad1, MT-Xmxi1-DBM, MT-ΔNXmxi1, EnR-Xmxi1 or Max as indicated in the upper right hand corner. Antisense probes used are indicated in the lower left hand corner. The injected side is always on the right and all embryos are shown as dorsal views, anterior down with the exception of C and N, which are ventral views.

As all members of the Mad family have closely related SID repressor and bHLHZip domains, we therefore asked if the overexpression phenotype observed is shared with other members of the Myc/Max/Mad Network or unique to Xmxi1. The bHLH and SID domains of Xmad1 exhibit 76% and 80% identity compared to the corresponding domains in Xmxi1, but the overall homology is much lower (53%). Moreover, in contrast to Xmxi1, which is found primarily in proliferating tissue, Xmad1 is predominately found in post mitotic cells (Jürgenset al., 2005).

Overexpression of MT-Xmad1 (500 pg) did not alter the expression of N-tubulin or Sox3 (N-tubulin, 83%, n=34; Sox3, 83%, n=18) (Figure 20D, E). These results suggest that the overexpression phenotype is unique to Xmxi1. In addition, the activity of Xmxi1 was found to be dependent on its ability to bind DNA. Overexpression of MT-Xmxi1 containing a point mutation in the basic domain that is expected to disrupt DNA binding (MT-Xmxi1-DBM) did not induce ectopic activation of Sox3 and inhibition of N-tubulin expression (no effect, N-tubulin 86%, n=87; Sox3, 82%, n=93) (Figure 20F, G) (Fisher et al., 1993; Prochownik et al., 1998). Moreover, coinjection of MT-Xmxi1 together with Max, its putative binding partner, did not alter the effect of Xmxi1 on primary neurogenesis (inhibition of N-tubulin, 80%, n=21, ectopic Sox3, 76%, n=21) (Figure 20H, I). All these experiments support the observed ectopic activation of Sox3. Inhibition of neuronal differentiation by MT-Xmxi1 is most likely not due to an unspecific activity, such as the binding to non-target E-box sequences, the sequestration of Max or other bHLH transcription cofactors (Figure 21).

To determine if the SID repressor domain was required for Xmxi1 function in the context of primary neurogenesis, the SID repressor domain was removed. Overexpression of mRNA encoding MT-ΔNXmxi1 (500 pg) also did not alter the expression of Sox3 or N-tubulin, demonstrating the necessity of the repressor domain (no effect, N-tubulin, 92%, n=25; Sox3, 80%, n=50) (Figure 20J, K). Correspondingly, replacement of the SID domain with a heterologous repressor domain derived from the *Drosophila* Engrailed Repressor (EnR-Xmxi1) (Jaynes and O'Farrell, 1991) functioned in a similar manner to MT-Xmxi1, inducing ectopic Sox3 expression concomitant with a

loss of N-tubulin and epidermal keratin expression (Sox3, 70%, n=46 ectopic; EpiK, 82%, n=11 reduced; N-tubulin, 78%, n=32 reduced) (Figure 20L, N).

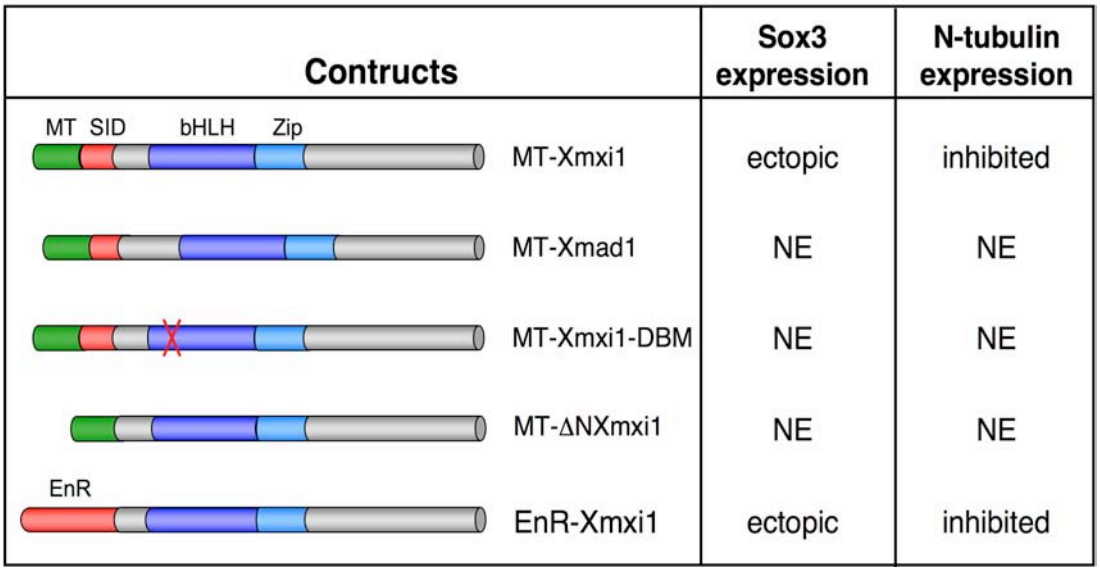

Figure 21 Summary of overexpression phenotypes of Xmxi1 constructs. Depicted are the overall features of the constructs as well as the influence on Sox3 and N-tubulin expression. Red indicates the repressor domain, blue the bHLHZipper domain and green the Myc epitope. NE, no effect.

3.1.8 Time window of Xmxi1 activity during primary neurogenesis

The competence of the neural ectoderm to respond to Xmxi1 was studied using an inducible version of Xmxi1 generated by fusion of the ligand binding domain of the human glucocorticoid receptor (GR) to the C-terminus of Xmxi1 (Xmxi1-GR) (Gammill and Sive, 1997). Temporal activation was achieved by treating injected embryos at various stages of embryonic development with dexamethasone. As shown in Figure 22, ectopic Sox3 and inhibition of N-tubulin by Xmxi1-GR was most effective if the treatment with dexamethasone was performed at the onset of gastrulation or earlier. At late gastrula stages, only a low number of embryos exhibited the described phenotype suggesting that the ability of Xmxi1 to induce a neural fate and inhibit neuronal differentiation is limited to early gastrula, when the neural plate is being established (Figure 22).

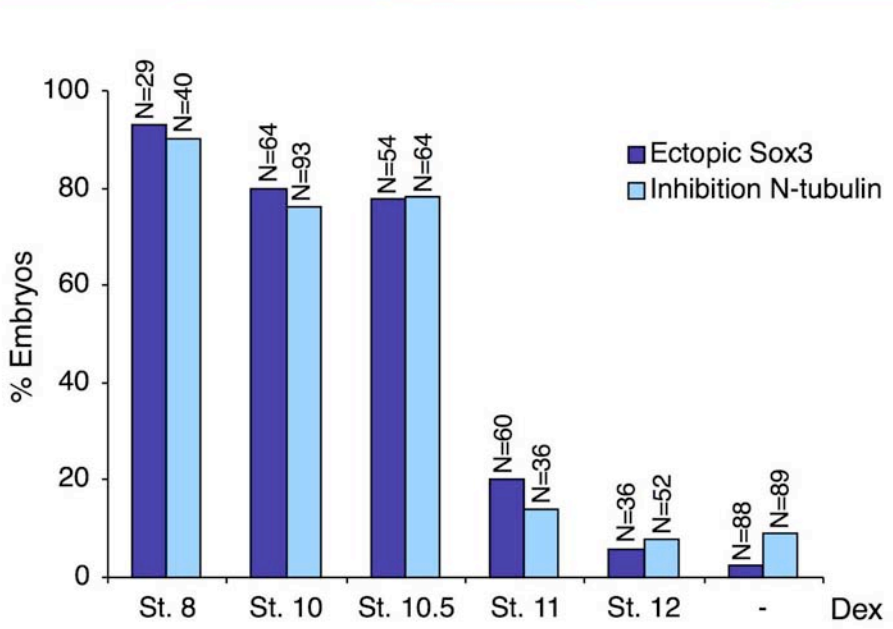

Figure 22 Competence of the neural ectoderm to respond to Xmxi1. A hormone inducible version of Xmxi1 (Xmxi1-GR, 500 pg) was injected in one blastomere of 2-cell stage embryos and activated at indicated developmental stages with dexamethasone. Graphical representation of the effect on Sox3 and N-tubulin expression as the percent of embryos exhibiting ectopic Sox3 with a loss of N-tubulin expression at stage 14.

3.1.9 Overexpression of Xmxi1 does not influence expression of early proneural transcription factors

The expansion of the neural plate and inhibition of neuronal differentiation is similar to the phenotype observed upon activation of Notch signaling (Coffman et al., 1993). However, the expression patterns of the Notch ligand X-Delta1 (100%, n=10) and ESR9 (75%, n=12), a direct Notch target gene, were not altered on the side injected with MT-Xmxi1 mRNA (Figure 23A, B). In addition, MT-Xmxi1 had no significant effect on early proneural markers such as X-ngnr-1 (83%, n=30), X-MyT1 (78%, n=32) and NeuroD (67%, n=27) (Figure 23C, E). The EnR-Xmxi1 afforded similar results as MT-Xmxi1, with the exception that NeuroD was suppressed in a significant percentage of the embryos (no change on the injected side, X-Delta1 94%, n=17; ESR9 74%, n=19; X-ngnr-1 100%, n=20, X-MyT1 88%, n=16 and down-regulated on the injected side NeuroD 67%, n=18) (Figure 23F, J).

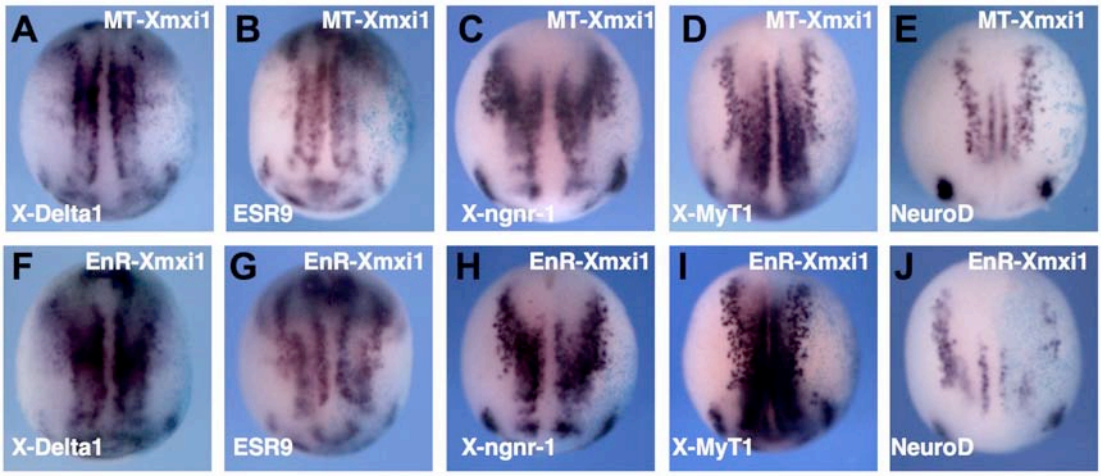

Figure 23 Overexpression of Xmxi1 does not influence neurogenic and early proneural genes. (**A-J**) Whole-mount in situ hybridization of stage 14 embryos injected with 500 pg of MT-Xmxi1 or EnR-Xmxi1. Antisense probes used are indicated in the lower left hand corner. The injected side is always on the right and all embryos are shown as dorsal views, anterior down.

3.1.10 Xmxi1 represses genes involved in cell cycle regulation

As the process of neuronal differentiation is tightly coupled with cell cycle exit and Xmxi1 ectopically activates Sox3, a marker that coincides with neural cell proliferation, the influence of Xmxi1 on the expression of factors known to regulate cell cycling in the context of primary neurogenesis was investigated. MT-Xmxi1 inhibited the expression of p21-activated kinase 3 (XPak3) (82%, n=55) and the cdk inhibitor $p27^{(Xic1)}$ (77%, n=44), which are required for cell cycle exit and differentiation of primary neurons (Figure 24A, B) (Souopgui et al., 2002; Carruthers et al., 2003; Vernon et al., 2003).

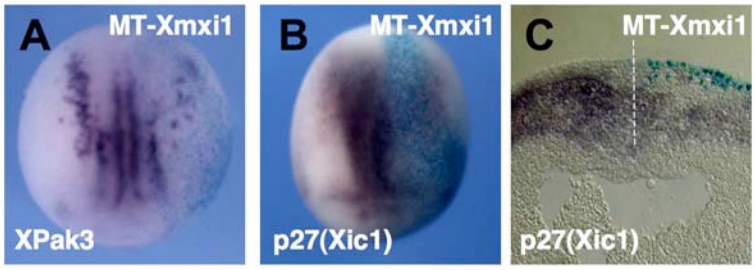

Figure 24 Xmxi1 inhibits genes required for cell cycle withdrawal. (**A**-**C**) Whole-mount in situ hybridization of stage 14 embryos injected with 500 pg of MT-Xmxi1. Antisense probes used are indicated in the lower left hand corner. The injected side is always on the right and all embryos are shown as dorsal views, anterior down. (**C**) Transversal section of B.

As shown in the transversal section in Figure 24C, MT-Xmxi1 inhibited $p27^{(Xic1)}$ in the neuroectoderm, as well as in the myotome, suggesting this may be a direct effect on $p27^{(Xic1)}$. The influence on these cell cycle regulating genes by Xmxi1 was also seen by overexpression of EnR-Xmxi1 (data not shown).

The results obtained by the gain-of-function experiments suggest that Xmxi1 expands the precursor population and inhibits differentiation events downstream of the early proneural transcription factors at the open neural plate stage. To gain additional support for this mechanism of action by Xmxi1, we employed the animal cap assay in combination with real-time RT-PCR. Injection of mRNA encoding for X-ngnr-1 in early cleavage embryos prior to explant preparation is sufficient to drive this nonneural ectoderm into differentiated neural tissue (Figure 25).

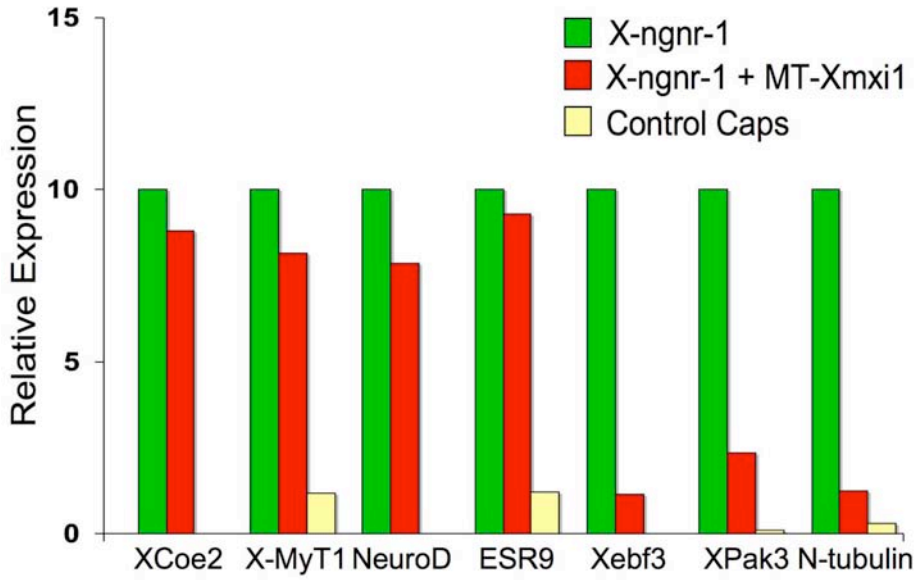

Figure 25 Xmxi1 blocks induction of late, but not early X-ngnr-1-induced target genes in animal caps. Real-time RT-PCR analysis of animal cap explants isolated from embryos injected with X-ngnr-1 (50 pg) alone or together with MT-Xmxi1 (500 pg) and cultured until sibling stage embryos reached stage 14. Expression levels were normalized to ODC and compared to the induction capacity of X-ngnr-1 injected animal caps, which were set to 10.

Coinjection of MT-Xmxi1 together with X-ngnr-1 did not alter the induction of early target genes of X-ngnr-1, such as XCoe2, X-MyT1, NeuroD or ESR9; however, X-ngnr-1-mediated activation of the late target genes XEbf3, XPak3 and N-tubulin was inhibited (Figure 26). Taken together, the results obtained thus far suggest that Xmxi1 maintains an early neuronal precursor state in neural ectoderm, resulting in the inhibition of neuronal differentiation at the open neural plate stage.

3.1.11 Xmxi1 inhibits neuronal differentiation only transiently

The inhibition of neuronal differentiation by SoxD is only transient (Yeo and Gautier, 2005; Mizuseki et al., 1998), therefore the ability of MT-Xmxi1 to influence neural and neuronal differentiation markers in both naive and neuralized animal caps was compared by use of animal caps that were cultured until siblings had reached stage 14 (early) or stage 20 (late) and analyzed by real-time RT-PCR. As shown in Figure 26A, in early animal caps MT-Xmxi1 weakly induces Sox3 and Nrp-1, but did not significantly alter the transcript levels of SoxD and NCAM. In contrast, the neural inducer noggin strongly activates all four panneural markers. Moreover, in early caps noggin and Xmxi1 alone or in combination did not induce X-ngnr-1 or N-tubulin. In late stage MT-Xmxi1-injected caps, the levels of Sox3 and Nrp-1 declined, but those of SoxD and NCAM were not altered. However, both X-ngnr-1 and N-tubulin were induced. The activation of X-ngnr-1 and N-tubulin was even stronger when Xmxi1 was coinjected with noggin. Animal caps injected with noggin alone did not strongly activate these markers.

To further confirm these results, we evaluated MT-Xmxi1-injected embryos at the early tailbud stage. As shown in Figure 26B-E, on the injected side, most embryos have small protrusions suggesting Xmxi1 may also increase proliferation. The ectopic Sox3 (45%, n=32) staining observed at the open neural plate stage persisted, with a reduced extent and percentage as compared to early neurula stages. The inhibition of N-tubulin was found to be transient, as wild-type levels were observed on the injected side of the neural tube.

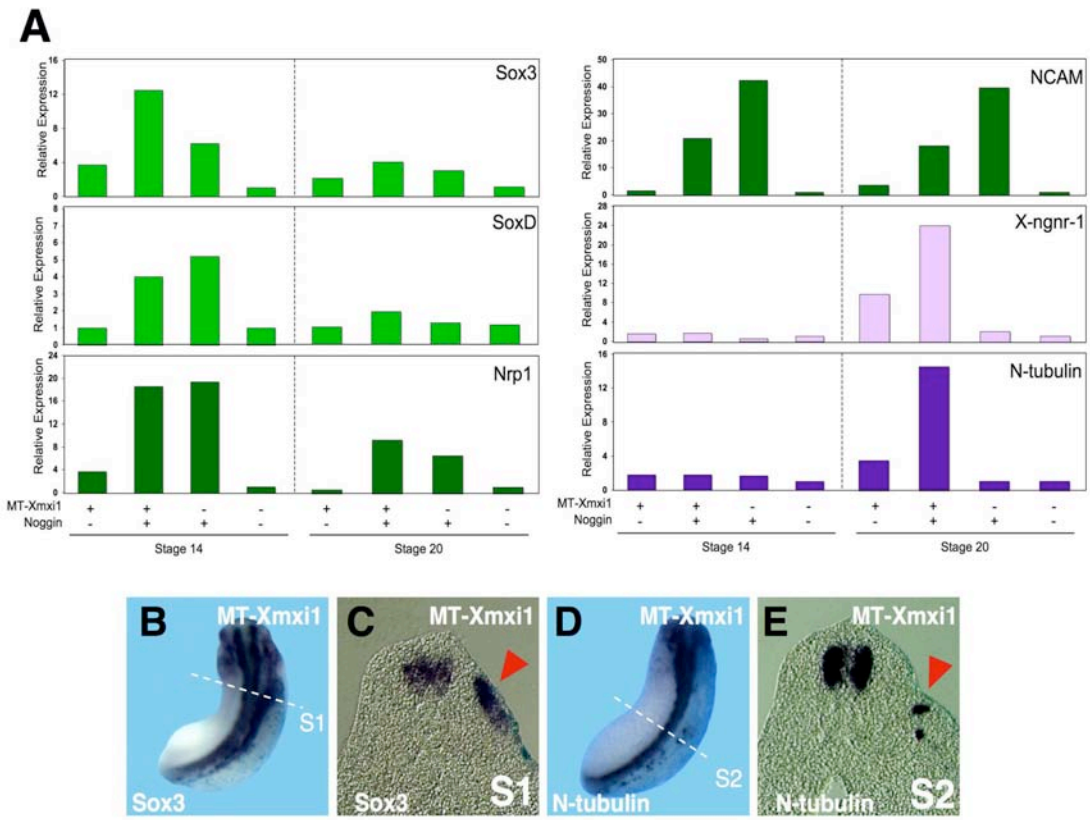

Figure 26 Xmxi1 induces delayed, ectopic neuronal differentiation. (**A**) Animal caps were isolated from embryos injected with noggin (25 pg) and MT-Xmxi1 (500 pg) as indicated. At the equivalent of stage 14 or 20, the caps were harvested and analyzed by real-time RT-PCR. Expression levels were normalized to ODC and compared to control caps, which were set to 1. (**B-E**) Whole-mount in situ hybridization of stage 14 and stage 22 embryos injected with 750 pg of MT-Xmxi1. At tailbud stages ectopic Sox3 is still present (B) and ectopic N-tubulin expression is also detected (D). (C, E) Transversal sections of B and D, respectively. Red arrowhead marks ectopic expression.

In addition, a limited amount of ectopic N-tubulin staining was detected (60%, n=25). Interestingly, as shown by Western blot analysis (Figure 27), by stage 22 the MT-Xmxi1 protein is no longer detectable suggesting that high levels of Xmxi1, similar to Sox3 may be incompatible with neuronal differentiation. At lower Xmxi1 concentrations (5-50 pg), an increase in neuronal differentiation was not observed (data not shown). However, at these lower concentrations, Sox3 was also not induced. Therefore, Xmxi1 can only induce a delayed neuronal differentiation in neuralized tissue consistent with the animal cap experiments.

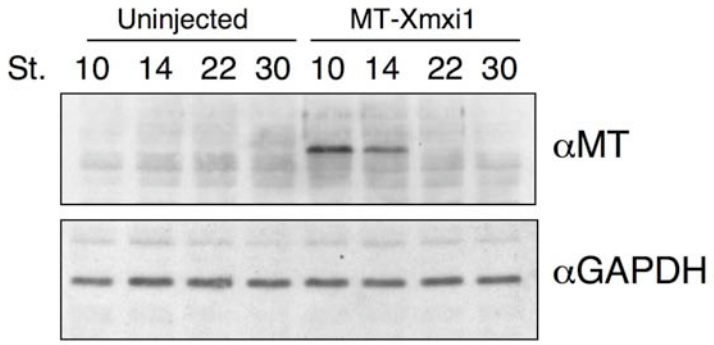

Figure 27 Stability of injected MT-Xmxi1. MT-Xmxi1 mRNA (500 pg) was injected into both blastomeres of the 2-cell stage *Xenopus* embryos and total cell extracts were prepared at the indicated stages. The lysates were separated by SDS-PAGE, and the tagged proteins detected by western blot analysis using an MT-antibody (9E10, Santa Cruz Biotechnology). After stripping, the membrane was re-probed using a GAPDH-antibody (ab9485, Abcam), which served as a loading control.

3.1.12 Xmxi1 induces proliferation

The inhibition of XPak3 and p27$^{(Xic)}$ as well as the increased Sox3 expression suggests a role of Xmxi1 in cell proliferation. Increased cell proliferation could be an explanation for the inhibition of neuronal differentiation at early neurula stages. A measurement for proliferation is the phosphorylation status of histone H3 (pH3). With the G2/M phase transition, mitotic chromatin condensation starts and the serine-10 of histone H3 is phosphorylated. This phosphorylation wave ends just prior to the formation of prophase chromosomes (Handzel et al., 1997; Saka and Smith, 2001). The number of pH3-positive cells in the neural plate of the injected side was compared with the uninjected side in serial sections.

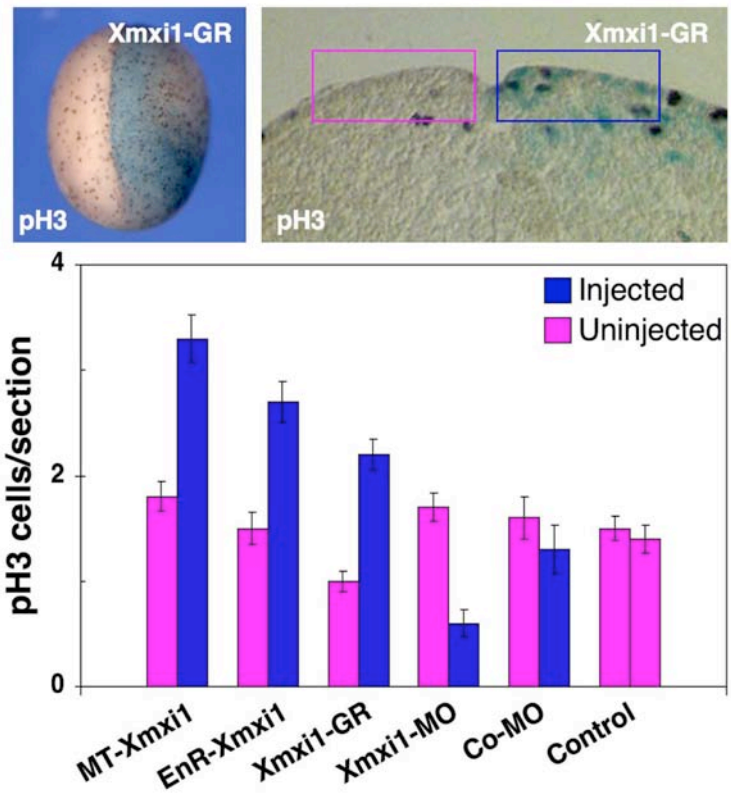

Figure 28 Xmxi1 induces proliferation in the open neural plate of *Xenopus* embryos. MT-Xmxi1 (500 pg), EnR-Xmxi1 (500 pg) and Xmxi1-GR (500 pg, induced at stage 10.5 with dexamethasone), Xmxi1-MO (12.5 ng) or the control-MO (Co-Mo) (12.5 ng) were injected in *Xenopus* embryos. Proliferation on the injected side compared with the noninjected side was measured by counting pH3 positive cells of 15 consecutive sections of five embryos in the open neural plate region. Shown is the average number of positive cells per embryo per section. Error bars indicate the standard error of the mean.

While sections from control embryos did not differ significantly in the number of pH3-positive cells on each side of the midline, overexpression of mRNA (500 pg) encoding for MT-Xmxi1 and EnR-Xmxi1 almost doubled the number of pH3-positive cells in comparison with the uninjected side. The hormone inducible version of Xmxi1 (Xmxi1-GR) also increased proliferation if induced at stage 10.5, where it ectopically activates Sox3 and inhibits N-tubulin expression (Figure 28 and Figure 23B). Moreover, injection of the Xmxi1-MO, but not of the control-MO significantly decreased cell cycling on the injected side. We also injected a lower concentration to determine if the effect of Xmxi1 on cell proliferation was maintained at lower concentrations. However, injection of a low dose of MT-Xmxi1 (50 pg) did not alter the number of pH3-positive cells on the injected side or the expression of Sox3 and N-tubulin (data not shown).

As Mxi1 overexpression results in the arrest of glioblastoma and prostate carcinoma cells in the G2/M phase, which would also result in an increase of pH3-positive cells, BrdU incorporation was measured to further evaluate the role of Xmxi1 on proliferation (Manni et al., 2002, Taj et al., 2001, Wechsler et al., 1997). During the BrdU assay, the pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) is incorporated into the replicating DNA in place of thymidine and can be detected by a specific antibody. Therefore BrdU is monitoring DNA synthesis, which is an indirect parameter of cell proliferation. Embryos were injected in the 2-cell stage and cultured until stage 15. BrdU was subsequently injected uniformly into the embryo and after incorporation for one hour, the embryos were collected.

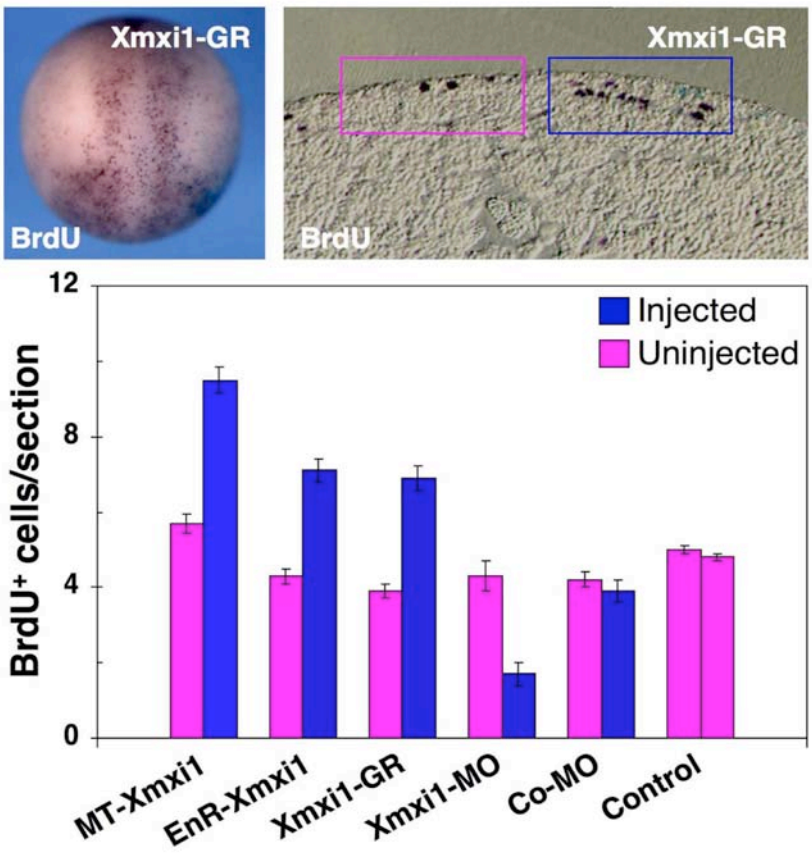

Figure 29 Xmxi1 induces proliferation in the open neural plate of *Xenopus* embryos. MT-Xmxi1 (500 pg), EnR-Xmxi1 (500 pg) and Xmxi1-GR (500 pg, induced at stage 10.5 with dexamethasone), Xmxi1-MO (12.5 ng) or the control-MO (Co-Mo) (12.5 ng) were injected in *Xenopus* embryos. Proliferation on the injected side compared with the noninjected side was measured by counting BrdU positive cells of 15 consecutive sections of five embryos in the open neural plate region. Shown is the average number of positive cells per embryo per section. Error bars indicate the standard error of the mean.

Consistent with the results obtained by pH3 staining, the MT-Xmxi1, EnR-Xmxi1 or Xmxi1-GR fusion constructs nearly doubled the number of

BrdU positive cells on the injected side, while the Xmxi1-MO, but not the control-MO, decreased the number of positive cells (Figure 29). These results suggest that Xmxi1 may maintain an undifferentiated neural state by activation of proliferation.

3.1.13 Xmxi1 functions independent from cell proliferation

The inhibition of neuronal differentiation by Xmxi1 could be the consequence of increased cell proliferation. Therefore we asked if the ectopic Sox3 and inhibition of N-tubulin expression, as observed upon overexpression of MT-Xmxi1, could be inhibited by forceful cell cycle arrest achieved through the treatment of the injected embryos with a cocktail of hydroxyurea and aphidicolin (HUA) (Harris and Hartenstein, 1991). Hydroxyurea inactivates ribonucleoside reductase by blocking the active site of the enzyme. This blocks synthesis of deoxynucleotides, which inhibits DNA synthesis and therefore forces cell cycle arrest and later apoptosis. Aphidicolin on the other hand is a reversible inhibitor of eukaryotic nuclear DNA replication. It inhibits alpha-type polymerase (also called primase), which initiates leading- and lagging-strand synthesis. The effectiveness of the inhibitors was verified by the failure of embryos to gastrulate when treated at stage 8 (Harris and Hartenstein, 1991). Embryos treated at stage 10 developed normally up to open neural plate stages, but exhibited strongly reduced pH3 staining further demonstrating the effectiveness of treatment (Figure 30).

Using the hormone inducible construct, Xmxi1-GR, we were able to block cell cycling before activating Xmxi1 activity. Ectopic Sox3 and inhibition of N-tubulin expression was still observed when the Xmxi1-GR injected embryos were treated with HUA at stage 10 and induced with dexamethasone at stage 10.5 (Figure 30) (-HUA, ectopic Sox3, 72%, n=19, inhibition N-tubulin 86%, n=31; +HUA, ectopic Sox3, 74%, n=15, inhibition N-tubulin 76%, n=24). These results suggest that both the Xmxi1-mediated induction of Sox3 and concomitant downregulation of N-tubulin do not directly depend on an increase in cellular proliferation.

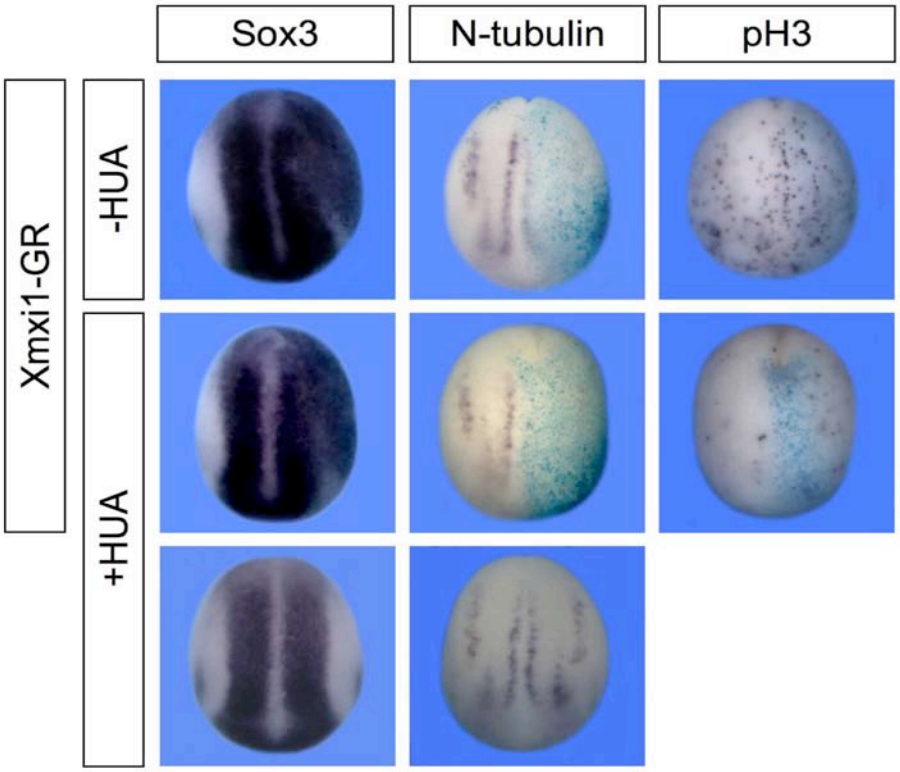

Figure 30 Forced cell cycle arrest does not alter the phenotype of Xmxi1 injected embryos. Whole-mount in situ hybridization or pH3 staining of stage 14 embryos injected with 500 pg of Xmxi1-GR as indicated to the left. Antisense probes used are indicated at the top. The injected side is always on the right and all embryos are shown as dorsal views, anterior down. Proliferation was blocked by HUA treatment at stage 10 and Xmxi1-GR was induced at stage 10.5 with dexamethasone.

3.1.14 Xmxi1 induces apoptosis

An alternative explanation for the absence of post-mitotic neurons upon overexpression of Xmxi1 could be due to the loss of these cells by apoptosis. The effect of Xmxi1 on programmed cell death during primary neurogenesis was therefore evaluated by TUNEL staining. During apoptosis “nicks” occur in the genomic DNA. Terminal deoxynucleotidyl transferase catalysis the binding of nucleotides (in the case of the TUNEL assay digoxygenin conjugated dUTP) to the free 3´-OH terminal of the DNA nicks, thereby labeling the apoptotic cell. As shown in Figure 31, injection of mRNA encoding for MT-Xmxi1 increased the amount of cells undergoing apoptosis. The increased number of apoptotic cells is most likely not sufficient to account for the loss of N-tubulin expression. To address if Xmxi1-induced apoptosis contributes to the activity of Xmxi1 during neurogenesis, MT-Xmxi1 was coinjected with the apoptosis inhibitor Bcl-2. Bcl-2 alone did not have an effect on Sox3 expression and slightly increased neuronal differentiation (Figure 31) (no

change Sox3 76%, n=24; increase N-tubulin 82%, n=23), as reported previously (Yeo et al., 2003).

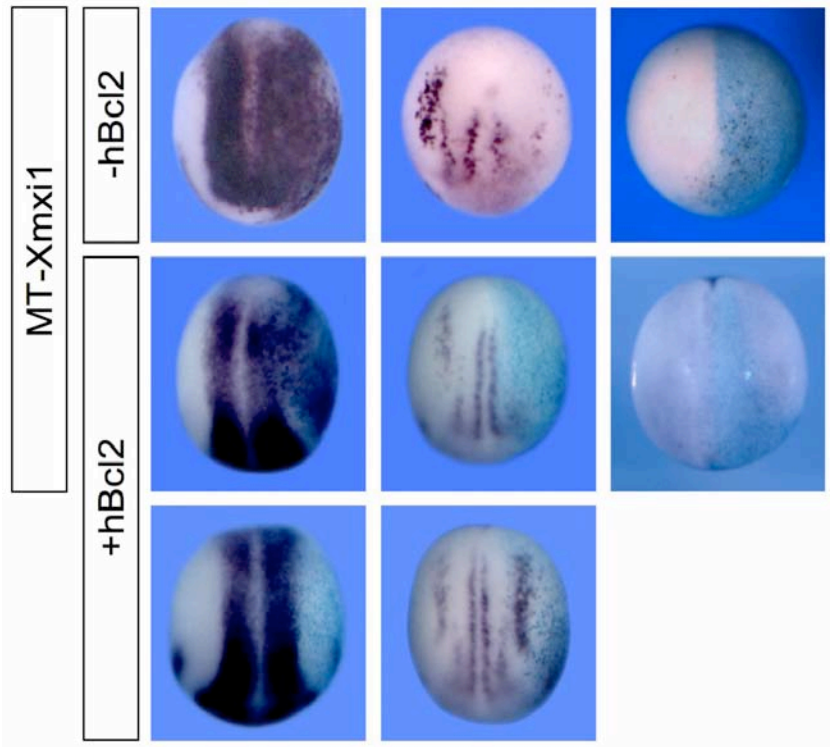

Figure 31 Inhibition of MT-XMxi1-induced apoptosis does not rescue loss of N-tubulin. Overexpression of MT-Xmxi1 (500 pg) led to an increase in apoptosis as seen by TUNEL staining. Coinjection of human Bcl2 mRNA (500 pg) blocked apoptosis but did not influence the effects of MT-Xmxi1 on Sox3 and N-tubulin. hBcl2 mRNA alone (500 pg) does not alter Sox3 expression but leads to an increase of N-tubulin positve cells within the regions of expression.

However, coinjection of human Bcl-2 (500 pg) with MT-Xmxi1 did not alter the ability of MT-Xmxi1 to inhibit N-tubulin expression, but was sufficient to block MT-Xmxi1-induced apoptosis (Figure 31) (-Bcl2, ectopic Sox3, 76%, n=34, inhibition N-tubulin 82%, n=25; +Bcl2, ectopic Sox3, 73%, n=32, inhibition N-tubulin 84%, n=25). Taken together, these results demonstrate that while both cell proliferation and apoptosis are induced by Xmxi1, both activities appear not to be necessary for the activation of Sox3 and inhibition of neuronal differentiation, suggesting Xmxi1 may have a direct influence on cell fate.

3.2 Identification of ESR target genes

Notch signaling has been shown to play an essential role in a multitude of developmental processes during embryogenesis. In the context of vertebrate neurogenesis, Notch signaling has been implicated in the maintenance of the progenitor state, the choice between neuronal and glial fate, as well as various aspects of postmitotic neuron activity (Louvi and Artavanis-Tsakonas, 2006). While it is established that the primary transcriptional targets of the Notch pathway are members of the Enhancer of Split Related (ESR) gene family, the gene targets for this important class of repressor proteins are not well defined. Thus, in order to further understand the genetic cascade of events induced by Notch, particularly in the context of primary neurogenesis where Notch restricts the number of neurons, we aimed to identify early target genes of ESR.

3.2.1 ESR1-VP16-GR induces primary neurogenesis

ESR1 is expressed within the territories of primary neurogenesis and overexpression inhibits neuron formation at the open neural plate stage (Schneider et al., 2001). Isolation of gene targets of a repressor is more difficult than the identification of a transcriptional activator, since these can be easier enriched in a preparation. Thus, we generated a putative antimorphic form of ESR1 by exchanging the WRPW repressor domain with the VP16 activator domain. A similar fusion protein using Xhairy1 was shown to activate gene targets normally repressed by the wild-type protein (Umbhauer et al., 2001; Sadowski et al., 1988). To control the onset of protein activity, ESR1-VP16 was fused to the glucocorticoid receptor ligand-binding domain allowing activation by the addition of dexamethasone (ESR1-VP16-GR) (Figure 32A). For comparative analysis, a hormone inducible ESR1 repressor construct was also prepared (ESR1 -GR).

To determine if ESR1-VP16-GR can indeed function as an antimophic form of ESR1-GR, 50 pg ESR1-GR or 50 pg ESR1-VP16-GR mRNA were injected into the animal half of one blastomere of 2-cell stage embryos. The injected embryos were induced at stage 10.5 with dexamethasone and

cultured to stage 25 (Figure 32B, C). As expected, ESR1-GR overexpression led to the inhibition of primary neurogenesis on the injected side, as marked by the loss of X-ngnr-1 (67%, n=18) and N-tubulin expression (63%, n=19) (Figure 32B). Overexpression of ESR1-VP16-GR afforded not only ectopic expression X-ngnr-1 (78%, n=23), a known target of ESR proteins, but also of N-tubulin (76%, n=17) (Figure 32B). This demonstrates that the antimorphic construct behaves opposite to the wild-type ESR1-GR protein and can therefore be used to identify downstream target genes of ESR proteins (Schneider et al., 2001).

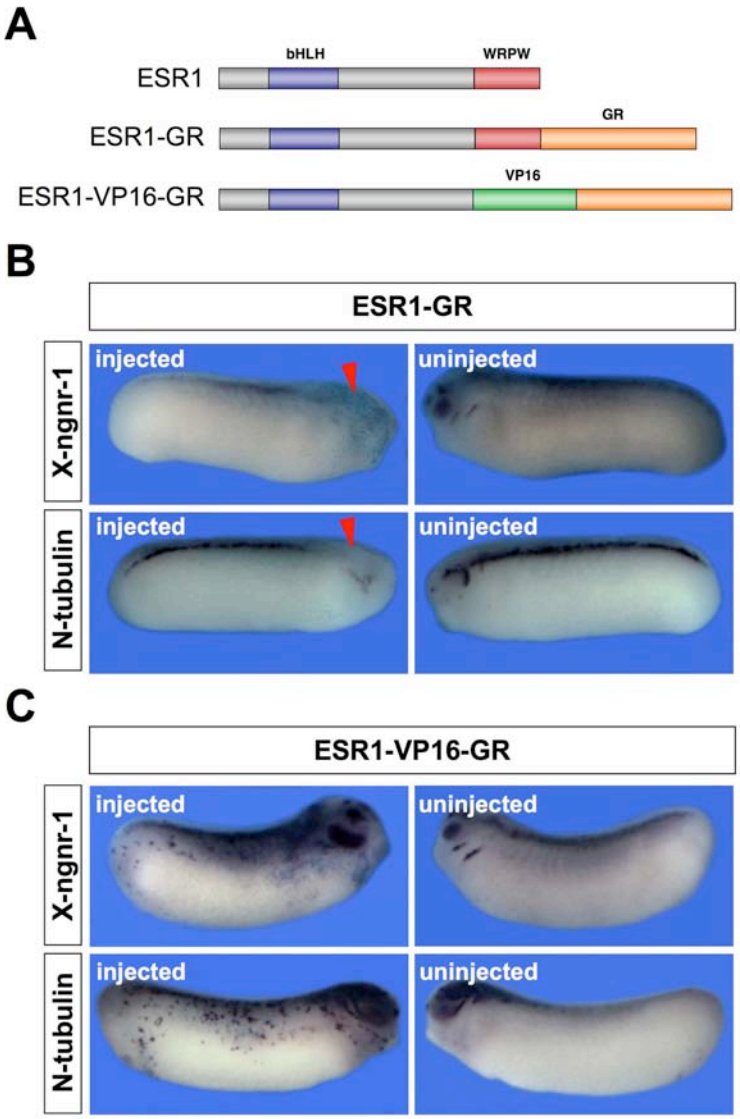

Figure 32 Induction of primary neurogenesis by ESR1-VP16-GR. (**A**) Scheme of the wild-type and hormone-inducible forms of ESR1. (**B-C**) Whole-mount in situ hybridization of stage 25 embryos injected with ESR1-GR (50 pg) or ESR1-VP16-GR (50 pg). Embryos were injected into the animal half of one blastomere of 2-cell stage embryos and induced with dexamethasone at stage 10.5. The antisense probes used are indicated to the left. The injected side (ß-gal, light blue) is shown in the left panel. (**B**) Overexpression of ESR1-GR leads to an inhibition of primary neurogenesis as seen by loss of X-ngnr-1 and N-tubulin expression (red arrowheads). (**C**) Overexpression of ESR1-VP16-GR leads to ectopic X-ngnr-1 and N-tubulin expression.

To isolate ESR1-VP16-GR activated genes, we used a PCR-based differential hybridization technique. Whole embryos are not desirable to use with this technique as they consist of a very heterogeneous cell population. Therefore, to reduce the complexity of the cell population, the animal cap assay was employed. First, ESR1-VP16-GR was tested in naïve animal caps to determine if it can induce primary neurogenesis in this tissue. In addition, as we aimed to identify early targets of ESR1, the temporal onset of X-ngnr-1 and other neuronal markers was determined by RT-PCR analysis. ESR1-VP16-GR was injected into the animal hemisphere of both blastomeres of 2-cell stage embryos, animal caps isolated, induced at stage 10.5 with dexamethasone and cultured for the indicated time points (Figure 33). In ESR1-VP16-GR injected animal caps, X-ngnr-1 transcripts could be detected one hour after induction with dexamethasone (Figure 33B). The X-ngnr-1 downstream transcription factors, X-MyT1 and NeuroD, were detected slightly later at 2 to 3 hours post-induction (Figure 33B).

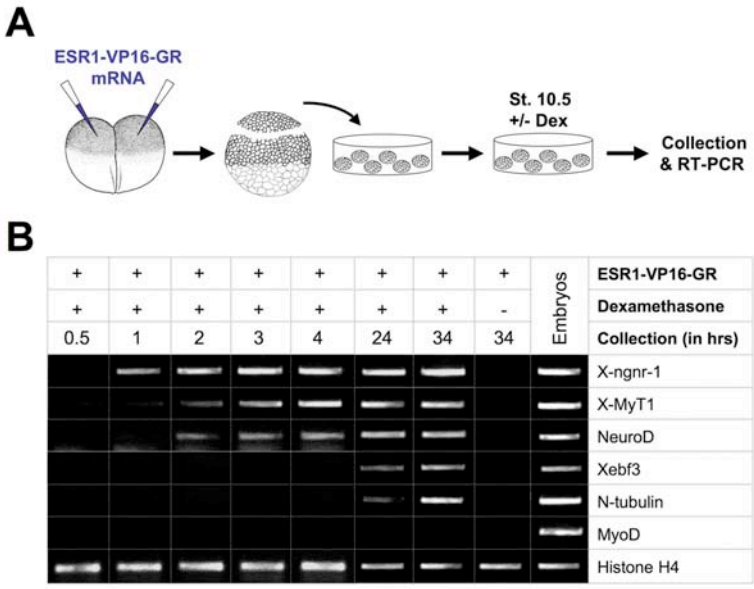

Figure 33 Time course of neurogenesis induced by ESR1-VP16-GR in animal caps. RT-PCR analysis of animal caps injected with ESR1-VP16-GR (50 pg), induced with dexamethasone at stage 10.5 and collected at indicated time points. ESR1-VP16-GR induces X-ngnr-1 already one hour after hormone treatment, while downstream proneural genes, such as X-MyT1 and NeuroD are detectable at considerable levels three hours after induction. The late acting proneural factor Xebf3 starts to be expressed 24 hours after treatment, as does N-tubulin. Histone H4 was used as loading control.

After a considerable delay, the late differentiation factor, Xebf3, as well as N-tubulin transcripts could also be detected demonstrating ESR1-VP16-GR is sufficient to induce neuronal differentiation in ectodermal explants (Figure 33B). As bHLH-Orange proteins are also involved in other

cellular contexts, such as myogenesis, the ability of ESR1-VP16-GR to induce MyoD was tested by RT-PCR to investigate the promiscuity of target gene activation (Cui, 2005). MyoD, a direct target of Xhairy-1, was not upregulated by ESR1-VP16-GR in animal caps. These results suggest, that ESR1-VP16-GR induces a specific set of bHLH-Orange target genes (Figure 33B) (Umbhauer et al., 2001).

3.2.2 cDNA library construction

Using the ESR1-VP16-GR injected animal caps, we wanted to identify ESR1 target genes by constructing an enriched cDNA library using PCR-based differential hybridization. Depicted in Figure 34 is the workflow for the preparation of the library.

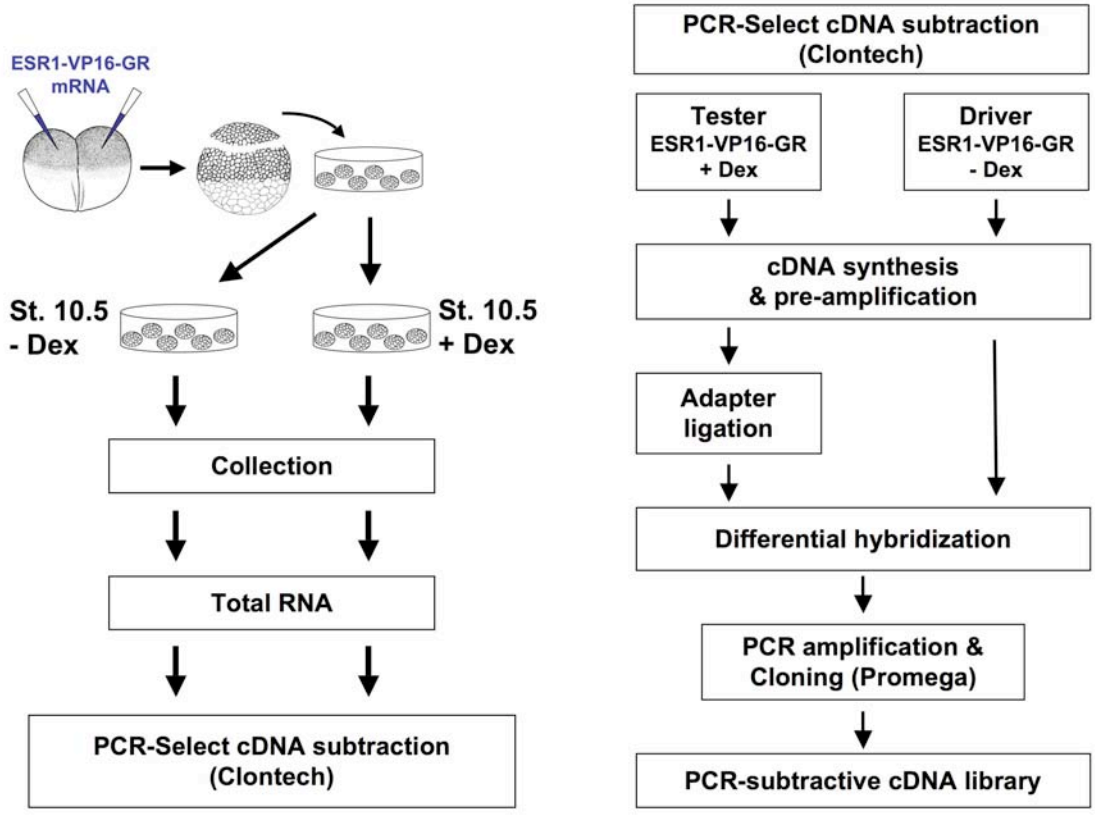

Figure 34 Workflow of cDNA library construction. ESR1-VP16-GR injected animal caps were divided into two populations, one of which was induced with dexamethasone at stage 10.5. After culturing for 1 hour, total RNA was prepared. After cDNA amplification and differential hybridization, PCR products were cloned into the pGEM-T vector (Promega) to construct the cDNA library enriched with ESR1-VP16-GR downstream target genes. For details see text.

The ESR1-VP16-GR injected animal caps were treated at stage 10.5 with or without dexamethasone and after an one-hour incubation, 80 caps of each pool were collected and total RNA prepared. The two different RNA

pools were compared using the PCR-Select cDNA subtraction kit (Clontech). To enrich the cDNA pools for mRNA sequences, the RNA populations were converted into cDNA using Oligo dT primers and pre-amplified using the SMART^{TM} PCR cDNA Synthesis Kit (Clontech). The induced pool (ESR1-VP16-GR, +Dex) is referred to as tester, as this pool includes additional (differentially expressed) cDNA molecules in comparison to the un-induced pool (ESR1-VP16-GR, -Dex), which is termed driver. The tester cDNA pool was ligated to adaptors for later PCR amplification, while the driver pool lacks adaptors. Tester and driver cDNAs were hybridized, and the hybrid sequences, which represent non-differentially expressed cDNAs, were removed. Consequently, the remaining unhybridized cDNAs represented genes that were expressed in the tester, but were absent from the driver. The differentially expressed cDNAs were subcloned into the pGEM-T vector (Promega), creating a cDNA library enriched for putative ESR1-VP16-GR target genes.

To validate the cDNA library construction, 192 random clones were analyzed by DNA sequencing. Surprisingly, X-ngnr-1 or other known ESR target genes were not identified. Therefore, RT-PCR analysis of 13 random clones was performed to determine if the library is actually enriched with differentially expressed cDNAs. As shown in Figure 35A, only one of 13 clones was upregulated in ESR1-VP16-GR induced animal caps. Transcripts of the other 12 clones were present in ESR1-VP16-GR injected as well as untreated control animal cap. Decreasing the cycle numbers of the PCR reactions did not result in a different distribution of transcripts, demonstrating that the PCR reactions were not past the linear range (data not shown).

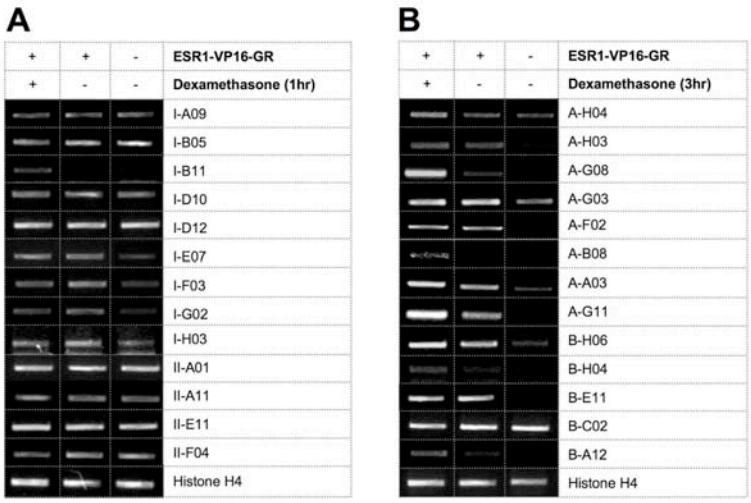

Figure 35 RT-PCR analysis of randomly picked clones identified in the one-hour and three-hour cDNA libraries. For 13 randomly picked clones of each cDNA library, RT primers were designed. (**A**) Using the same conditions of the one-hour cDNA library preparation, RT-PCR analysis revealed most clones not being differentially expressed. (**B**) RT-PCR analysis of 13 clones of the three-hour cDNA library indicated a successful library preparation since most clones were not expressed in the control caps and stronger expressed in the induced ESR1-VP16-GR animal caps.

Taken together, these results indicate, that within one hour, ESR1-VP16-GR cannot sufficiently induce target gene levels to construct a differentially expressed cDNA library. Therefore, in a second approach, the ESR1-VP16-GR injected animal caps were incubated with dexamethasone for three hours to increase target transcript levels. Among the 192 sequenced clones, again X-ngnr-1 as well as downstream targets such as X-MyT1 were not identified, although all transcripts are induced three hours after induction as shown by RT-PCR (Figure 35B).

However, two lines of evidence argue for a successful library preparation. It has been shown that there is a cross-regulation between members of bHLH-Orange proteins (Takada et al., 2005, Bianchi-Frias et al., 2004). Corresponding, within the 192 sequenced clones of the three-hour library, five ESR-type protein encoding cDNAs were identified. Moreover, the RT-PCR analysis of 13 random clones revealed that 8 out of 13 clones were represented at higher levels within the ESR1-VP16-GR induced animal caps as compared to the un-induced or control animal caps (Figure 35B). Transcripts of three additional clones were present in injected, un-induced animal caps and absent in control caps indicating some “leakiness” of the ESR1-VP16-GR construct (Figure 34B). The observation that some genes were activated in the absence of dexamethasone, suggests that these genes

are more sensitive to lower concentrations of ESR1-VP16-GR. Although the majority of clones were differentially expressed, two observations suggested the requirement for a second validation approach. First, the genes identified were not strongly differentially expressed. Second, 30% of the sequenced clones harbored no insert. Therefore a microarray-based differential hybridization approach was chosen, as a large number of clones can quickly be screened and differentially expressed clones identified.

3.2.3 Microarray analysis

To identify cDNA clones, which are indeed induced by ESR1-VP16-GR, 2,304 clones of the ESR1-VP16-GR target library, termed ESR library hereafter, were PCR amplified and spotted on glass slides. In addition, 9,216 PCR products of an oocyte cDNA library enriched for vegetally localized RNAs, termed vegetal library (Claussen and Pieler, 2004) as well as 15,912 PCR products of the RZPD clone set RZPDp988**,** termed RZPD library, were used. These libraries were spotted to identify additional ESR1-VP16-GR target genes and improve normalization of individual signal intensities in the subsequent computational analysis. The ESR library was constructed using total RNA isolated from ESR1-VP16-GR injected animal caps induced with dexamethasone in comparison to total RNA isolated from ESR1-VP16-GR injected un-induced animal caps. The differentially expressed clones within the ESR library are therefore a mixture of genes responsive to ESR1-VP16-GR as well as to dexamethasone itself. To eliminate genes, which are induced by dexamethasone alone, the hybridization targets for the microarray analysis were derived from a different RNA preparation than those used for library construction. Total RNA derived from induced ESR1-VP16-GR injected animal caps (ESR-AC) and control animal caps induced with dexamethasone (CC-AC) was used as starting material (Figure 36). Both RNA populations include the transcripts upregulated by dexamethasone alone, and therefore these transcripts will not be identified as differentially expressed clones during the microarray analysis. To increase the statistical significance, a total of ten hybridizations was performed following the swap design (Kerr and Churchill, 2001).

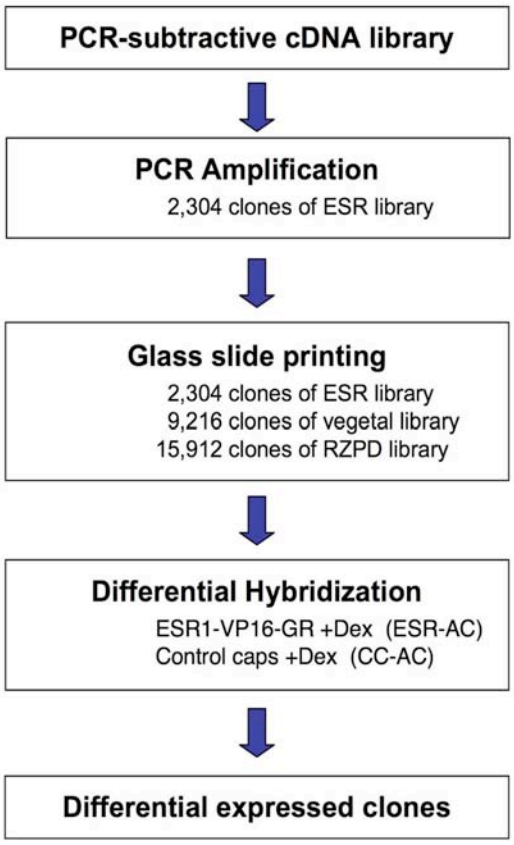

Figure 36 Workflow of microarray based ESR1 target gene identification. For details see text.

After computational analysis, the microarray results were represented as scatterplots (Figure 37) (Landgrebe et al., 2004). The logarithm of the signal intensity ratio of CC-AC to ESR-AC (c-value) was plotted against the probability (p-value). In a simplified view, a clone with a high c-value is strongly differentially expressed. If the c-value is positive, the clone is over represented in the CC-AC pool, thus repressed by ESR1-VP16-GR. Clones with a negative c-value are over represented in the ESR-AC pool and thus activated by ESR1-VP16-GR. The p-value refers to the probability that the different measurements of the signal intensity ratios of a spot are statistically significant. The smaller the p-value, the better the reproducibility of the result.

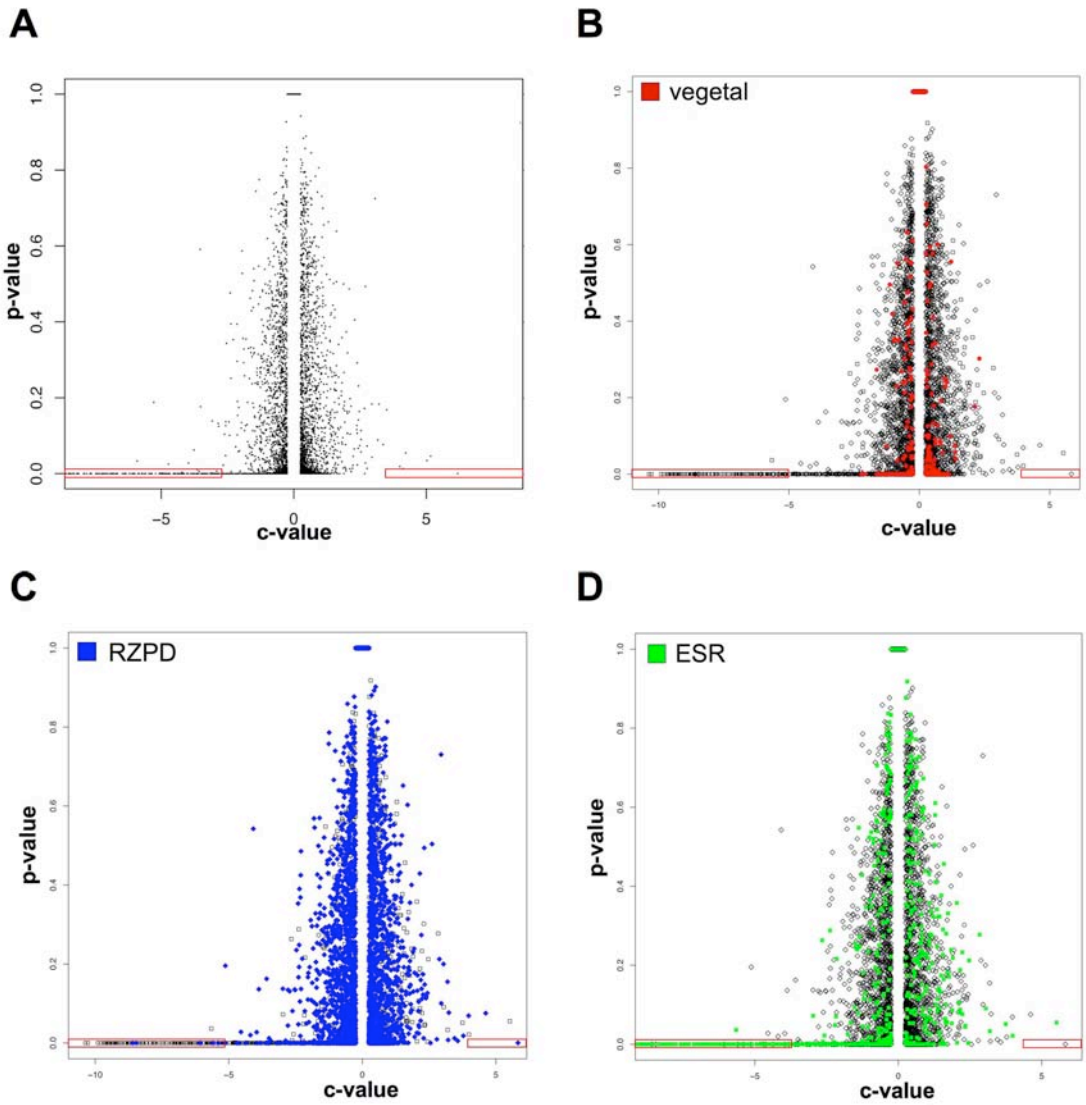

Figure 37 Scatterplots of microarray result. The signal differences of each cDNA clone are shown on the x-axis as $\log_2$ (CC-AC/ESR-AC) (c-value). Positive numbers indicate that the corresponding clone is downregulated by ESR1-VP16-GR, whereas negative values indicate an upregulation. The p-value is plotted on the y-axis. Small numbers indicate a high significance that the corresponding c-value is true. Clones with a c-value between -1 and 1 are considered not differentially expressed and are not shown. (A) All 27,442 clones are shown. The candidate clones are boxed with a cut off of $p<0.0001$ and +/-3. (B-D) Each individual library is colored, vegetal (red), RZPD (blue) and ESR (green). While the vegetal and RZPD libraries are uniformly distributed, a considerably higher portion of the ESR library is located in the lower left hand corner. Therefore, this library is enriched for ESR1-VP16-GR target genes. An enlargement of the candidate clones is shown in Figure 38.

Clones upregulated by ESR1-VP16-GR are located in the lower left hand corner of the scatterplot, whereas downregulated clones are located in the lower right hand corner. The distribution of the cDNA clones of each library in comparison to all signals is shown in Figure 37B-D. Clones of the RZPD library as well as of the vegetal library are distributed equally along the x- and y-axis (Figure 37B, C). In contrast, a large number of clones identified in the ESR library is present in the lower left hand corner (Figure 37D), indicating that these clones are strongly upregulated by ESR1-VP16-GR. This

result further supports the idea that the library itself is enriched in differentially expressed clones.

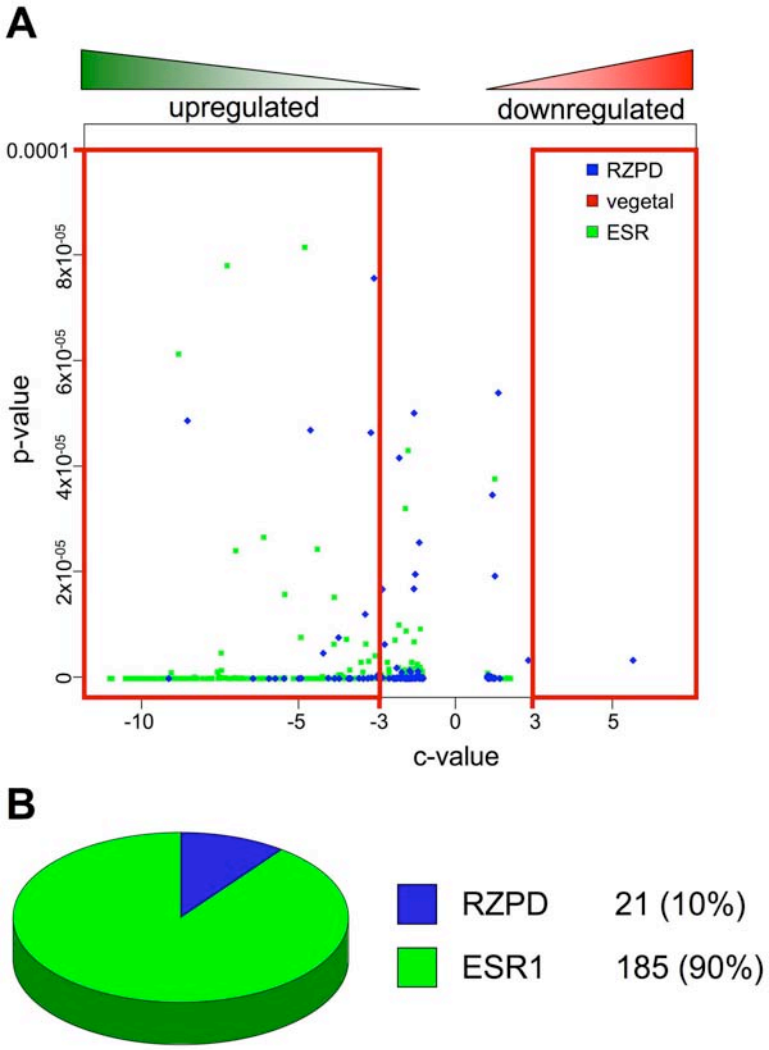

Figure 38 Summary of microarray result. (A) Scatterplot of cDNA clones within a range of p<0.0001. Clones with a c-value between -1 and 1 are not shown. (**B**) Distribution of clones identified in the microarray in respect to the different libraries. 90% of the candidate clones are identified within the ESR1 library, arguing for a enrichment of this library with ESR1-VP16-GR target genes.

Using a p-value of less than 0.0001 and an c-value of +/- 3, a total of 207 candidate clones for differential expression were identified. 206 were induced by ESR1-VP16-GR and only one clone (from the RZPD library) was found to be downregulated (Figure 37, Figure 38A). Comparing the number of clones identified in each library, 185 clones or 90% were identified in the ESR library, while only 21 clones (10%) were identified in the RZPD library. No positive clones were identified in the vegetal library (Figure 38). In relation to the total number of clones spotted for each library, only 0.08% (21 of 15,912) of the RZPD library clones were identified and 0% (0 of 9,216) of the vegetal

library, while 8% (185 of 2,304) of all ESR library clones were differentially expressed.

3.2.4 ESR1-VP16-GR target genes

In a first step, all 207 differentially expressed clones were sequenced and compared to the available databases, including the *Xenopus tropicalis* EST and genomic databases (BLAST, http://www.ncbi.nlm.nih.gov/BLAST; NIBB, http://xenopus.nibb.ac.jp; Xt genome, http://genome.jgi-psf.org/Xentr4/; Xt EST, http://www.sanger.ac.uk/). A total of 55 different genes could be identified by assigning clones to the same contig and by sequence alignments with known genes (Table 1). For simplicity, pseudoalleles were not taken into account, so that ESR9 in Table 1 represents all cDNA clones, which aligned to ESR9a and ESR9b. Several cDNA clones harbored the same insert, therefore representing one unique clone in multiple copies. The total number of clones found for each gene, as well as the total number of unique clones are listed in Table 1.

Among the 34 known genes identified, 12 fall into the context of Notch signaling, most of which are represented multiple times (Figure 39). In addition to the known genes, 20 cDNA clones could be linked to known EST sequences and only one cDNA clone could not be annotated (Figure 39). Four cDNA clones did not harbor an insert. These are false positives and considering their c-values, which were close to the cut-off of +/- 3, increasing the candidate pool by using a broader c-value would most likely not be beneficial.

No	Clone ID	Highest BLAST hit / identified gene		AccNo	Library	(max.) Insert Size	Total # of cDNA clones	Unique cDNA clones	(max.) c-value	RT PCR Result	ESR1-VP16-GR Result
1	-	ESR4		AF137073	He/R	600	36	9	-10.1	+	++
2	-	ESR8			He	460	28	8	-9.7	+	++
3	-	ESR9		AJ009282	He/R	700	27	7	-10.8	+	++
4	-	XHes2		DQ156231	He/R	500	19	7	-9.6	+	++
5	-	ESR10		AJ009285	He	680	17	5	-9.7	+	++
6	-	X-Delta-2		U70843	He	500	8	6	-9.0	+	++
7	-	XHR-1	HES-related 1	AB071433	He/R	630	7	6	-6.4	+	S
8	-	SgK	Serum glucocorticoid regulated kinase	BC073077	He	570	4	3	-6.2	+	++
9	-	ESR3/7		BC072958	He/R	590	4	2	-4.8	+	++
10	-	X-Delta-1		L42229	He	600	3	2	-7.3	+	++
11	-	ESR5		AF137072	He/R	500	3	2	-6.3	+	++
12	-	ESR11			He	500	2	2	-6.5	+	++
13	-	XGadd45 gamma		BC078567	He/R	800	2	2	-5.9	+	++
14	-	Olig3	Oligodendrocyte transcription factor 3 (*Xenopus tropicalis*)	BC082516	He	508	2	1	-3.9	+	S
15	He 132	EST		BP695029	He	293	1	1	-9.9	+	++
16	He 154	xPRMT1	Protein arginine methyltransferase 1	AB085173	He	400	1	1	-9.2	+	ND
17	He 117	CAF1-150	Chromatin assembly factor 1 p150 subunit	AF222339	He	319	1	1	-9.1	+	++
18	He 267	EST		AAH67978	He	330	1	1	-9.0	+	++
19	He 313	EST		BC087479	He	400	1	1	-8.6	ND	ND
20	R 8584	EST		BC084852	R	500	1	1	-8.4	+	ND
21	He 712	ESR2		BX846504	He	249	1	1	-7.5	+	++
22	He 224	EST		BJ642962	He	870	1	1	-7.4	+	++
23	He 272	EST		BC082208	He	288	1	1	-7.2	+	+
24	He 105	Matr3	Matrin 3 (*Xenopus tropicalis*)	BC076655	He	475	1	1	-7.2	+	ND
25	He 116	XCAP-H	13S condensin XCAP-H subunit	BC068643	He	339	1	1	-6.9	ND	++
26	He 217	p120		AF150744	He	200	1	1	-6.4	ND	++
27	He 195	EST		BJ639156	He	600	1	1	-6.4	ND	ND
28	He 180	CROP	Cisplatin resistance-associated overexpressed	NM_001016304	He	1000	1	1	-6.0	ND	S
29	He 733	CPSF-100	Cleavage and polyadenylation specificity factor 100 kDa subunit	AF139986	He	437	1	1	-5.0	ND	++
30	He 1302	EST		BP723878	He	350	1	1	-5.0	ND	++
31	He 2209	Sall4	Sal-like 4 (*Xenopus tropicalis*)	NM_001001458	He	281	1	1	-4.9	ND	++
32	He 1783	EST		BP730455	He	512	1	1	-4.7	ND	+
33	He 188	Smarcd1	similar to SWI/SNF related	BC045009	He	596	1	1	-4.7	ND	+
34	R 6322	EST		AAI08580	R	765	1	1	-4.5	ND	++
35	He 192	Fru-1,6-P_2	Fructose-1,6-bisphosphatase	BC053784	He	129	1	1	-4.2	ND	+
36	R 3306	Id4	Inhibitor of differentiation 4	AAP34250	R	670	1	1	-4.0	ND	++
37	He 2281	Foxi1	Forkhead-box protein I 1	BC042303	He	578	1	1	-3.9	ND	++
38	He 1538	EST		CA971845	He	256	1	1	-3.8	ND	+
39	He 989	EST		BC048226	He	325	1	1	-3.8	ND	+
40	R 3988	DLL-1	Distal-less homeobox gene 1	AAB27236	R	800	1	1	-3.8	ND	+
41	R 4644	POU 2		AAH76747	R	960	1	1	-3.8	ND	++
42	He 2025	EST		CB561947	He	490	1	1	-3.7	ND	++
43	He 714	EST		BJ057335	He	133	1	1	-3.7	ND	++
44	He 247	CKB	Creatine kinase, brain	BC042282	He	487	1	1	-3.7	ND	+
45	R 10387	Ensa	Endosulfine alpha	AAH44986	R	580	1	1	-3.6	ND	++
46	He 2169	No significant homology			He	500	1	1	-3.6	ND	-
47	He 1028	Hoxd13		AY167742	He	362	1	1	-3.4	ND	++
48	He 1547	EST		BP676976	He	328	1	1	-3.4	ND	+
49	R 9413	EST		BC057749	R	580	1	1	-3.3	ND	ND
50	He 208	SIP1	Survival of motor neuron protein interacting protein 1	AF027151	He	262	1	1	-3.2	ND	++
51	He 459	EST		BC059972	He	394	1	1	-3.2	ND	++
52	R 14995	EST		BF612822	R	360	1	1	-3.1	ND	ND
53	R 9328	EST		BQ400749	R	750	1	1	-3.0	ND	++
54	He 2379	Abcb4	ATP-binding cassette, sub-family B (MDR/TAP), member 4 (*Xenopus tropicalis*)	BC063924	He	700	1	1	-3.0	ND	ND
55	R 507	EST		BC084892	R	480	1	1	6.2	ND	-

Table 1 List of candidate genes identified by microarray analysis. After sequence analysis, 55 unique genes/ESTs could be identified out of the 207 cDNA clones, which were differentially expressed by ESR1-VP6-GR. The library, in which the gene was identified is listed. The total number of clones as well as the number of unique clones are listed for each gene. The length of the longest cDNA clone is listed as well as the maximal c-value. The result of the RT-PCR analysis of clones with a c-value smaller than -7 and the whole-mount in situ hybridization analysis in ESR1-VP16-GR injected embryos is shown. The table is sorted descending by total number of unique clones, followed by total number of clones followed by the c-value. R, RZPD library; He, ESR1 library; ND, not determined; S, shift; ++, strongly induced in whole embryos; +, weakly induced in whole embryos or induced in animal caps.

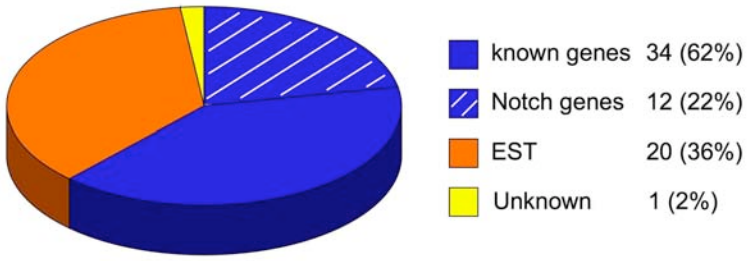

Figure 39 Summary of the 207 candidate clones by sequence analysis. This is a summary of Table 1 in respect to the result of the sequence analysis. In total, 34 known genes could be identified within the 207 differentially expressed candidate clones. Among those, 12 genes fall into the context of Notch signaling. In addition, 20 candidate clones could be assigned to already known expressed sequence tags (EST), while one clone could not be annotated. The 4 candidate clones, which harbored no insert, were not considered.

3.2.5 Notch signaling genes are targets of ESR1

Within the 207 differentially expressed clones, twelve genes were identified that were reported to be involved in Notch signaling. These include the two Notch ligands (X-Delta-1 and X-Delta-2) as well as the Notch effectors XHes2, XHR-1, ESR2, ESR3/7, ESR4, ESR5, ESR8, ESR9, ESR10 and ESR11. With the exception of ESR11, all ESR genes were found in multiple copies, with a c-value smaller than -7, indicating strong differential expression.

As shown in Figure 40, all Notch pathway genes, found to be candidate ESR1 targets in the microarray approach, were upregulated only in the ESR1-VP16-GR induced animal cap population, as analyzed by RT-PCR. In comparison to the initial RT-PCR analysis, the microarray experiment identified strongly upregulated clones (compare Figure34B and Figure 40). For RT-PCR analysis of genes represented in multiple copies and having small c-values, less cycles were necessary to detect the transcripts, suggesting that they were more abundant in the cDNA preparation, than cDNA clones represented in a single copy. bHLH-Orange proteins that were not found in the microarray analysis, such as ESR6 or Hes6, were also not regulated by ESR1-VP16-GR in the animal cap assay, strengthening that the

identified bHLH-Orange genes are true positives in the animal cap system (Figure 40).

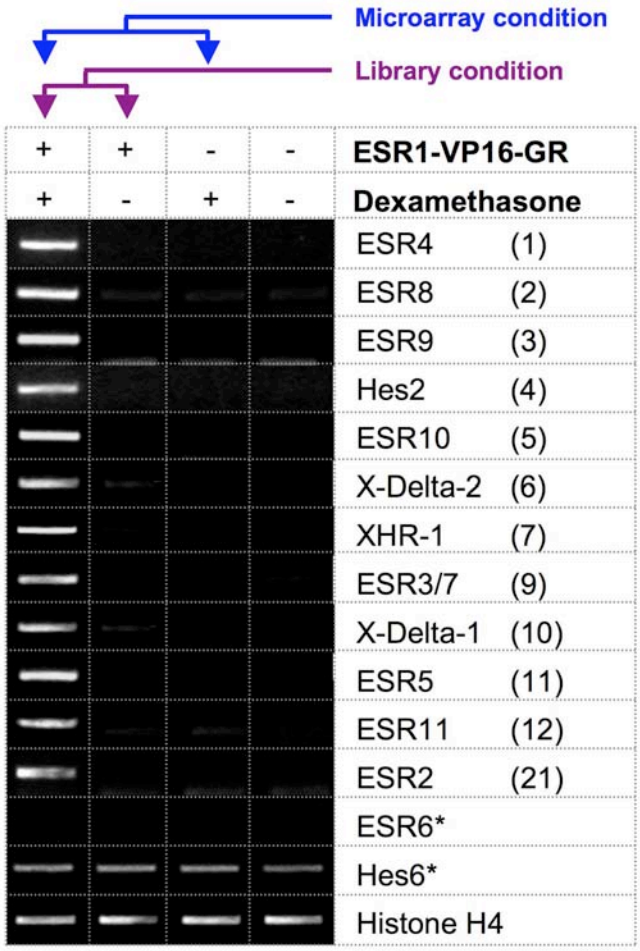

Figure 40 Regulation of Notch pathway genes by ESR1-VP16-GR in animal caps. Animal caps of ESR1-VP16-GR injected embryos and control embryos were cut at blastula stage, treated with or without dexamethasone at stage 10.5 and collected 3 hours later. Clones with a smaller c-value than -7 were analyzed. All clones, which could be detected in whole embryos, were only upregulated in induced ESR1-VP16-GR injected animal caps. Asterisks indicate Notch pathway genes not found in the microarray analysis. The number of each clone (according to Table 1) is shown in parenthesis.

ESR proteins are reported to inhibit transcription of target genes as well as the activity of bHLH proteins through heterodimerization (Davis and Turner, 2001). To analyze the requirement of DNA binding of the candidate genes, a DNA binding mutant of ESR1-VP16-GR (ESR1-VP16-DBM-GR) was constructed by mutating the basic region (Ström et al., 1997). If ESR1 directly represses transcription of the identified Notch pathway genes, the DNA binding mutant of ESR1-VP16-GR should not upregulate their transcription in the animal cap assay. In addition, ESR1-GR was constructed to demonstrate the requirement of the activation domain. As shown in Figure 41, DNA binding as well as the activation domain were required for ESR1-VP16 to

activate all tested Notch pathway genes. These results suggest that activation of genes by ESR1-VP16-GR is direct and not for example the result of sequestering a repressor of the upregulated genes.

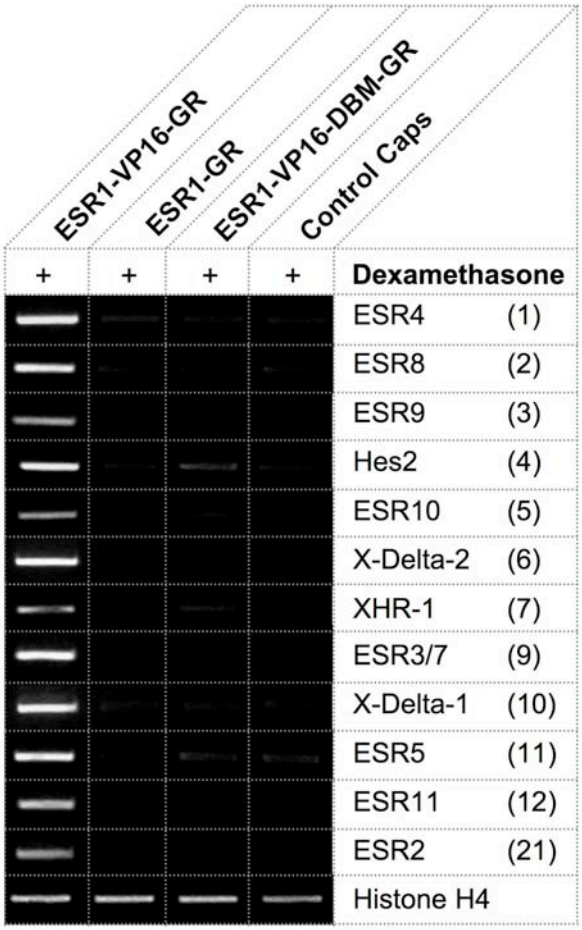

Figure 41 Notch pathway genes are transcriptional targets of ESR1. Animal caps of mRNA injected embryos and control embryos were cut at blastula stage, treated with and without dexamethasone at stage 10.5 and collected 3 hours later. Only ESR1-VP16-GR robustly induces the identified Notch pathway genes. The number of each clone (according to Table 1) is shown in parenthesis.

To further confirm that the identified Notch signaling genes are regulated by ESR1, ESR1-VP16-GR (50pg) or ESR1-GR (50pg) mRNA was injected into the animal hemisphere of one blastomere of the 2-cell stage embryo, induced with dexamethasone at stage 10.5 and cultured until open neural plate stage. As shown in Figure 42A, all identified ESR1 target genes were upregulated by ESR1-VP16-GR in *Xenopus* embryos. Furthermore, all analyzed genes were repressed upon overexpression of ESR1-GR (Figure 42B). The antagonistic regulation of the genes by ESR1-GR and ESR1-VP16-GR overexpression supports that these genes are targets of ESR1 *in vivo*.

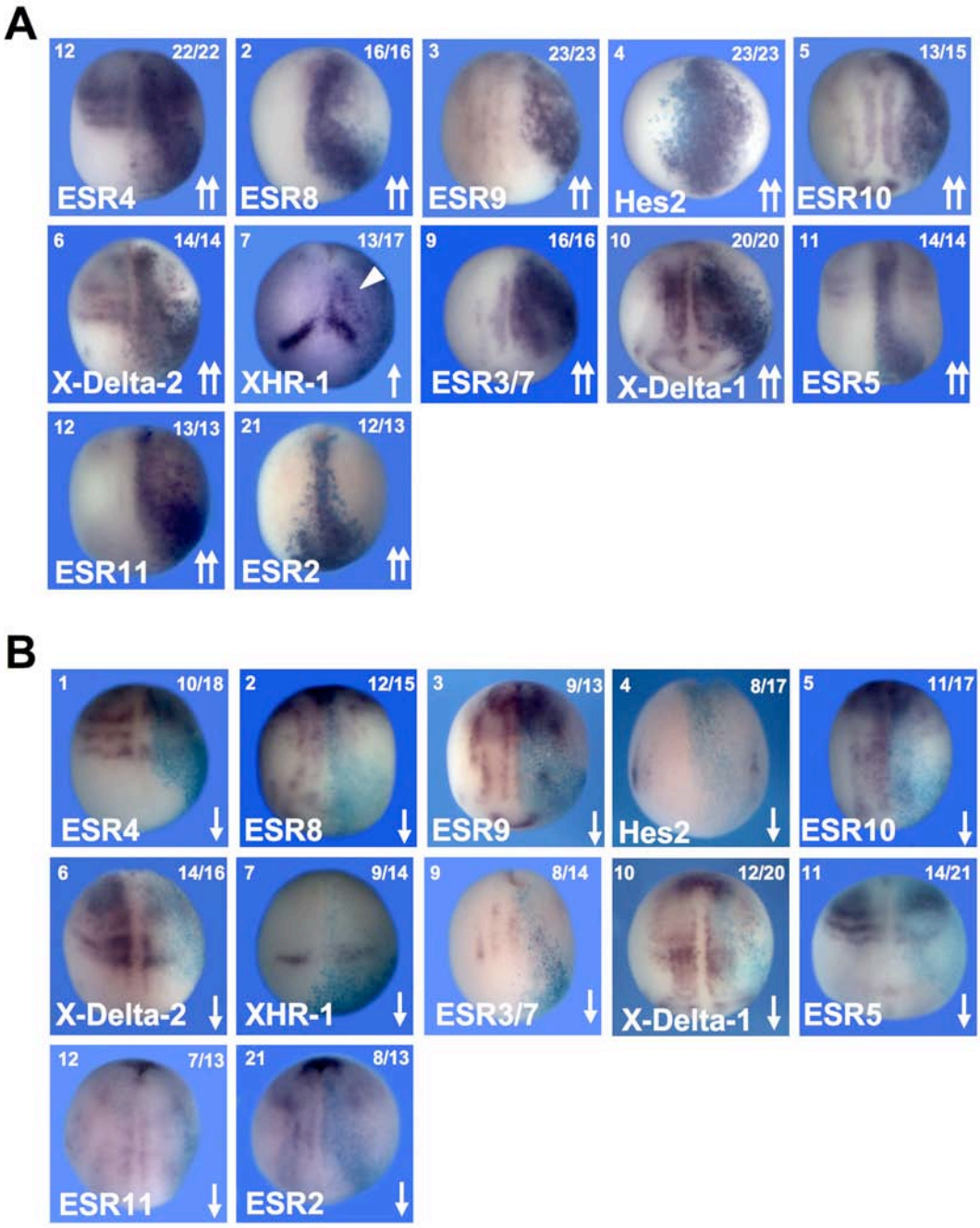

Figure 42 Regulation of Notch pathway genes by ESR1 in whole embryos. Whole-mount in situ hybridization of stage 14 embryos injected with ESR1-VP16-GR (50 pg) (Panel A) or ESR1-GR (50 pg) (Panel B) RNA into one blastomere of the 2-cell stage and induced at stage 10.5 with dexamethasone. The injected side (ß-gal, light blue) is on the right and embryos are shown as a dorsal view, anterior down. The analyzed clones are indicated to the left with their number according to Table 1 (upper left hand corner) as well as their names (lower left hand corner). The score of upregulation or downregulation is indicated in the lower right hand corner and the numbers of embryos exhibiting the phenotype in respect to the total number of analyzed embryos is shown in the upper right hand corner. (**A**) Overexpression of ESR1-VP16-GR leads to an upregulation of all marker genes. Arrowhead indicates ectopic expression of XHR-1. Two arrows pointing upwards indicate strong ectopic expression, wereas one arrow pointing upwards indicate mild ectopic activation. (**B**) Conversely, overexpression of ESR1-GR leads to an inhibition of marker gene expression. Arrow pointing downwards indicate repression of endogenous expression.

3.2.6 Other ESR1 target genes

In addition to Notch pathway genes, serum glucocorticoid regulated kinase (SgK), XGadd45γ and olig3, were identified in multiple copies and strongly induced by ESR1-VP16-GR in the animal caps as shown by RT-PCR (Figure 43). Genes identified only once in the microarray approach, but strongly differentially expressed (c-value < -7) were also tested by RT-PCR analysis. All of the resulting 8 genes were upregulated in the induced, ESR1-VP16-GR injected animal caps (Figure 43).

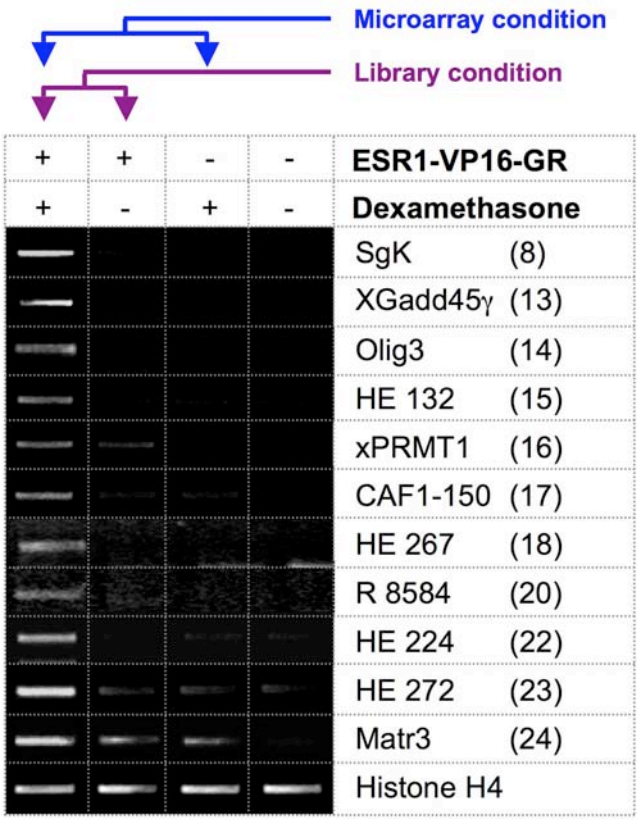

Figure 43 Regulation of highly differentially expressed clones by ESR1-VP16-GR in animal caps. Animal caps of ESR1-VP16-GR injected embryos and control embryos were cut at blastula stage, treated with and without dexamethasone at stage 10.5 and collected 3 hours later. Clones with a smaller c-value than -7 were analyzed. All clones, which could be detected in whole embryos, were only upregulated in induced ESR1-VP16-GR injected animal caps. The number of each clone (according to Table 1) is shown in parenthesis.

As single copy clones were much weaker differentially expressed than those clones that appeared in multiple copies, the regulation of all non-Notch pathway genes by ESR1-VP16-GR overexpression was analyzed by whole-mount in situ hybridization and not by RT-PCR. Since most cDNA inserts were smaller than 500 base pairs, longer cDNA clones were obtained from the German Resource Center for Genome Research (RZPD,

http://www.rzpd.de/) or National Institute for Basic Biology (NIBB, http://xenopus.nibb.ac.jp/) and were used for probe generation. As shown in Figure 44, 24 clones were strongly and 7 weakly upregulated. Eight cDNA clones did not show any staining or were ubiquitously expressed. Only two cDNA clones were downregulated, of which one was also identified to be downregulated in the microarray analysis (No. 55) (Figure 44).

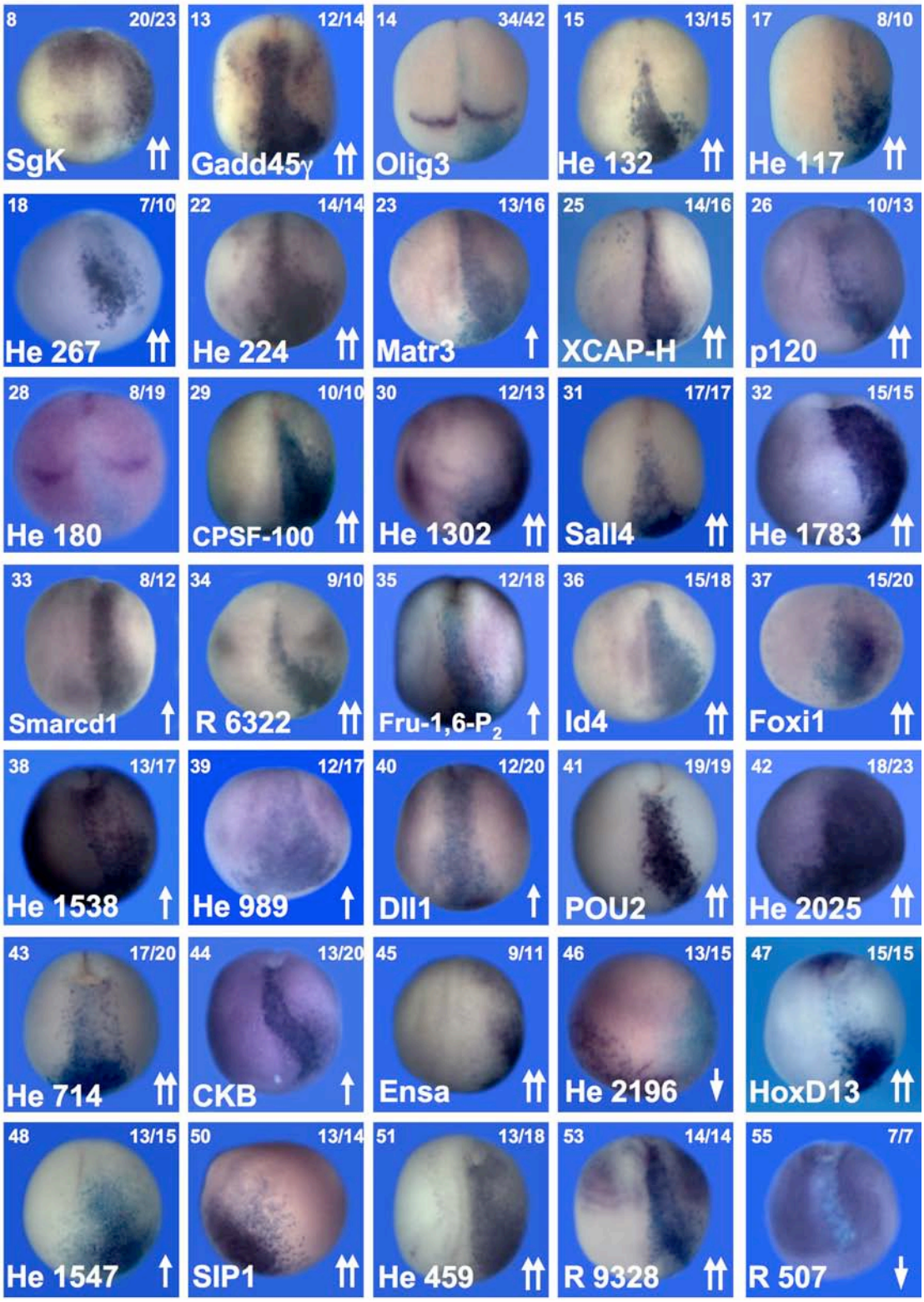

Figure 44 Regulation of ESR1-VP16-GR target genes in whole embryos. Whole-mount in situ hybridization of stage 14 embryos injected with ESR1-VP16-GR RNA into one blastomere of the 2-cell stage and induced at stage 10.5 with dexamethasone. The injected side (ß-gal, light blue) is on the right and embryos are shown as a dorsal view, anterior down. The analyzed clones are indicated to the left with their number according to Table 1 (upper left hand corner) as well as their names (lower left hand corner). The score of upregulation or downregulation is indicated in the lower right hand corner and the numbers of embryos exhibiting the phenotype in respect to the whole number of analyzed embryos is shown in the upper right hand corner. Two arrows pointing upwards indicate strong ectopic expression, wereas one arrow pointing upwards indicate mild ectopic activation. Arrow pointing downwards indicate repression of endogenous expression.

3.2.7 Temporal expression analysis of selective clones

Among the Notch signaling pathway genes identified in this study only the expression analysis of ESR2 and ESR11 is not yet described in the literature. Therefore, we analyzed the expression of the two genes by whole-mount in situ hybridization. ESR2 was first weakly detected at stage 13 in two bilateral stripes in the open neural plate (Figure 45A). By stage 15, the ESR2 transcripts were detected in a pattern similar to X-Delta-1 expression (Figure 45B) (Chitnis et al., 1995). In addition to the expression in the longitudinal stripes on both sides of the midline, ESR2 is weakly expressed in the trigeminal and olfactory placodes, as well as a region, which will give rise to the ventral forebrain (Figure 45B) (Bellefroid et al., 1996). At stage 37, transcripts could be detected in the eye, central nervous system and the tailtip (Figure 45C).

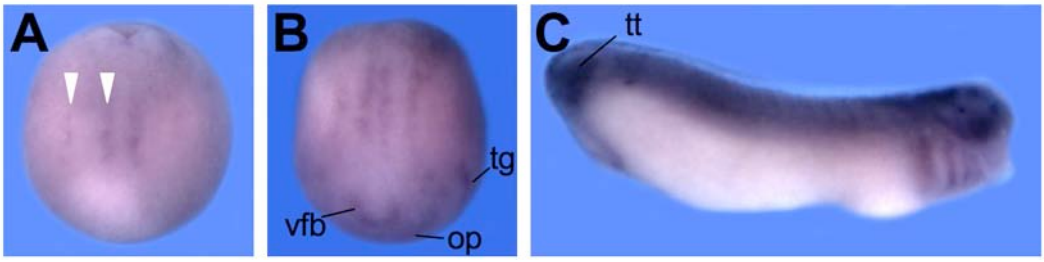

Figure 45 Spatial expression analysis of ESR2. Whole-mount in situ hybridization of ESR2. (**A**) Stage 13, dorsal, anterior down. Arrowheads mark longitudinal expression domains. (**B**) Stage 14, dorsal, anterior down. (**C**) Stage 37 lateral, anterior to the right. tg, trigeminal placode; vfb ventral forebrain; op olfactory placode; tt, tailtip.

The first transcripts of ESR11 could be detected at the lateral margins of stage 13 embryos (Figure 45A). By stage 14, the pattern is restricted to the territories of primary neurogenesis similar to those of ESR2 and X-Delta-1 (Figure 46B). ESR11 was also found to be expressed in the pronephros anlagen, with the first weak expression at stage 19 (Figure 46C, D). A strong expression could also be detected within the region of the dorsolateral line placodes (Figure 46C) (Schlosser and Northcutt, 2001). With ongoing development, ESR11 continues to be expressed in the pronephros, the central nervous system, and the eye (Figure 46E). In a transversal section of a stage 30 embryo, expression of ESR11 in the eye is restricted to the peripheral edge of the retina, which will give rise to the ciliary marginal zone, in which active proliferation occurs (Perron et al., 1998) (Figure 46F).

Correspondingly, the expression in the neural tube is located in the proliferating neural progenitors of the ventricular zone (Figure 46G).

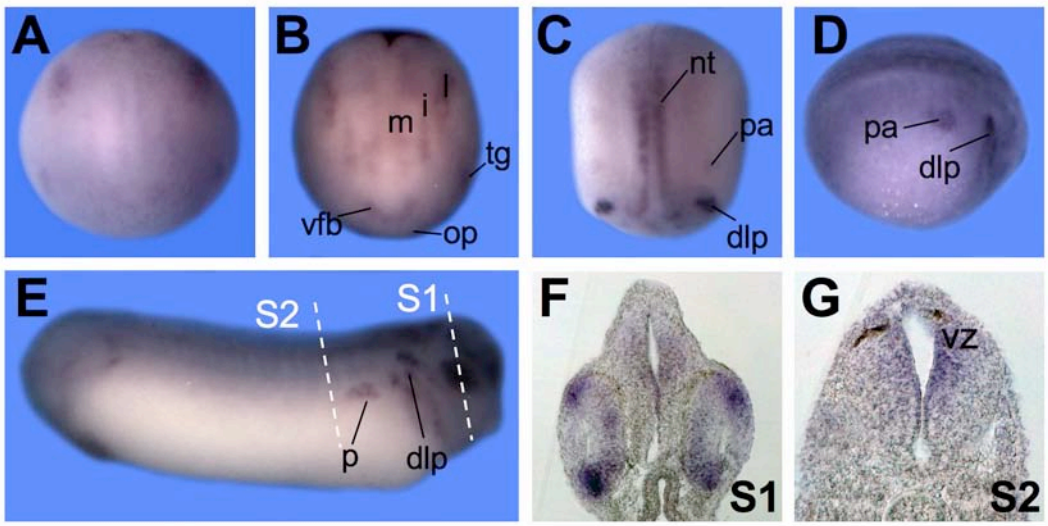

Figure 46. Spatial expression analysis of ESR11. Whole-mount in situ expression analysis of ESR11. (**A**) Stage 13, dorsal, anterior down. (**B**) Stage 14, dorsal, anterior down. (**C**) Stage 19, anterior down. (**D**) Stage 21, anterior to the right. (**E**) Stage 27, lateral, anterior to the right. (**F**) S1 transversal section. (**G**) S2 transversal section. m, medial; i, intermediate; l; tg, trigeminal placode; vfb ventral forebrain; op olfactory placode; nt, neural tube, pa, pronephros anlage; dlp, dorsolateral line placodes; p, pronephros; vz, ventricular zone.

In addition to Notch signaling pathway genes, two additional *Xenopus* genes, which have not been described yet, were found multiple times in the microarray analysis: Serum glucocorticoid regulated kinase (SgK) and clone He 435, which is most homologous to *Xenopus tropicalis* Oligodendrocyte transcription 3 (Olig3), (see Table 1).

SgK was first detected at stage 12 in a region above the dorsal lip of the embryo and distinct cells in the endoderm (Figure 47A). While the expression is mostly absent from the neural plate, weak expression was detected throughout the nonneural ectoderm in a punctuated pattern (Figure 47B). SgK is expressed in the posterior tip of the embryo and in a half-moon shape area in the anterior region of the embryo, most likely the presumptive cement gland region (Figure 47C). The most posterior expression domain extends anteriorly in two longitudinal stripes, while the anterior expression persists (Figure 47D, E). At stage 23, the expression in individual cells of the nonneural ectoderm is most prominent, while in the anterior an expression domain around the cement gland forms (Figure 47E, F). At stage 32, SgK expression could be detected in individual cells and a specific region of the midbrain (Figure 47G).

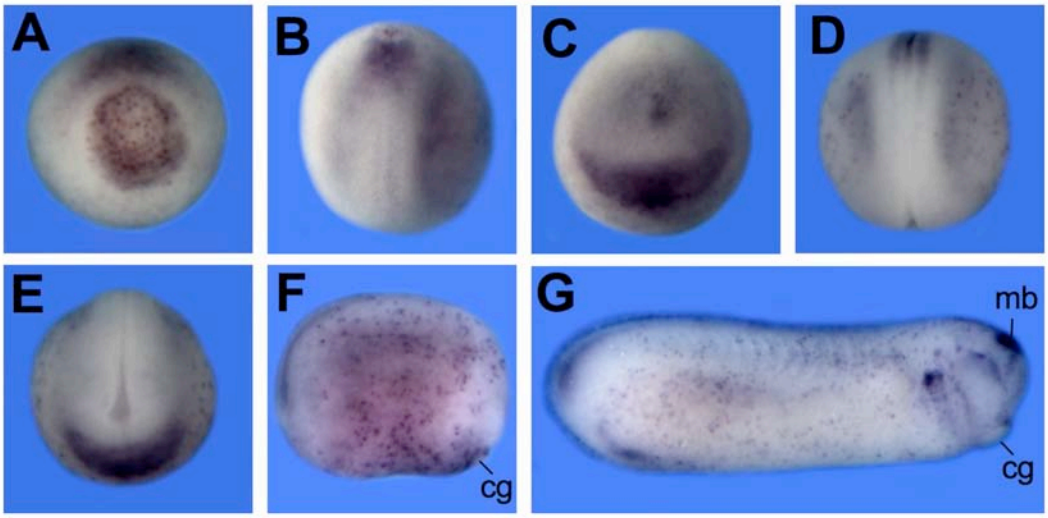

Figure 47 Spatial expression analysis of SgK. Whole-mount in situ expression analysis of SgK. (**A**) Stage 12, vegetal, dorsal up. (**B**) Stage 16, dorsal, anterior down. (**C**) Embryo in B, anterior, dorsal up. (**D**) Stage 18, dorsal, anterior down. (**E**) Embryo in D, anterior, dorsal up. (**F**) Stage 23, lateral, anterior to the right. (**G**) Stage 32, anterior to the right. Cg, cement gland; mb, midbrain

The early expression pattern of Olig3 is reminiscent of XHR-1, as it is expressed at stage 12 in two transversal stripes located in the anterior neural ectoderm (Figure 48A). The expression persists during late gastrula (Figure 48B). At stage 17, the expression narrows as the neural plate starts to fold (Figure 48C). By stage 19, Olig3 is also expressed in two longitudinal stripes in the folding neural tube (Figure 48D). Anteriorly, Olig3 could be detected in two specific regions, which most probably correspond to the midbrain-hindbrain boundary or hindbrain areas (Figure 48D). At stage 27, Olig3 is strongly expressed in the hindbrain as well as weakly in the neural tube (Figure 48G). In a transversal section of a stage 27 embryo, Olig3 transcripts could be detected in the dorsal hindbrain region as well as in the dorsal neural tube. This expression correlated with the expression domains found in mouse, where Olig3 is expressed in the most dorsal part of the hindbrain and neural tube (Takebayashi et al., 2002).

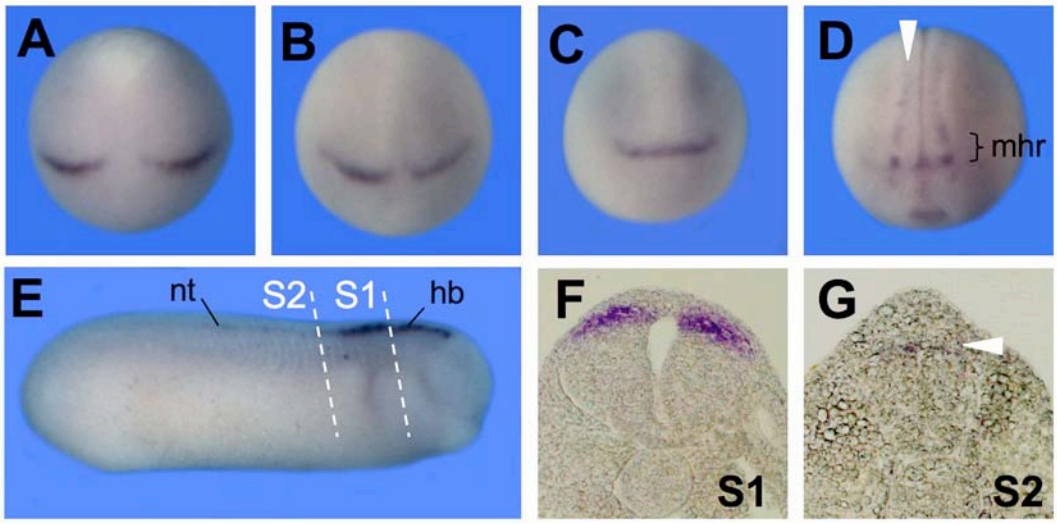

Figure 48 Spatial expression analysis of Olig3. Whole-mount in situ expression analysis of Olig3. (**A**) Stage 12, dorsal, anterior down. (**B**) Stage 13, dorsal, anterior down. (**C**) Stage 17, dorsal, anterior down. (**D**) Stage 19, dorsal, anterior down. White arrow head marks longitudinal stripe of expression. (**E**) Stage 27. Lateral, anterior to the right. (**F**) S1, transversal section. (**G**) S2, transversal section. White arrow marks expression in most dorsal domain of the neural tube. mhr, midbrain-hindbrain region; nt, neural tube; hb, hindbrain

Among the genes, which were identified only once in the microarray analysis, the expression of R 9328 expression was similar to the expression of ESR4 and ESR5 (Figure 49; also Figure 42) (Jen et al., 1999). R 9328 was upregulated by ESR1-VP16-GR, but sequence alignments with the known ESR proteins revealed no significant homology. As seen in Figure 49, R 9328 transcripts could be first detected at stage 11.5 in a ring around the blastopore, with the exception of the most ventral lip (Figure 49A). At stage 12, the expression persisted in the posterior regions of the embryos with one transversal anterior stripe of expression (Figure 49B). This pattern is reminiscent of the early phases of segmentation within the presomitic mesoderm (Jen et al., 1999). This expression pattern continued throughout development as the paraxial mesoderm undergoes somitogenesis in a rostral-caudal direction (Figure 49C, D).

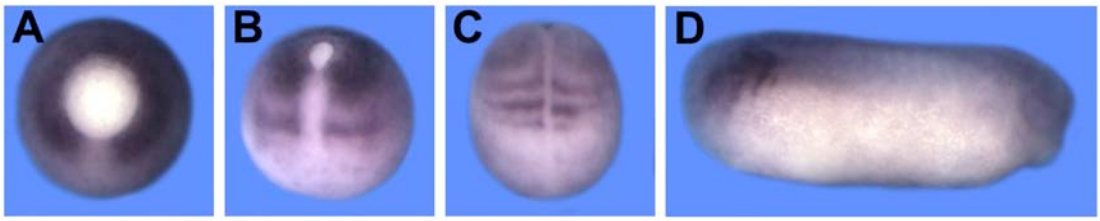

Figure 49 Spatial expression analysis of R 9328. (**A**) Stage 11.5, vegetal, dorsal up. (**B**) Stage 12, dorsal, anterior down. (**C**) Stage 14, dorsal, anterior down. (**D**) Stage 25, lateral, anterior right.

We could also identify three additional genes, which are expressed within the territories of primary neurogenesis. By stage 11, clone He 1302 was

expressed broadly throughout the posterior ectoderm and by stage 12 two stripes of expression extend posterior (Figure 50A, B). During neurula stages, the expression was detected within the three longitudinal domains of primary neurogenesis (Figure 50C). The anterior expression was restricted to a group of cells associated with the olfactory placode, and by a central transversal stripe of cells that corresponds to areas in the ventral forebrain region (Figure 50D). By stage 22, He 1302 is expressed in the trigeminal ganglion and the neural tube (Figure 50E). In a transversal section of a stage 37 embryo, expression of He 1302 was restricted to the marginal zone of the neural tube, in which post-mitotic neurons are located (Figure 50G).

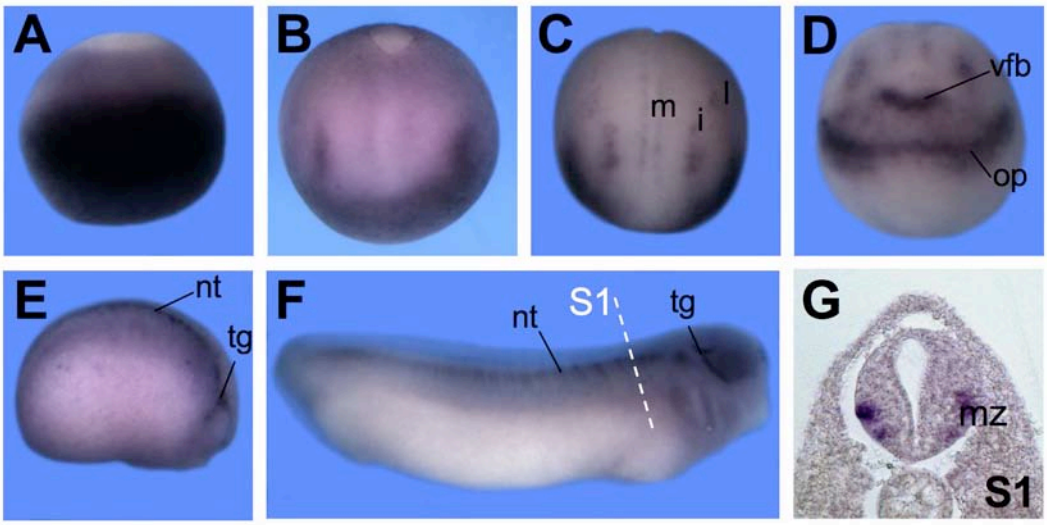

Figure 50 Spatial expression analysis of He 1302. (**A**) Stage 11, dorsal. (**B**) Stage 12, dorsal, anterior down. (**C**) Stage 13, dorsal, anterior down. (**D**) Stage 14, anterior. White arrow head markes anterior neural rinch. Black arrow head markes trigeminal placodes. (**E**) Stage 22, lateral, anterior right. (**F**) Stage 37, lateral, anterior right. (**G**) S1 transversal section. m, medial; i, intermediate; l, lateral; vfb ventral forebrain; op olfactory placode; nt, neural tube, tg, trigeminal ganglion; mz, marginal zone

Expression of clone He 459 could be detected around stage 11 broadly throughout the posterior ectoderm (Figure 51A). At stage 12, two expression domains that extended into two posterior stripes in the anterior neural plate region were detected (Figure 51B). While the strong expression in the anterior ectoderm persists, expression within the longitudinal stripes increased (Figure 51C, D). By stage 20, the expression in the posterior region of the embryo declined and only expression in the region of the midbrain and hindbrain as well as the dorsolateral line placodes was strongly detected (Figure 51E, F). At stage 37, He 459 was expressed in specific regions of the brain as well as the otic vesicle (Figure 51G).

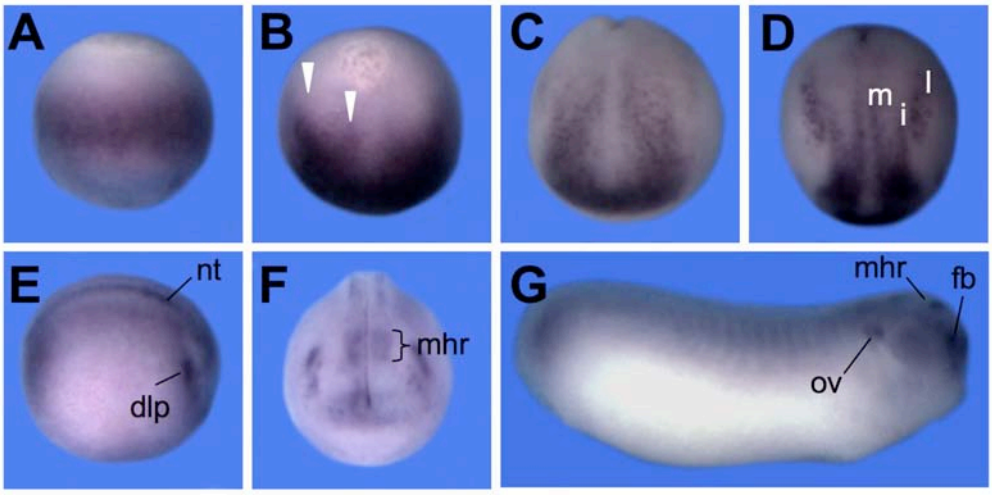

Figure 51 Spatial expression analysis of He 459. (**A**) Stage 11, dorsal. (**B**) Stage 12, dorsal, anterior down. (**C**) Stage 13, dorsal, anterior down. (**D**) Stage 14.5, anterior. (**E**) Stage 20, lateral, anterior right. (**F**) Same as (E), anterior. (**G**) Stage 37, lateral, anterior right. m, medial; i, intermediate; l, lateral; nt, neural tube; dlp, dorsolateral line placodes; mhr, midbrain-hindbrain region; ov otic vesicle, fb, forebrain.

In contrast to the broad domains of He 1302 and He 459, Endosulfine alpha (Ensa) could be first detected at stage 12 in two longitudinal domains. (Figure 52A). By stage 13, Ensa is weakly expressed in the presomitic mesoderm as well as the territories of primary neurogenesis in the posterior and anterior neural plate (Figure 52B-E).

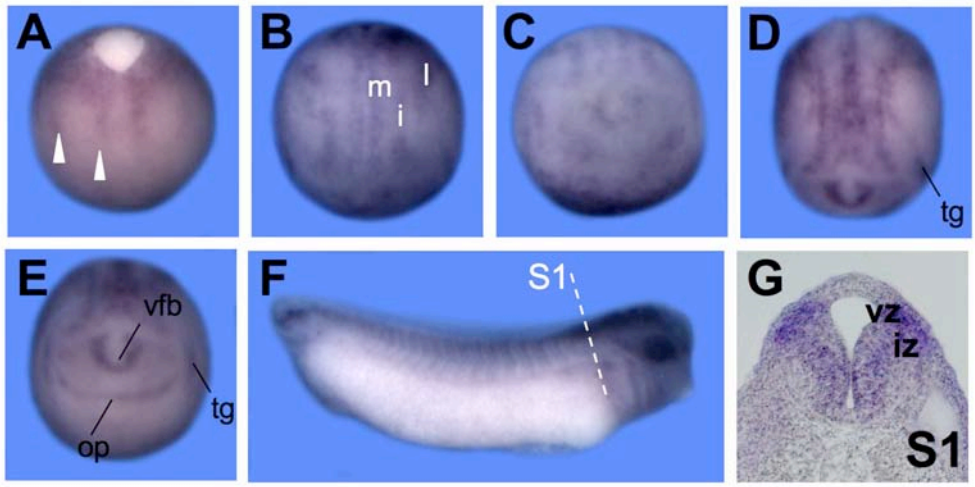

Figure 52 Spatial expression analysis of Ensa. (**A**) Stage 12, dorsal, anterior down. Arrowheads mark expression in longitudinal stripes. (**B**) Stage 13, dorsal, anterior down. (**C**) Same as (B) anterior. (**D**) Stage 15, dorsal, anterior down. (**E**) Same as (D) anterior. (**F**) Stage 37, lateral, anterior right. (**G**) S1 transversal section. m, medial; i, intermediate; l, lateral; nt, neural tube; vfb, ventral forebrain; op, olfactory placode; vz, ventricular zone; iz, intermediate zone.

Ensa continues to be expressed in the central and peripheral nervous system including the eye. In a transversal section of a stage 37 embryo, this expression in the neural tube is located in the proliferating neural progenitors

of the ventricular zone and the intermediate zone, where cells start to differentiate (Figure 52G).

4 Discussion

4.1 Xmxi1 in the context of primary neurogenesis

Xmxi1 is a novel *Xenopus* Mad family member recently identified in our laboratory (Souopgui et al., 2003). It is expressed broadly throughout the domains of primary neurogenesis during early embryogenesis and at later developmental stages, the expression is maintained throughout the CNS. Consistent with the early expression of Xmxi1 transcripts during *Xenopus* primary neurogenesis, as part of this thesis work have found that Xmxi1 plays an essential role in the formation of a mature neural fate, which can be acted upon by factors that induce neuronal differentiation (Klisch et al., 2006). A summary of the obtained results and the relationship of Xmxi1 with known components of the neurogenesis network are shown in Figure 53.

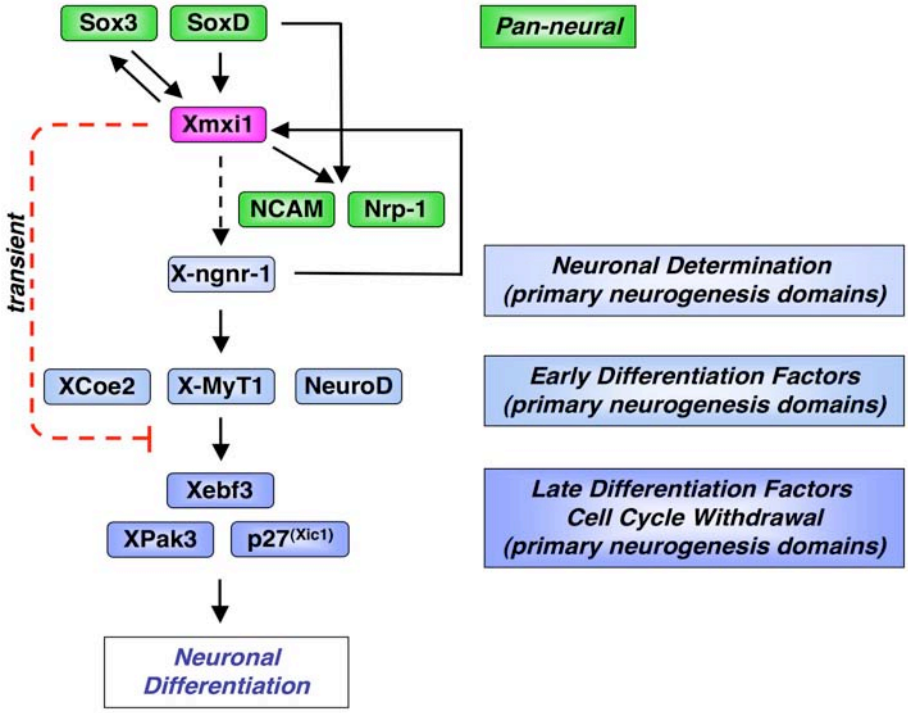

Figure 53 Scheme representing the role of Xmxi1 during primary neurogenesis in *Xenopus*. Neural induction by BMP inhibitors leads to the activation of several pan-neural expressed genes including Sox3 and SoxD. In turn, SoxD can activate Xmxi1, which is required for SoxD-induced activation of NCAM, Nrp-1, X-ngnr-1 and neuronal differentiation. Xmxi1 transiently inhibits neuronal differentiation downstream of the proneural genes (red dashed arrow), which may be the result of Sox3 activation. At later stages, Xmxi1 can induce neuronal differentiation (black dashed arrow). Xmxi1 is also activated by proneural genes such as X-ngnr-1 and NeuroD. In the absence of Xmxi1, X-ngnr-1 does not completely neuralize the ectoderm and neuronal differentiation is inhibited.

Consistent with an early role in the context of neurogenesis, Xmxi1 was positively regulated by the panneurally expressed HMG-box transcription factors Sox3 and SoxD. Through loss-of-function experiments, Xmxi1 was shown to be required for the endogenous expression of X-ngnr-1 and consequently neuronal differentiation, as well as for SoxD-induced activation of Nrp-1, NCAM and X-ngnr-1. While Xmxi1 is required for neurogenesis, overexpression of Xmxi1 at high concentration inhibits neuronal differentiation, which may be the result of Sox3 induction and maintenance of an early neural cell fate. X-ngnr-1 and its early proneural target genes, such as X-MyT1 and Ebf2 are not inhibited by overexpression of Xmxi1, but the expression of late acting differentiation genes such as Ebf3 and those required for cell cycle withdrawal of the progenitors including XPak3 and $p27^{(Xic1)}$ are inhibited. Suppression of neuronal differentiation is only transient and at later developmental stages, the expanded precursor population, perhaps due to a decline in concentration of Xmxi1 or Sox3, can undergo neuronal differentiation. In addition, Xmxi1 is also positively regulated by X-ngnr-1 and NeuroD. While Xmxi1 may have an additional role downstream of X-ngnr-1, the study supports the requirement of Xmxi1 in establishing a mature neural fate, which is required prior to the initiation of neuronal differentiation. This hypothesis is supported in animal cap experiments whereby in the absence of Xmxi1, the complete neuralization of the ectoderm by X-ngnr-1 as marked by Nrp-1 and NCAM is inhibited, as well as neuronal differentiation.

Several prepattern genes have been identified that bridge the events of neural induction with the downstream acting proneural genes. However, the epistatic relationship and the molecular mechanism by which these factors act is for the most part unknown. Members of the SoxB1 and Zic families have been found to participate in the early establishment and stabilization of a neural fate. SoxD and members of the Xiro family, in contrast, act as late stabilizing genes, positively regulating the proneural genes and promoting neuronal differentiation (Penzel et al., 1997; Kuo et al., 1998; Brewster et al., 1998; Mizuseki et al., 1998; Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998). As only a restricted number of cells within the neuroectoderm will exit the cell cycle and commence differentiation (Hartenstein, 1989), one intriguing

question is how the three bilateral longitudinal primary neurogenesis domains are selected by these broadly expressed prepattern genes. Zic2 inhibits neuronal differentiation and may contribute to this process, particularly at late neurula stages where it is expressed in the posterior region of the neural plate in domains that alternate with stripes of the proneural genes (Brewster et al., 1998). However, during gastrulation and early neurula stages, Zic2 is expressed broadly throughout the prospective neural ectoderm (Brewster et al., 1998), suggesting the involvement of additional factors.

Most identified proneural genes exhibit coordinated expression in the discrete domains of primary neurogenesis. The temporal expression of the genes within these domains correlates with their function. Early expressed genes tend to act as determination factors while later expressed genes function in differentiation. Moreover, a refinement of the proneural domains is also observed. For example, the expression of X-ngnr-1 is much broader than downstream differentiation factors such as X-MyT1, which is expressed in slightly larger domains than N-tubulin (Bellefroid et al., 1996). As described above, it remains elusive as to how the known prepattern genes can account for the restricted expression of the proneural genes. Based on the expression of Xmxi1 and its requirement for SoxD function, Xmxi1 is an ideal candidate to participate in this process.

In naive animal caps, the effectiveness of Xmxi1 in promoting ectopic neuronal differentiation was low. However, in neuralized animal caps, the ability of Xmxi1 to induce neuronal differentiation was much stronger, suggesting the existence of co-acting factors such as SoxD and other prepattern genes that act synergistically or in parallel. The gain-of-function phenotype of Xmxi1 was similar to that of SoxD, i.e the transient inhibition of neuronal differentiation at the open neural plate stage with ectopic neurons at the tailbud stage (Yeo and Gautier, 2005; Mizuseki et al., 1998). Albeit, SoxD is much more effective in inducing ectopic neuronal differentiation compared with Xmxi1.

In Xmxi1-injected embryos, at the open neural plate stage, an inhibition of neuronal differentiation was observed concomitant with ectopic activation of a marker of proliferating neural progenitor cells, Sox3. A correlation of ectopic

Sox3 activation with inhibition of neuronal differentiation at the open neural plate stage followed by ectopic neurogenesis at later stages has been observed during primary neurogenesis in *Xenopus* (Bellefroid et al., 1998; Voigt et al., 2005). Electroporation of Sox2 and Sox3 in the chick neural tube has also been shown to inhibit endogenous and X-ngnr-1-induced neurogenesis (Bylund et al., 2003; Graham et al., 2003). The association of Sox3 with proliferating cells has given rise to the idea that Sox3 promotes proliferation at the expense of differentiation. However, a direct interaction of Sox3 with the cell cycle machinery has not been established. Moreover, forced cell cycle arrest with HUA could not rescue the ectopic Sox3 expression and suppression of neuronal differentiation suggesting Xmxi1, and possibly Sox3, have a direct influence on neural cell fate specification.

4.2 Xmxi1 as part of the Myc/Max/Mad Network

The observation that Xmxi1 induces proliferation was somewhat surprising as previous studies have demonstrated that Mxi1, like other members of the Mad family, can induce growth arrest and can antagonize Myc activity when overexpressed in cell lines (Zervos et al., 1993; Schreiber-Agus et al., 1995; Wechsler et al., 1997). From these studies and numerous others, a model of the Myc/Max Network has been established, in which the Mad family of transcriptional repressors are thought to function exclusively to attenuate and restrict Myc activity. However, emerging evidence, predominately from in vivo experiments, suggests that this elegant but simplistic model needs to be reevaluated. The different Mad knockout mice (Mad1, Mxi1 and Mad3), with the exception of Mnt, exhibited only subtle effects on growth and differentiation and did not give rise to tumors, as would be predicted if the Mads were to function as Myc antagonists in vivo (Sadaghiani and Thiebaud, 1987; Toyo-oka et al., 2004, Grandori et al., 2000; Hurlin et al., 2003). In addition, the Mad and Myc proteins are most likely to have both shared and unique target genes (O'Hagan et al., 2000). While the expression of some members of the Mad family, such as Xmad1, coincides with cells that have undergone terminal differentiation (Gehring et al., 2000; Bejarano et al., 2000; Roussel et al., 1996; Roy and Reisman, 1995; Pulverer et al., 2000; Jürgenset al., 2005). Mxi1, in contrast, is found in both

proliferating (together with Myc) and differentiating cells (Zervos et al., 1993; Larsson et al., 1994; Hurlin et al., 1995). The situation for Mxi1 is further complicated by the finding of multiple isoforms differing in their ability to act as transcriptional repressors and to inhibit Myc activity (Engstrom et al., 2004; Dugast-Darzacq et al., 2004).

The distinct domains of expression during *Xenopus* embryogenesis described in this study and that of the previously described Xmads suggests that members of the Mad family of transcriptional repressors may have additional functions independent from the ability to antagonize Myc activity (Newman et al., 1999; Jürgenset al., 2005). Moreover, the expression of Xmxi1 at neurula stages does not coincide with Myc expression, which is found in the premigratory neural crest cells and the anterior neural plate (Bellmeyer et al., 2003), further suggesting a function for Xmxi1 during *Xenopus* embryogenesis other than acting as a Myc antagonist.

4.3 Identification of ESR target genes

To gain insight into the molecular mechanisms by which Notch signaling influences cell fate decisions, particularly in the context of primary neurogenesis, we aimed to identify early gene targets of ESR1. Towards this end, a hormone inducible antimorphic ESR1 protein was generated (ESR1-VP16-GR), which upon overexpression afforded an activation of primary neurogenesis in whole embryos as well as in animal caps. Using this construct, a cDNA library was generated by PCR subtractive hybridization that was enriched in ESR1-VP16-GR target genes (ESR library). In a microarray-based secondary validation, 2,304 clones of the ESR library were analyzed in addition to another 25,128 cDNA clones from two unrelated libraries. Using this approach, 207 differentially expressed clones were identified. 206 clones were identified being upregulated, while only one clone was identified to be downregulated. This outcome is consistent with the notion that an activating form of a repressor protein was used, which should primarily activate transcription in the limited time window analyzed. Within the ESR library, 185 of the 207 differentially expressed clones (90%) were identified. In comparison to the total number of ESR clones analyzed, 8% of the clones of the ESR library were strongly differentially expressed. In a recent study aimed to identify targets of XHR-1 in the midbrain-hindbrain boundary, a large set of unrelated clones were screened by use of a macroarray (Takada et al., 2005). In this screen, 75 out of 48,000 cDNA clones were found to be gene targets of XHR-1, so that only 0.16% of all clones were candidate genes (Takada et al., 2005). Using the PCR subtractive approach, we could increase the candidate pool by fifty to hundred folds.

4.4 Putative ESR1-VP16-GR gene targets

In a first step, all 207 differentially expressed clones were sequenced and a total of 55 non-redundant genes/ESTs could be identified. Among these 34 were identified as known genes and 20 as EST sequences, only one clone did not show significant homology to sequences within the databases. However, it

cannot be ruled out, that some cDNA clones are indeed known genes, since some inserts were very short and may be part of an untranslated region.

Of the 55 identified genes, 42 were upregulated by ESR1-VP16-GR *in vivo* (Figure 54). Three clones, all expressed in the midbrain-hindbrain region, were shifted posteriorly, while one of these, XHR-1, also showed weak ectopic expression in the open neural plate. Only 2 genes, one of which was identified to be downregulated in the microarray analysis, are inhibited in whole embryos upon ESR1-VP16-GR overexpression. Eight genes could not be analyzed by whole-mount in situ hybridization, most likely due to an insufficient length of the in situ probe. This demonstrates that the genes identified using our approach are potential targets of ESR1 *in vivo*.

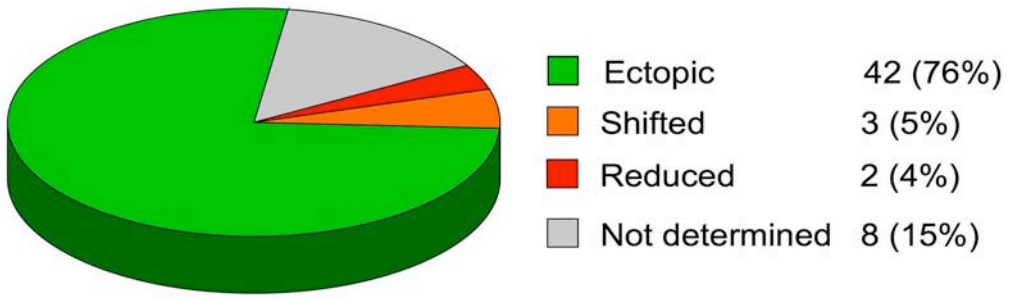

Figure 54 Pie diagram of ESR1-VP16-GR overexpression. Shown is the result of the overexpression phenotype of ESR1-VP16-GR (50 pg). The predominant number of genes identified within this screen were ectopically upregulated by ESR1-VP16-GR, while only 2 clones were inhibited, of which one was identified within the microarray to be downreagulated as well.

4.5 Cross-regulation of ESR genes in *Xenopus*

Within the 55 differentially expressed genes/ESTs, twelve genes could be associated with Notch signaling, including the Notch ligands X-Delta-1 and X-Delta-2, and the Notch effectors ESR2, ESR3/7, ESR4, ESR5, ESR8, ESR9, ESR10, ESR1, Hes2 and XHR-1 (Chitnis et al, 1995; Jen et al., 1997; Shinga et al., 2001; Deblandre et al., 1999; Jen et al., 1999; Sölter, 2006; Li et al., 2003; Turner, unpublished). We could demonstrate, that all Notch components are upregulated by ESR1-VP16-GR in whole embryos and ectodermal explants. Conversely, all were downregulated by ESR1-GR in whole embryos. Furthermore, an intact DNA binding domain as well as an

activator domain are necessary for the induction by ESR1-VP16-GR in animal caps.

Consistent with previous results in *Drosophila* and *Xenopus*, we thus observed a cross-regulation of Notch effectors. Using a chromatin profiling technique it has been shown in *Drosophila*, that Hairy binds to the promoter of E(spl)m8, one Enhancer of split gene, demonstrating a direct transcriptional cross-repression among Notch effectors (Bianchi-Frias et al., 2004). Using cycloheximide treatment in *Xenopus*, it was shown that ESR1, ESR9 and XHR-1 directly repress each other *in vivo* (Takada et al., 2005). A regulatory loop was also demonstrated for Hairy and ESR1, which results in a cell context specific response to Notch signaling (Cui, 2005). These observations suggest, that the cross-regulation of Notch effectors is conserved and a complex network of cell autonomous and non-cell autonomous interactions may exist.

Although several bHLH-Orange proteins were identified in our screen, it appears that only a subset of bHLHL-Orange proteins is activated by ESR1-VP16-GR. bHLH-Orange proteins, which are most likely not regulated by Notch signaling, were not identified in our microarray-based ESR target screen. Two examples are ESR6 and Hes6, which were also not induced by ESR1-VP16-GR in animal caps (see Figure 40, Table 1). ESR6 is expressed within the superficial layer of the ectoderm with the onset of transcription (late blastula) and is thought to be involved in the inhibition of primary neurogenesis within this layer (Chalmers et al., 2002). Although ESR6 can be regulated by Notch signaling, this might be not the case *in vivo*, since ESR6 expression is detected earlier than Notch and Delta expression (Deblandre et al., 1999; Chalmers et al., 2002). Hes6 is not regulated by Notch signaling and was shown to promote neurogenesis (Koyano-Nakagawa et al., 2000). The difference in regulation by Notch signaling might be a reason for the differences in cross-repression among bHLH-Orange proteins.

The cross-regulation of Notch effectors could be required to cell-specifically segregate the diverse molecular events during development, in which Notch signaling is involved. (Louvi and Artavanis-Tsakonas, 2006). As the Notch receptor is widely expressed during embryogenesis (Chitnis et al.,

1995), a separation of Notch activities has to be achieved by different means. The results of our screen and other published reports support a model, in which a Notch effector inhibits Notch activities, which are not involved in its own specific cellular context. In this view, ESR1 might inhibit the onset of the segmentation program within the proneural territories by specifically inhibiting the ligand X-Delta-2, as well as the Notch effectors ESR4 and ESR5, which have been shown to be involved in segmentation (Jen et al., 1997, Jen et al., 1999). During segmentation, ESR9 and ESR10 exhibit a cyclic expression, which is independent of *de novo* protein synthesis (Li et al., 2003). By a combination of Notch effectors, including ESR1, this oscillation clock could be maintained by repression and derepression mechanisms within cells rather than cell-to-cell signaling. ESR1 might also influence the positioning the midbrain-hindbrain boundary. This is indicated by the posterior shift of XHR-1 expression by ESR1-VP16-GR overexpression. It has been shown that XHR-1 and ESR1 and ESR9 are part of a negative feedback loop and repress their transcription vice versa (Takada et al., 2001).

4.6 ESR1 gene targets in the context of primary neurogenesis

The idea of negative feedback loop among Notch signaling components to establish or maintain an inhibitory state, is supported by the fact, that X-Delta-1 is a target of ESR1. It is thought, that a certain threshold of proneural gene activity leads to the stabilization of the neuronal precursor state and therefore these cells can further differentiation, while neighboring cells stay uncommitted. ESR1 could participate in this selection, as the ESR1 expressing cell lowers the Notch signaling activity in the neighboring cell by inhibition of X-Delta-1. The neighboring cell can accumulate proneural activity and undergo differentiation.

Several Notch effectors are expressed within the domains of primary neurogenesis and have been found to be putative target genes of ESR1 *in vivo*, such as ESR2, ESR3/7, ESR8, ESR9, ESR10 and ESR11. A cross-repression among ESR proteins would make sense, if these proteins were differentially expressed. To test this possibility, whole-mount in situ hybridizations using two differently labeled probes, might resolve the question

if these ESR proteins are expressed within the same cell or in adjacent cells. Although these genes could have redundant functions in a cell and the strong expression of one member might be sufficient to transduce Notch signaling leading to an inhibition of primary neurogenesis. Several observations point to the fact, that each member has a specific role, which in sum leads to the restriction of neuronal precursors. They differ in at least two aspects, one being their responsiveness to Notch signaling.

For example, ESR1 is upregulated by Notch input, while ESR10 is activated by Notch input and proneural gene activity, such as X-ngnr-1 (Lamar and Kintner 2005). Another example is XHairy2, which, although activated by Notch signaling, is also upregulated by the homeobox gene Xrx1 (Davis et al., 2001; Andreazzoli et al., 2003). Notch effectors also differ in their binding partner specificity. Some bHLH-Orange proteins, such as *Xenopus* Hes-related 1 (XHRT1), can homo- and heterodimerize with each other, while others, such as ESR9, do not (Taelman et al., 2004). Instead, ESR9 heterodimerizes with bHLH activators, such as X-ngnr-1 and NeuroD, which is not the case for XHRT1 (Taelman et al., 2004). Distinct dimerization properties between bHLH-Orange proteins have also been reported in other species (Leimeister et al., 2000; Dawson et al., 1995). The results of our screen support a transcriptional cross-regulation among bHLH-Orange proteins, most likely specific for each member. Further studies have to be performed, by which the overexpression or knock-down phenotypes of specific Notch effectors are globally analyzed.

Owing to the multiple Notch effectors upregulated by ESR1-VP16-GR, it is not surprising that there is a developmental delay of primary neurogenesis in the animal cap system. This delay might be achieved primarily through inhibiting proneural activity through heterodimerization rather than transcriptional repression, since proneural gene transcripts, such as X-ngnr-1, X-MyT1 and NeuroD can be detected in ESR1-VP16-GR injected animal caps. But how the animal cap cells circumvent the inhibition of the multiple ESR genes, which are induced by ESR1-VP16-GR, remains unknown. However, the animal cap assay in combination with antimorphic ESR constructs is an attractive system to further dissect the temporal activation of

marker gene expression, and should help to resolve this question in the future.

It is known that ESR1 overexpression inhibits X-ngnr-1 in whole embryos (Tealman et al., 2004). Our results suggest, that ESR1 might also directly influence additional steps of neurogenesis. XlPOU2 is a prepattern gene, induced by neural induction. POU2 is regulated by other prepattern genes, such as Zic1 and Zic3 and can itself induce X-ngnr-1 (Witta et al., 1995; Matsuo-Takasaki et al., 1999). We identified POU2 in our screen to be ectopically activated by ESR1-VP16-GR demonstrating that ESR proteins influence neurogenesis prior to neuronal determination. XGadd45γ, also found in our screen, is involved in cell cycle withdrawal of neuronal precursors (de la Calle-Mustienes et al., 2002). Repression of very early acting transcriptional activators, as well as late acting cell cycle withdrawal genes, is one way to keep cells, which have high levels of ESR1, in an uncommitted proliferative state. By maintaining cell proliferation, Notch can influence the cell fate as reported for glia specification in the retina (Ohnuma et al., 2002). While POU2 is not activated by X-ngnr-1, overexpression of X-ngnr-1 ectopically activates XGadd45γ (de la Calle-Mustienes et al., 2002). Thus, the identification of XGadd45γ might be a secondary effect due to the proneural activity in the ectodermal explants. The activation of XGadd45γ by ESR1-VP16-GR in the presence of cycloheximide, which blocks *de novo* protein synthesis, will determine, if the interaction is direct.

Although X-ngnr-1 could be detected by RT-PCR analysis one hour after induction of ESR1-VP16-GR injected animal caps, it was not found among our candidate clones. The early onset of transcription hints that X-ngnr-1 is a potential direct target of ESR1. One reason why X-ngnr-1 was not identified in our screen could be the differences in transcript levels. Using RT-PCR analysis, X-ngnr-1 transcripts needed five to six additional cycles to be detected in comparison to ESR genes. The different expression levels are also reflected in the number of clones for each identified gene. Only 14 out of 55 identified genes were present multiple times, demonstrating that the library is not saturated. By screening additional clones, also low abundant genes may be identified in future.

4.7 New putative ESR1 target genes

Among the identified target genes the spatial expression patterns of two ESR family members, ESR2 and ESR11, have not been precisely described. ESR2 is weakly expressed in a similar pattern as X-Delta-1, as it can be detected at the open neural plate stages in the territories of primary neurogenesis and in the presomitic mesoderm. ESR2 could therefore be involved in lateral inhibition during primary neurogenesis as well as segmentation. ESR11 can be first detected at stage 13 with a weak expression in a stripe like fashion in the posterior neural plate. At around stage 19 to 20, ESR11 transcription can be detected in the pronephros anlagen, an indication, that ESR11 might play a role during pronephros development and patterning as reported for other Notch effectors (Tealmann et al., 2006).

In addition to these Notch effectors, a cDNA clone was isolated, R 9328, which is expressed in a similar pattern to ESR4, arguing for a role in the segmentation process (Jen et al., 1999). Isolation of a full-length clone, will give further insights, if it is a known ESR member, an addition ESR member, or a new gene.

Two clones are expressed in the anterior neuroectoderm in a similar pattern to that of XHR-1, an early marker of the midbrain-hindbrain region and also found in the screen (Shinga et al., 2001). These include the clone He 180 and a clone, which is highly homologous to the *Xenopus tropicalis* bHLH transcription factor Olig3. Upon overexpression of ESR1-VP16-GR, the expression domain of XHR-1 is shifted posteriorly with a weak ectopic expression in the open neural plate. This shift was also observed for the expression domains of Olig3 and He 180. Therefore, these genes and also ESR1 may play a role during midbrain-hindbrain development as reported for XHR-1 (Shinga et al., 2001). In addition, Olig3 could be detected in a longitudinal stripe located to both sides of the midline. As reported for mouse Olig3, *Xenopus* Olig3 is most likely involved in the specification of a subset of dorsal neurons (Takebayashi et al., 2002; Müller et al., 2005).

While 22 clones did not show any visible endogenous expression at the open neural plate stage, three novel genes showed weak expression in the characteristical stripes of primary neurogenesis, namely He 1302 (No. 30), endosulfine alpha (Ensa) (No. 45) and He 459 (No. 51). He 1302 and He 459 expression can be detected as early as stage 10 to 11 broadly in the neuroectoderm similar to that of Sox2, while the expression is restricted during gastrulation to the territories of primary neurogenesis (Mizuseki et al., 1998). While the expression of He 1302 persists in parts of the nervous system and is later found in the marginal zone of the closed neural tube, He 459 expression concentrates to specific brain regions. Ensa expression can be detected in a stripe-like pattern from stage 12 onwards and closely resembles X-Delta-1 expression. In the closed neural tube, transcripts are found throughout the whole neural tube as well as the eye. Important to note is that these genes are expressed early in the territories of primary neurogenesis and the characterization of these genes may give further insights in the molecular events, which govern neuronal development.

Out of 55 identified genes, 42 were unregulated by ESR1-VP16-GR overexpression but 22 did not show an endogenous expression at open neural plate stages. This does not exclude that these genes are not expressed within the territories of primary neurogenesis, but might be due to the low abundance of the transcripts. In future, screening for full length mRNA sequences of each gene we identified and performing detailed expression analysis will help us to further increase the knowledge the molecular mechanisms involved in Notch mediated repression.

5 Conclusions

In this thesis work two aspects of primary neurogenesis in *Xenopus laevis* were investigated. In the first part, we could assign a developmental function to Xmxi1, a member of the Mad family of transcriptional repressors. Xmxi1 was found to be necessary for the early neuronal precursor cell population. Through loss-of-function and gain-of-function experiments, we could demonstrate, that Xmxi1 is essential for panneural genes to induce proneural identity. In contrast to the mammalian Mxi1, which in cell culture inhibits proliferation, we could show that Xmxi1 induces proliferation during primary neurogenesis. Elucidation of the molecular mechanisms by which the transcriptional repressor Xmxi1 mediates its functions will expand our understanding on the events, which govern neurogenesis. On the one hand, Xmxi1 acts as a repressor of genes involved in cell cycle withdrawal, and on the other hand as an activator of panneural gene transcription. Identification of interaction partners and target genes should afford insights into this dual function.

In the second part of this work, we identified several candidate targets genes of the Notch effector ESR1. Among these genes, some may be involved in the specification and differentiation of the midbrain-hindbrain, somites and primary neurons. In future, the characterization of these genes will help to understand, how Notch signaling can influence such a large number of developmental processes in different ways. In particular, how the different developmental processes are temporally and spatially separated, is unknown. In the context of primary neurogenesis, it will be of interest to determine, how the proneural machinery escapes Notch signaling to undergo neuronal differentiation. Future experiments, which are based on this work, should shed insight in the complex interplay of Notch repression at the level of its effectors, the ESR proteins.

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7 Appendix

7.1 Candidate genes and their corresponding cDNA clones

Table 2 List of candidate genes and their corresponding clones. All 207 differentially expressed clones are listed by their homology to known sequences in the public databases. In the first row, the corresponding gene/EST with accession number is listed. The ranking is based on the total number of clones assigned to the gene/EST. Genes/ESTs with the same number of clones are sorted by ascending c-value. The template for whole-mount in situ hybridization (WMISH) is listed with the corresponding RNA polymerase for antisense RNA preparation. Clones starting with a "XL" are obtained from the National Institute for Basic Biology (NIBB, http://xenopus.nibb.ac.jp/), clones starting with "RZPD" are obtained from the Resource Center for Genome Research (RZPD, http://www.rzpd.de/). In addition, RT-PCR primer sequences are listed. He, cDNA library enriched for ESR1-VP16-GR target genes; R, RZPD library.

1 Enhancer of split related 4 (ESR4) (AF137073)

36 Clones (*c-value/p-value*):

He 383 (-3.166/4.39E-08); He 426 (-3.611/1.04E-07); He 487 (-9.298/1.24E-09); He 588 (-6.328/4.39E-09); He 600 (-7.013/7.30E-10); He 610 (-9.084/9.65E-10); He 729 (-6.926/1.76E-07); He 814 (-4.789/5.10E-10); He 933 (-4.671/4.54E-10); He 1175 (-5.472/3.30E-09); He 1193 (-10.094/2.79E-09); He 1235 (-10.003/2.80E-09); He 1247 (-5.082/3.22E-09); He 1332 (-9.003/6.83E-10); He 1349 (-9.810/1.32E-09); He 1375 (-5.215/1.80E-04); He 1403 (-9.140/2.37E-08); He 1449 (-6.650/6.73E-10); He 1451 (-7.696/9.65E-10); He 1485 (-8.112/8.43E-10); He 1532 (-5.745/5.07E-09); He 1563 (-9.764/1.32E-09); He 1608 (-3.388/6.83E-10); He 1682 (-9.973/3.51E-09); He 1916 (-8.657/2.37E-09); He 1928 (-6.692/1.74E-08); He 1983 (-7.100/1.12E-09); He 2103 (-6.406/1.69E-08); He 2144 (-4.724/8.04E-05); He 2190 (-4.924/9.81E-10); He 2288 (-8.191/3.13E-09); He 2383 (-8.683/6.05E-05); He 2390 (-4.288/1.37E-09); R 7008 (-3.662/7.64E-06); R 14836 (-3.352/9.65E-10)

WMISH template (*Template / RNA polymerase*):

RZPDp988E04134D / T7

RT-PCR primers:

for: 5'-AACTCTGCTGACAGTATCTGTGAG-3'

rev: 5'-GAGTCCTTTGTGTTGACTTTCTGG-3'

2 Enhancer of split related 8 (ESR8)

28 Clones:

He 101 (-7.198/7.33E-08); He 119 (-8.965/7.89E-10); He 125 (-10.256/9.65E-10); He 226 (-7.008/1.06E-09); He 307 (-5.409/5.04E-09); He 371 (-5.586/2.10E-09); He 376 (-9.108/9.65E-10); He 531 (-3.470/1.37E-09); He 534 (-9.390/4.39E-09); He 560 (-8.593/1.67E-08); He 674 (-7.931/1.94E-09); He 859 (-9.679/8.19E-10); He 947 (-8.181/2.74E-07); He 956 (-5.588/1.38E-08); He 1068 (-5.837/6.18E-08); He 1085 (-4.735/5.65E-10); He 1262 (-3.080/6.77E-08); He 1346 (-6.790/5.65E-10); He 1500 (-8.419/4.19E-09); He 1555 (-7.253/1.41E-07); He 1654 (-9.320/8.19E-10); He 1736 (-7.896/3.50E-09); He 1881 (-6.724/9.33E-08); He 2042 (-4.512/5.96E-04); He 2080 (-4.718/8.19E-10); He 2093 (-5.35071.57E-05); He 2175 (-6.025/8.19E-10); He 2274 (-5.994/6.78E-09); He 2320 (-3.428/1.28E-08); He 2392 (-7.812/9.65E-10)

WMISH template:

Sölter, 2006

RT-PCR primers:

for: 5'-GCTACTAAAACTGGTGAGATGTC-3'

rev: 5'-CTGTTGGTCACAGGGGACTGTAC-3'

3 Enhancer of split related9 (ESR9) (AJ009282)

27 Clones:

He 333 (-10.347/6.57E-10); He 400 (-9.521/9.65E-10); He 491 (-3.107/2.81E-09); He 504 (-5.359/3.06E-08); He 504 (-5.359/3.06E-08); He 778 (-8.119/9.65E-10); He 907 (-4.797/5.71E-09); He 1025 (-6.848/3.97E-04); He 1081 (-9.662/2.81E-09); He 1303 (-9.735/2.05E-09); He 1316 (-7.079/1.84E-09); He 1300 (-10.056/1.32E-09); He 1541 (-10.770/9.65E-10); He 1677 (-10.839/7.20E-10); He 1693 (-9.262/1.24E-09); He 1766 (-9.919/2.03E-09); He 1774 (-6.725/9.81E-10); He 1940 (-7.433/1.52E-09); He 2016 (-3.499/6.94E-09); He 2061 (-4.865/1.46E-09); He 2074 (-7.675/4.27E-08); He 2077 (-9.942/3.66E-09); He 2157 (-8.904/7.39E-10); He 2311 (-8.740/7.93E-09); He 2378 (-9.028/6.64E-08); R 10338 (-5.847/8.19E-10); R 14818 (-8.990/8.19E-10)

WMISH template:

Sölter, 2006

RT-PCR primers:

for: 5'-GCTTCCAAATATGCACAATCATCC-3'

rev: 5'-CCAGTCCCAGGAGTTGTGCATTT-3'

4 XHes2 (DQ156231)

19 clones:

He 865 (-5.714/6.73E-10); He 166 (-8.914/1.10E-06); He 509(-9.578/3.50E-09); He 557 (-3.327/7.06E-10); He 629 (-4.467/4.71E-10); He 1226 (-5.062/3.24E-09); He 1229 (-5.007/1.78E-09); He 1238 (-8.653/7.30E-10); He 1274 (-4.222/2.80E-09); He 1425 (-4.018/5.65E-10); He 1444 (-3.041/2.40E-08); He 1632 (-4.142/1.32E-09); He 1755 (-9.174/5.65E-10); He 1971 (-8.508/7.89E-10); He 2066 (-6.806/7.20E-10); He 2119 (-6.827/6.38E-08); He 2340 (-9.608/1.52E-09); He 2369 (-4.735/2.80E-09); He 2370 (-7.632/8.19E-10)

WMISH template:

Sölter, 2006

RT-PCR primers:

for: 5'-CTCCGGTCCAGGCACAAAATCCC-3'

rev: 5'-TCTGTGGCTGGAGGGTTGGTTCTG-3'

5 Enhancer of split related 10 (ESR10) (AJ009285)

17 clones:

He 112 (-8.919/1.02E-09); He 291 (-6.088/4.83E-09); He 413 (-9.817/8.19E-10); He 501 (-8.456/2.76E-08); He 703 (-3.805/7.20E-10); He 768 (-10.011/2.70E-08); He 953(-8.133/9.78E-10); He 1153 (-9.687/1.52E-09); He 1432 (-4.851/7.66E-06); He 1785 (-4.400/7.24E-10); He 1889 (-6.204/2.09E-09); He 2018 (-4.128/2.40E-08); He 2139 (-7.333/1.52E-06); He 216 (2-6.015/2.63E-05); He 2224 (-4.372/1.55E-08); He 2393 (-8.291/9.65E-10)

WMISH template:

Sölter, 2006

RT-PCR primers:

for: 5'-CAACCAAATGTAAACCGTTATCC-3''

rev: 5'-TGGTGGTGCGGTGCCTTTGGTAG-3

6 X-Delta-2 (U70843)

8 Clones:

He 472 (-9.015/1.24E-09); He 677 (-4.041/1.43E-07); He 877 (-3.312/1.60E-06); He 1373 (-9.080/8.19E-10); He 1435 (-3.584/6.25E-07); He 1765 (-3.869/4.32E-09); He 2239 (-5.358/4.39E-09); He 2350 (-8.329/4.54E-10)

WMISH template:

Jen et al., 1997

RT-PCR primers:

for: 5'-CTGCAAGGATCTGAAAGTGAGG-3''

rev: 5'-GGGAAGATTAGACCTCAGTGGC-3

7 HES related 1 (XHR-1) (AB071433)

7 clones:

He 479 (-3.461/9.65E-10); He 1192 (-6.398/2.60E-09); He 1201 (-5.384/9.65E-10); He 1577 (-4.501/9.15E-09); He 1945 (-6.103/6.02E-09); R 14741 (-4.142/4.72E-06); R 14803 (-5.647/1.30E-09)

WMISH template:

RZPDp988B07134D / T7

RT-PCR primers:

for: 5'-AGGCACCTTGAATGCTATGG-3'

rev: 5'-GGACTCCTTCCACTCTGTGC-3'

8 Serum glucocorticoid regulated kinase (SgK) (BC073077)

4 clones:

He 1093 (-4.841/7.65E-06); He 1829 (-3.492 2.76E-07); He 1911 (-6.189 2.21E-09); He 2391 (-5.726/9.78E-10)

WMISH template:

XL041n1 / T7

RT-PCR primers:

for: 5'-TTTTCTCACACACCCAACCA-3'

rev: 5'-GGATGAAGGACCAAGGTTGA-3'

9 Enhancer of split related 3/7 (ESR3/7) (BC072958)

4 clones:

He 1714 (-4.317/1.38E-08); He 1828 (-3.292/1.19E-04); He 2255 (-4.234/3.66E-09); R 8617 (-4.835/5.56E-08)

WMISH template:

RZPDp988G0177D / T7

RT-PCR primers:

for: 5'-GAAATCCCTCATAACAATGACATC-3'

rev: 5'-CTACCAATGCCATTTTCAGGTTTG-3'

10 Enhancer of split related 5 (ESR5) (AF1370729)

3 clones:

He 440 (-5.023/1.65E-08); R 1996 (-6.346/7.89E-10); R 9314 (-5.372 8.19E-10)

WMISH template:

RZPDp988G0417D / T7

RT-PCR primers:

for: 5'-CAACCAAATGTAAACCGTTATCC-3'

rev: 5'-TGGTGGTGCGGTGCCTTTGGTAG-3'

11 X-Delta-1 (L42229)

3 clones:

He 330 (-7.344/4.76E-06); He 1299 (-4.329/2.41E-05); He 1510 (-5.350/1.38E-07)

WMISH template:

(Chitnis et al., 1995)

RT-PCR primers:

for: 5'-CCGTGGTGAGTCCAAGACAATG-3'

rev: 5'-GACGTTGAGTAGGCAGAGTCTG-3'

12 Enhancer of split related 11 (ESR11)

2 clones:

He 954 (-6.470/4.58E-10); He 2206 (-6.097/1.48E-09)

WMISH template:

XL109h14 / T7

RT-PCR primers:

for: 5'-TGTCACAAAAATGCCCTTGA-3'

rev: 5'-CAGGCCTGTATGGATTGACC-3'

13 Growth-arrest-and-DNA-damage induced gene gamma (XGadd45γ) (BC078567)

2 clones:

He 202 (-5.874/6.56E-09); R 8228 (-4.911/2.65E-09)

WMISH template:

IRBHp990E064D / T7

RT-PCR primers:

for: 5'-GCTGCTGATGAATACGACGA-3'

rev: 5'-CATAGACTTTGCGGCTTTCC-3'

14 Oligodendrocyte transcription factor 3 (Olig3) (BC082516)

2 clones

He 435 (-3.889/7.18E-09); He 457 (-3.617/7.02E-07)

WMISH template:

IRAKp961C23170Q / T7

RT-PCR primers:

for: 5'-CTCCATGAACTCCATGCTGA-3'

rev: 5'-GGGGAAATAATGGCTGTGAA-3'

15 EST (BP695029)

Clone:

He 132 (-9.9/1.85E-08)

WMISH template:

XL476e20ex / T3

RT-PCR primers:

for: 5'-CAAAGCCAAAAACCCTCTGA-3'

rev: 5'-TCCCATCTTCTAAGAGCCTCA-3'

16 Protein arginine methyltransferase 1 (xPRMT1) (AB085173)

Clone:

He 154 (-9.177/8.65E-10)

WMISH template:

XL240b19ex / T7

RT-PCR primers:

for: 5'-TAGCCACAAATGGGGAATGT-3'

rev: 5'-TCCGGAGAGACCTGCTAAAA-3'

17 Chromatin assembly factor 1 p150 subunit (CAF1-150) (AF222339)

Clone:

He 117 (-9.129/1.21E-09)

WMISH template:

XL085d19 / T7

RT-PCR primers:

for: 5’-TGCAGCCTGTAGTGATTGGT-3’

rev: 5’-TTCCATTGACATTCCCATGA-3’

18 EST (AAH67978)

Clone:

He 267 (-9.049/9.65E-10)

WMISH template:

XL304p04ex / T3

RT-PCR primers:

for: 5’-TGACAAAGAGCCAAGCTCAA-3’

rev: 5’-TTGCAAAGTGCTGTTGAAAAA-3’

19 EST (BC087479)

Clone:

He 313 (-8.607/1.51E-08)

WMISH template:

XL185p12 / T7

RT-PCR primers:

for: 5’-GGCATGCAGCCATCATTA-3’

rev: 5’-CAGAGTGGGACATGAGGGAT-3’

20 EST (BC084852)

Clone:

R 8584 (-8.403/4.81E-05)

WMISH template:

RZPDp988D0477D / T7

RT-PCR primers:

for: 5’-CACACCATGTGCTGTTTGTTT-3’

rev: 5’-CCGGTGGGAAGTTTTTAAGG-3’

21 Enhancer of split related 2 (ESR2) (BX846504)

Clone:

He 712 (-7.467/1.24E-06)

WMISH template:

XL102p06 / T7

RT-PCR primers:

for: 5’-TTGCTACCCTAGGCTGAACG-3’

rev: 5’-AGAAATCCTGAGAACATTCAAGAGA-3’

22 EST (BJ642962)

Clone:

He 224 (-7.412/2.98E-07)

WMISH template:

He 224 / SP6

RT-PCR primers:

for: 5’-CAGTGTCTGATTGGTGGCAG-3’

rev: 5’-ACCTGACTGGCTTGTCCTTG-3’

23 EST (BC0822089)

Clone:

He 272 (-7.249/5.40E-08)

WMISH template:

XL205h08 / T7

RT-PCR primers:

for: 5'-CGTTTTTGGTGTGTGTCACC-3'

rev: 5'-AGGACGCCCAGTAATGTCAG-3'

24 Matrin 3 (*Xenopus tropicalis*) (Matr3) (BC076655)

Clone:

He 105 (-7.160/7.70E-05)

WMISH template:

XL205h08 / T7

RT-PCR primers:

for: 5'-AAAAGCGGGACTGCATACAC-3'

rev: 5'-CCCTTGGTTCATTCTTCCAA-3'

25 13S condensin XCAP-H subunit (XCAP-H) (BC068643)

Clone:

He 116 (-6.890/2.38E-05)

WMISH template:

XL221b05ex / T3

26 p120 (AF150744)

Clone:

He 217 (-6.397/9.65E-10)

WMISH template:

XL448o23ex / T3

27 EST (BJ639156)

Clone:

He 195 (-6.381/4.39E-08)

WMISH template:

XL201c03 / T7

28 Cisplatin resistance-associated overexpressed (CROP) (NM_001016304)

Clone:

He 180 (-6.046/2.30E-08)

WMISH template:

He 180 / T7

29 Cleavage and polyadenylation specificity factor 100 kDa subunit (CPSF-100) (AF139986)

Clone:

He 733 (-4.987/2.90E-09)

WMISH template:

RZPDp988E02125D / T7

30 EST (BP723878)
Clone:
He 1302 (-4.967/4.02E-09)
WMISH template:
XL280n24ex / T3

31 Sal-like 4 (*Xenopus tropicalis*) (Sall4) (NM_001001458)
Clone:
He 2209 (-4.875/8.19E-10)
WMISH template:
XL332n14ex / T3

32 EST (BP730455)
Clone:
He 1783 (-4.696/4.19E-09)
WMISH template:
XL480i17ex / T7

33 Similar to SWI/SNF related (Smarcd1) (BC045009)
Clone:
He 188 (-4.677/7.24E-10)
WMISH template:
He 188 / T7

34 EST (AAI08580)
Clone:
R6322 (-4.543/4.63E-05)
WMISH template:
RZPDp988G0557D / T7

35 Fructose-1,6-bisphosphatase (Fru-1,6-P_2) (BC053784)
Clone:
He 192 (-4.170/3.49E-07)
WMISH template:
XL059f06 / T7

36 Inhibitor of differentiation 4 (Id4) (AAP34250)
Clone:
R3306 (-3.982/7.96E-08)
WMISH template:
IMAGp998I058296Q / T7

37 Forkhead-box protein i 1 (Foxi1) (BC042303)
Clone:
He 2281 (-3.867/7.82E-09)
WMISH template:
He 2281 / SP6

38 EST (CA971845)
Clone:
He 1538 (-3.808/6.40E-06)
WMISH template:
IMAGp998H1213766Q

39 EST (BC048226)
Clone:
He 989 (-3.797/1.52E-05)
WMISH template:
XL449l06ex / T7

40 Distal-less homeobox gene 1 (DLL-1) (AAB27236)
Clone:
R 3988 (-3.788/9.65E-10)
WMISH template:
IMAGp998H108306Q / T7

41 POU 2 (AAH76747)
Clone:
R 4644 (-3.332/5.13E-08)
WMISH template:
IMAGp998F249301Q / T7

42 EST (CB561947)
Clone:
He 2025 (-3.748/2.26E-07)
WMISH template:
XL322i16ex / T3

43 EST (BJ057335)
Clone:
He 714 (-3.660/6.49E-07)
WMISH template:
XL034l23 / T7

44 Creatine kinase, brain (CKB) (BC042282)
Clone:
He 247 (-3.650/3.14E-04)
WMISH template:
XL411e09ex / T7

45 Endosulfine alpha (Ensa) (AAH44986)
Clone:
R 10387 (-3.644/5.79E-09)
WMISH template:
IMAGp998G0612227Q / T7

46 No significant homology

Clone:
He 2169 (-3.598/2.65E-09)
WMISH template:
He 2169 / SP6

47 Hoxd13 (AY167742)
Clone:
He 1028 (-3.415/7.32E-06)
WMISH template:
IRBHp990H0249D / T7

48 EST (BP676976)
Clone:
He 1546 (-3.391/8.04E-08)
WMISH template:
XL424h18ex / T3

49 EST (BC057749)
Clone:
R 9413 (-3.284/4.54E-10)
WMISH template:
IMAGp998B1111131Q / T7

50 Survival of motor neuron protein interacting protein 1 (SIP1) (AF027151)
Clone:
He 208 (-3.248/9.81E-10)
WMISH template:
XL294p22ex / T3

51 EST (BC059972)
Clone:
He 459 (-3.165/7.19E-09)
WMISH template:
XL260d20ex / T3

52 EST (BF612822)
Clone:
R 14995 (-3.077/1.75E-04)
WMISH template:
IMAGp998A188376Q

53 EST (BQ400749)
Clone:
R 9328 (-3.021/4.92E-08)
WMISH template:
IMAGp998D0710950Q / T7

54 ATP-binding cassette, sub-family B, member 4 (*Xenopus tropicalis*) (Abcb4) (BC063924)

Clone:
He 2379 (-3.003/5.18E-04)
WMISH template:
XL102p06 / T7

55 EST (BC084892)
Clone:
R 507 (6.181/1.78E-04)
WMISH template:
RZPDp988C037D / T3

pGEMT-T vector
4 Clones:
He 474 (-3.681/4.69E-09); He 496 (-3.023/6.11E-09); He 979 (-3.441/3.50E-04); He 2252 (-3.372/3.37E-09)

7.2 RT-PCR primers used during initial analysis of one- and three-hour libraries

Table 3 Overview of RT-PCR primers used during the initial analysis of the one-hour and three-hour library.

1 hour library:

I-A09
for: 5'-TGCTGAGCTGAAGCAAAAGA-3'
rev: 5'-GTCACTTTCCCAGAGCTTGC-3'

I-B05
for: 5'-TCCCACTGAAGCTACTGCCT-3'
rev: 5'-CCAGAGCTCAAGGAAACGAG-3'

I-B11
for: 5'-TGGCTTGTGAAATGTTCTGG-3'
rev: 5'-CAGGAAGTTGTGGTTCAGCA-3'

I-D10
for: 5'-TGCTGAGCTGAAGCAAAAGA-3'
rev: 5'-GTCACTTTCCCAGAGCTTGC-3'

I-D12
for: 5'-AAAAGGCTTATTCCCACGCT-3'
rev: 5'-CGTCAGATTGGTATGGGCTT-3'

I-E07
for: 5'-TGGAAAGGAACAATCGGAAG-3'
rev: 5'-GAAAAGATGGCAGGACCAAA-3'

I-F03
for: 5'-TTTTGCTATGCATTCCTCCC-3'
rev: 5'-TAGAGCAAAGGCACGTGATG-3'

I-G02
for: 5'-GAGTTCAAAAGCGCCCATAG-3'
rev: 5'-GTGGTGCAGCTATCCAGAGA-3'

II-A01
for: 5'-ACACAGCTGCGTTTTCAGTG-3'
rev: 5'-TAGAGAGCAGGGATCATGGG-3'

II-A11
for: 5'-TTCTTTGAGAAGAACAGCCCA-3'
rev: 5'-GAACCAAGTTGCAAAGAGCC-3'

II-E11
for: 5'-TATCTGCAGAATCTCGCCCT-3'
rev: 5'-GGCAATTTTACCACAGCATGA-3'

II-F04
for: 5'-ACACAGCTGCGTTTTCAGTG-3'
rev: 5'-TAGAGAGCAGGGATCATGGG-3'

3 hour library:

A-H04
for: 5'-TGCTCTGTCCATGTGCTTTC-3'
rev: 5'-CATGTTACAGTGTGCCCCAG-3'

A-H03
for: 5'-CTGCGGCAGTGTATACGAAA-3'
rev: 5'-TGGTTCGCGTTTCTTCTTCT-3'

A-G08
for: 5'-AGGAGAGGAGGAAGGATGGA-3'
rev: 5'-TGAGGATCAATAGGAACCGC-3'

A-G03
for: 5'-GGCCAAAGTCAGCATGGTAT-3'
rev: 5'-AGAGGAAGCCCACCCTATGT-3'

A-F02
for: 5'-GAGGAGACCCTACTGGGGAG-3'
rev: 5'-CATAATTTCCTGGGGGACCT-3'

A-B08
for: 5'-GTTTTCCCGCATGTATTGCT-3'
rev: 5'-GGCCAAACTTGAGAAGACCA-3'

A-A03
for: 5'-TCCCACACACTGCCAGATAG-3'
rev: 5'-TGAAAGTGCACCAGACTTGC-3'

A-G11
for: 5'-TTTCCCAGAGCTTTTCTGGA-3'
rev: 5'-TTTCAGCAAGATGGCAACAG-3'

B-H06
for: 5'-AACCAAGATGCTGGTTCCAC-3'
rev: 5'-AGACGCGCAATTGAAAAGAT-3'

B-H4
for: 5'-ATGGCTCCATAGGTTTCACG-3'
rev: 5'-GAGATGGGTGAACCGTGACT-3'

B-E11
for: 5'-TGCATGTTTGGCACACTTCT-3'
rev: 5'-CGGACTTTCCCATTCTGTGT-3'

B-C02
for: 5'-ACCCTGGTTGGCATAGTCAG-3'
rev: 5'-CGGGTGAAGAGATCACAAT-3'

7.3 DNA sequences of candidate clones

Table 4 List of all 207 candidate clones with their corresponding DNA sequence. The vector of each clone is listed. The sequence is shown 5' -> 3' in accordance with the sequencing primer used. He, clone of the ESR1-VP16-GR library; R, clone of the RZPD library. The RZPD clones are listed with vector backbone and accession number.

Clone He 101 pGEM-T (SP6 ->)

5'-ACAAGGCAGAGGAAGATTTTTCTCCTATATCTGTGTTCATCATGAAAATACATATAT
TATCATTCCAGCTGCTGGAAATAGAGGCAATCCCTTCCCAAAAGAAATGAGGGAGCC
ATTTCTATCTATAGACAGGCAATAATATGCCTAACAATTAATATAAATATAACAGACATT
CAGTCAGTGCTGT-3'

Clone He 105 pGEM-T (SP6 ->)

5'-ACGCGGGGGGGATTAGAGTCCAGGCCGGAGCCGAGAAGCTGCATGTTAATGGCG
TTGTGGGACGTTTGGCGGCTGCGTTAAGAAAAGCGGGACTGCATACACCATAGGAG
TTCTCGGTTTCTGCTCCCAAGAAGATTCCTCAAAAGTTCCTTTCCGGCTAACAAAATT
CTATATTTATAGTTCGGGACTTGATATTTGTGCCATTTGGCATTTTAAAAATCAAGGAA
AAAAATAGTGCTACAATGTCAAAGTCTTTTCAACAGTCATCCTTAAGCAGAGACTTGC
AAGCCCGTCATAGGAGTGATACTACAGGAACAGGTCTTCTTACTGCTAATCAGTCATT
ATCTATTCCATCTTCTCTTGGAAGAATGAACCAAGGGACTAGCCGTATTGCTAGTTTG
ATGAGCTGTGGAACAATTTCTAAATTAAATCAGCCGGGATCTCATATATCTAGTTCCT
CTTCTCACAGCTTGCC-3'

Clone He 112 pGEM-T (SP6 ->)

5'-ACTGTTAAATGCTGCAAATATTGCATATTACTAAGAAATGTAACAGTTATTATACAGT
TAAATACTGCAAATGTTGCATGTTACCTAAAAAATATAACATTTCAATACTGCAAATATT
GCATATTACTAAGAAATGTAAGTTATTATACTGTTAAATACTGCACATATTGCATATTAC
TAAGAAATGTAACAGTTAATATACTGTTAAATACTGCAAATATTGCATTTTACAAAGAA
ATATAACAGTTATACAGTTAAATACTGCAAATATTGCATATTACGTAAAAATATAACATT
TTCAATACTGCAAATATTGCATAGT-3'

Clone He 116 pGEM-T (SP6 ->)

5'-ACCGATGTCTCCATTTCCGATCTGAGTTAGCTGTGCTCCTTTTCTTTCCAATCTGCA
AGCTTCACGATGTTCTGCAAACTCTCCAATATCTCCAGCATCCATCCCTTCGCNAACA
TCTGCATCGAATTCATCAGCTACAGGAGCTTCTGACTCCACAAAGTCATCCTCCACCT
CTGCATTGATGTCAGAGACGTGGTCGCTCTTCTTAAACTTGTCCATTAATAATGAGAA
GTTCTCGTTTTGGGCATCACTGTTCCACTGCATGAATCGGAACCCAGAGAGGGATGG
ACATAGCGGACGCTTTTCCACACACTGTAAAAACAGGGATTTAAGTTCCGT-3'

Clone He 117 pGEM-T (SP6 ->)

5'-ACAGATCCAGAGAACCAAAAATTTAGACAGAAGCTGAAGGCTAAGGAGTGGTATG
AGTTGCAAACAAACGGCAAGAAGATCCGTGCAATGCAGCCTGTAGTGATTGGTTGTG
TGTGGTGGGACAGTAAAGCTTCAGAGATTAGTCTTCTGCAAAAATTTTCTGCCTGTAT
TCTGGAGTCACCTGCAGTTGATGAAGAGCTTGCTCAAGAGATCAGCTCTGCTCAAAG
CCTTAAGGACAGGCAAATACTCTCCAAACTTGTTCCTCTACTTCATGGGAATGTCAAT
GGAAGTAAGATAATGATACAAGAATTTCAGGAGT-3'

Clone He 119 pGEM-T (SP6 ->)

5'-ACGCGGGAGCAGCACCGAAACAGTAACTGAGCGCAATGGCACCTTGCACTATTAG
CCAGCAGCCTGCTAACAGCATTAGAAAGATAAGAAAACCAGTGGTTGAAAAGATGAG
GAGAGATCGAATTAACAGCAGCATCGAGCAGCTCAGGATGTTACTGGAGAAAGAGTT
TGAGAAAAATCATCTCCCGTCCAAACCAGAGAAAGCTGACATTCTGGAAATGGCAGT
CAGCTTCTTGCAGCAGCACATAGCTACTAAAACTGGTGAGATGTCAGGCAAATCCTA
CAAGGAGGGATACTCCAAATGTGTTGAAGATTCGCTTCATTTCTTGTCCGTTCACAAT
CAAGGAAGCCTGCTGAAACATTTCCATGGTCATCAGTTTAACGCAGGAGAGACCCAT
CAGCTGTGCCCTGT-3'

Clone He 125 pGEM-T (T7 ->)

5'-ACGCGGGACACAGCAAAGCCTTGTAGTGGCAGCAGCACAGCTCTCCCTGACAGTA
ACTGAGCACAATGGCACCTTGCAGCATTAGCCCACAGCCCGCTAGCACCAATAGAAA
GATGAGAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGGATTAACAGCAGCATCGA
GCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCATCTCCCATCCAAACC
AGAGAAAGCTGACATTCTGGAGGTGGCAGTGAGCTTCCTACAGCAGCAATTAGTTAC
CAAATCTGCTGATATATCAGGCAAATCCTACAAGGAGGGATATTCCAAATGTGTTGAA
GAGTCGCTGCATTTCTTGTCGGCTTGCAATCATCACAGTGAAGGAAACCTGCTCAAC

ATTTNCATGGTCATCAGTGTNCCTCAGTAGAACCCCCCAGTCTCAGCTGCTCCNTGG
ACAGTCCCNTTTNCCAACCCTGTCCCNANAGNAAANTTTTNGGGAACCATGGTGAAA
GGGT-3'

Clone He 132 pGEM-T (SP6 ->)

5'-ACATCCCCCAGCGCAAAGCCAAAAACCCTCTGAAGAAAGTAGGTCTGGCATGGGC
CATTGGCTTTCCATCTGGCATCCTGCTCTTCCTGCTGGCAAAGAGGGAGGTGGATAA
AAAGCGTCTGGACCAGCTCAAAGTCCGGCAGAGAATGAAAGAAGCCAATCTCGGGG
AGTATGACAGACCAAGATTCAACAAGACAACCTAGCTGTCATACCATTTTTTTGGGTG
GGGGGGCACATTGAGGCTCTTAGAAGATGGGATTAAATGATTCACAGCTTTTATTATA
AAATATTGT-3'

Clone He 154 pGEM-T (SP6 ->)

5'-ACAACTTAAGAGCCCCCAACAGTTGTCCTGCCTTTAGCAGGTCTCTCTGGAGGCA
CTCCTTTTTGTTTTAGTAGGGAATTCAGTCAGAATGGATTGCTAGAATTTAGGACTTCA
TTTTGACTGTAGAGAACCCTAAACCTCCATTACGGAGTGGCCTAAGGGGTGTCTACA
CTGCAAGAGTATTCAGCCCCTGCTGGCAGCAAAGTGTTAAGCTCCAAAGTGTGCATT
CCCATTTTCACTTTTTTTTTTTTATAAATGGACACTATNTGGGTGGGATTTCNGTCCTG
TTAACAGGGGGCNGGNATCATCAGTATCACTGTTTATCCCTCAGGGGGNGCAAAACC
TCCATCCCATTGNGGGCAAATGGGNCACTCACTTGGCTTGGGGNTTTGNACCCCG-3'

Clone He 166 pGEM-T (SP6 ->)

5'-ACTCCAAGCTGGAAAAAGCTGATATTTTGGAAATGACAGTGAGATTCCTAAGAGAC
ATCCCTCCGGTCCAGACACAAAATCAAGCAGATCGCTACAAGGAAGGCTACAGAGCA
TGTGTAGAGCGTCTGAGCGCAATCCTTGGTAAATCTCATGTGCTGACCGGAGAGGCG
AGTAACCGCCTTCTAGAATACCTGCAGAGGAGCCCTGAGCTGTGCAGTTCAGATTGT
AATCATCCTCCCAAGCCCCAACGTCCTAGGATTGTGCTCCAAGTGAGTCCCAGAACA
TCTCAGTTTGGATCTCCTCTCCAGAACCAACCCTCCAGCCACAG-3'

Clone He 180 pGEM-T (SP6 ->)

5'-ACAGTAGAAGAATTAAAGGAAAAAATGAGAAAAAAAATCTTCAGACCCAGATCGGGA
TGAGTGTTCAAAGAAAGAGCGAGATGATAAAGATCGTGAGAAAGAAAAGGAACAGGA
GAGAGAAGAGAGAGAAAAAAAAAGACGGAGAGAGGAGGAAGCTAAAGAAAAGGAGA
AAAATCGTGAGAGGGAGAAACGCAAAAGAAGCTGCTCTCGTAGCAGAAATTCAAGCA
GAACATCCGACAGACGCGGAAGCAGAACCCGTGACCATAAGAGGTCAAGGAGTAGA
GATCGGAAGAGAAGCAGGAGTAGAGAGCGCCAAAGAAGCCAAAGTCACAACAGAAC
AGAAAGAAAGCACAGATCCCGAAGCAGGGAAAAGAGGAGGTCTAAAAGCAGAGAAC
GGAAATCATACAAACACAAAAGCAGAAGTAGGGAGAGGGACCGGGACAGAAAATCTA
AGGAAAAAGAAAAACGAAGCTCTGATGACAAAAAAAGCAGAGAATCCAGCAGTCGAG
AGAAACCGAGTGAATTTAGAAAAACTGAATCAAGGGAATCCGTTGCACAGGTTGAGG
TCAATGGGGCGAACGAAGGCAATAAATCTGAAGGTGACACTCAGTCCAATTAAAACT
GATCTTTGACCTCTGATCAGGCAGAGGGCTGCACTACAGATACCTGAGAGCCGCCTG
CCCAAGTCGTCTTAACTGAAGACCTTCTTTAAATGAGGTGACAGAAAGCTGTAATGAA
AATTTAAAGCTGTTAGCATTGCATTTATACAGTTGAGTTCAAAACCGTTGTAATGTTTA
TAGTGAATTAGTGCACATGTCCTTCCATTGTCAGTCCTGTTGCAAGCACCAGGNTAGT
CTCTTNTTTGACCTAGAACCTGCTTGCNCATTGTTTAAANTATTTTTTTTTGGACTNGN
AACNCAAGTGATTGTTTGTGNACCACGTTANTTTNTTTTCNCCNTTNACCTNNTCCTC
ACTNTNAATAAGCTTTTATTTTGTTGT-3'

Clone He 188 pGEM-T (SP6 ->)

5'-CACGCGTTGGGNGCTCTCCCATATGGTNCGNNCTGCAGGCGGCCGCACTAGTGA
TTAGCGTGGTCGCGGCCGAGGTACATATTAGAAAACAAGGAGGACAATCCCCCCCA
CANACNTTTGNGNCCNANNAAAAATNNAANTAANNGGAGNGANANTANNGGGCCGT
NACTGTTNCTGCATGNTAANGCACATATGTNTACTGCNNATAAATNNTTTATGANTCC
TACTTTGCTAGGGCAAGCCAACACATTNCTAGTAACACCGTACCTGCCCGGGCGGCC
GTNNAGCCCTATAGTGANTCGTATNATANTCCCGCGNCCATGGNNGCCGGTGAGCA
TGCTCACGTCGGGCCCAATTNNCCCTATNGTGAGTCNTATTAAGCACCAGCACATCT
GCTTGCAAGTGACGTGTTCCTTTGTGCAGGATTGAGGTCATTAGATTTCAATGATGAA
GGAATCTCGTGAGGGCGGCAGGCATACCGCGCAGTGGAAATGTACCTGCCCGGGC
GGCCGCTCGAAATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAA
TTCGCCCTATAGTGAGTCGTATT-3'

Clone He 192 pGEM-T (SP6 ->)

5'-GGGCCGNAGTNCGCNGCTNCCCGGACCGCCATGGNCCGCGGGNATTAGCGTGG
TCGCGGCCGAGGTACTATATNCAAGNCACTGACTCAAGTCCATTAGTAGCCATTCCT
CCAGCCTGCTCCATAATGTAAGCCATTGGGTTGCACTCATACAGAAGTCTCAGCTTG

CCCTTTGGGCTCTTTACATTGGCTGAGTACCTGCCCGGGCGGCCGCTCGAAATCACT
AGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCAT
AGCTTGAGTATTCTATAGTGTCACC-3’

Clone He 195 pGEM-T (SP6 ->)

5’-CCACGCGTTGGGNGNCTCTCCCCATATGGTNCGACCTGCAGGNCGGCCGCACTA
GTGATTAGCGTGGTCGCGGCCGAGGTACAAGAGAGCCGCTTATTTTTGATACTCGTT
AATTGTTGTAAACGTTCCTGTAATAAGATGTTCAGTGCCGTGATGGTCTCGCTTTGGA
GCTCCGGTGATTGGCGTGGAGCCCCAGACGTTGCCTATCCACGGTTTAGTGTATTCA
TTTTGTTTGTCCCTTGCTAAAACATAAAATGCCTGTAAAAAGAGTAGGGATGCACCGA
ATACACTATTCTGGATTCGACAGAACCCCCGAATCCTTCGCGAAAGATTCAGCCGAAT
ACCGAATCCGAATTTACATATGCAAATTAGGGGTGGGAAGGGAAAACATCTTTAACTT
CCTTCTTTTGTGACAAAAAGTCACTCAATTACCCTCCCTGCCCTTAATTTGAATATCTA
AGTTCGGATTCGGCTCAGATGGGCAGAAGGATTCATCCGAATCCTGGTGAAAAAGGC
CGAATCCCGACCGAATCCTGGATTCGGTGCATCCTTAAAAAAGAGCCGTGCGTTTAG
AGTACCTGCCCGGGCGGCC-3’

Clone He 202 pGEM-T (SP6 ->)

5’-ACANCACTGACTGTAATGTACATGTTATATTATATATTATTTCGATTAGGCAGTATAT
ATTAGCCTGTCTATAGTATAGAAATGCGCTCCCTCATATTCTTTTGGGAAGGGATTGC
CTCTATTTCCAGCAGCTGGAATGATAATATATGTATTTTCATGATGAACACAGATGTAG
GAGAAAAATCTTCCTCTGCCTTGT-3’

Clone He 208 pGEM-T (SP6 ->)

5’-CCACGNCGTTGGGNANCTCTCCCCATATGGTNCGACCTGCAGGNCGGCCGCACT
AGTGATTTCGAGCGGCCGCCCGGGCAGGTACCCNTCAGGAGCAGGCTGGCATCCN
GACAGANATATGCTAACGGTCTGTTTCTTTCANAANTTTTTGGGATCAATCTGTGCAA
TGACTACATCANGACAACNTGCTGCTTCAATCTGGACTCTCCGCANATACTCCTGAG
GGGTCCGAGGTGGNACGGAGGGATCATANTCNTNGGGAAGATCANAGGCCTTAACC
GGTAACANCCTGNGCATCAGCTCTTCGAGGCCCCGAATCCATGCTTGTCCGGATCTC
CCTGCGTACCTCGGCCGCGACCACGCTAATNNNGCNGCCATGGCGGCCGGGAGCA
TGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTAACCGGGAGCATGCGA
CGTCNGGCCCAATTCGCCCTATAGTGAGTCGTATTAA-3’

Clone He 217 pGEM-T (SP6 ->)

5’-CACGTCGTTGGGGGNCTACTCCCCATATGGTCCGACCTGCAGGNCGGCCGCACT
AGATGATTAGCGTGGTCGCGGCCGAGGTACAAAACTGNNAGNGTGTCNCCTTTCTNC
AGCAAATGTCTGNACATGCTTTTNCAAAATATCTCTGGGTCNNTNGNCCACTTTATTG
NAGGTTCAACTTAATTCNTGANACCAACANANNATTTTGGGGGAATGTNATAAAAGGN
NCACNTGCTNCTCTTGGATTAATAACTNCTAACGACTAANTNCCTGGGNATAAANAAA
GCAGAGATNNGATTGACTGCTATGGCTTAAATATTTNCGCTCCTTTTATTACATTGNAT
ATGTAACATATTGNCTGNNGTGGTGCTCATAGNAATGGNACCTGCCCGGGCNGCCN
CTCCAATCCCGCGGCCATGGCNGCCGGTANCATGCGACNTCTGNCCCNANNCNNNC
TATATGGAGNCGGATNAAGACTAGAGAGGCTACCGGAAGTTGGAATTGACCCAAAAT
GATCGGAGAGTGGATCTTCGTAACTCCTTAACCTTCTGCGAGGGTCTCCTCCATTAG
CCCCTCTCCTCCCAGTAGTAGCATAATCAGGCCCATCTATAAATCCTCCCAGACTCCT
TCGATCATCTTCCAATCCATAGGCATCTTCAGAGCCATATATCCTTCCTGAAAGTAGA
TCAGACTGGCTGCCTCCAAGACCACCACCCCTAACCTGTGGTTGGGGCCCATATCCA
GGGCCGCGGAAAGGATCAACACTGCGATACCGCTGATTTGGCCTGGACAGACTACT
ATAACCAGGGTGTGGAGACTGGGAAATCAGTGGCATTG-3’

Clone He 224 pGEM-T (SP6 ->)

5’-GCGGCCGGGCAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATT
AAACNTAACAGAGCAGAAAACAGTAGATCTGTAAGTAGCAATCTAAGGGTATATTTTC
TATAAAACAGTCTCTATGCACAGTTTATGCACCAGTGTCTATATGTTTTATAGATTTTTA
TTAATCTTATGTGCTGGTTGTGCCGGAGACAAAGCAAACAATATGAGAGTCACTAGGA
ATACCTCAGTGTCTGATTGGTGGCAGATAAACCGTGTTCCTGTTATCCATAGGCTTCT
GNNTTTNTGGTCAGGGTAATTCCTATAATATTCTGATCCAGTGAATAAAGAATGTTGG
CAAAGTATTGAAATTATATGGCCAGCTATGTTTGATCTGCTCCCTTCACTATAAACAG
GCAGATGTTAAAGTAATTATTGTCTGGGAGTTGTAAGTGATGGACCCATCATTTATTC
ACCTTTACGGAAAGCCTTATATTTGGTAACTATTGCACTGAGCAAGGACAAGCCAGTC
AGGTTGACATTACACCTTACACATTGTAGGTTGTANCACAAAAAAACCACAATCTCTA
ATTTATAATTAACAGGGTTGTTCACCTTTGAGATAACTTTTANTATGACGTANAGCGTG
ATATTCTGAGATAATTTGCAATTTTTCATTTTTTATTATTGAAGGTTTTTGAGTTATTTAG
CTTTTTATTCATCAACTCTTCAATTTGCATTTTAAGCAATCTGGTAACT-3’

Clone He 226 pGEM-T (SP6 ->)

5’-ACAGGGCACAGACTGANGGGTCTCTCGCTGCGATAAACTGATGACCATGGCAAAT
GTNTNAGCANGCTTCCTTGATTGTGAACGGACAANAAATGAAGCNAATCTTCAAAACA
TTTGGAGTATCCCNCCTTGTATGATTTGCCTGACATCTCACCAGTTTTAGTAGGCTAT
GTGCTGCTGCAAGAAGCTGACTGCCATTTCCGGAATGTCACCTTTCNCTGGTTTGGA
TGGGAGATGATGTTTCTCAAACTCTTTCTCCAATAACATCCTGAGCTGCTCGATGCTG
CTGTTAATTCNATCTCTCCTCATCTTTTCAACCACTGGTTTNCTTATCTTTCTAATGCT
GTTAGCAGGCTGCTGACTAATACCTGCAAGGNGCCATTGCGCTCAGTTACTGNATTC
GGTGCTGCTGCGGCTTNANCTGNTTCCTGTCTCCCCGCGT-3’

Clone He 247 pGEM-T (SP6 ->)

5’-GGGNGACTCCCCCATATGGTCCGACCTGCAGGCGGCCGCACTAGTGATTAGCGT
GGTCGCGGCCGAGGTACCAAGGTTGGGTGGACAGGTAAGAACATAACCAAGATGCT
GGTTCCACATGAATGCAGATCCTTTGTTCTTGAAAAGAGTTTCAATTTTTGTCAGGCC
AGTGTAAAACCTCTTGAATACTTCCTTCATGTTACCACCCTTTTGCATGGAGATCACA
CGAAGATGGTCTTCCTCATTGATCCAGACAAGGAAGGTCTTGTTGTCGTTGTGCCAG
ATTCCTCTGACATCAGGCCAGTCCCGGGCCATTCCAGAAGCCAAGAGGAGAGGAGA
AACTGGCTTATCAAACAGGAAGTGGTCATCGATAAGCTGCTGCTGCTCCTGGTCAGT
CATGCTATTCAGAGCGTAGTATTTGCCTTTTAGGTCCCCGTCCAAGCTAGCAAGTGCT
TCTATTGACATCTTTTCAATTGCGCGTCTTTCTCCACGGCTGCAGTGAGGTGGGAGG
CTGAATCCGCGAATACTCCTTCCAGTTCTGACACGAGAGCTGAGTACCTGCCCGGGC
GGCCGCTCGAAATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAA
TTCGCCCTATAGTGAGTCGTATTAAA-3’

Clone He 267 pGEM-T (SP6 ->)

5’-ACCAGTCATTTACAGATGCAAAACAAGCTCTTATGAAGTAGATTAAACCAGCTTTTA
AATTTGACAAAGAGCCAAGCTCAAAAGCTTACTAGAGAGAATAAAGAAAATAATTGCC
TGTGTTCATAAAATCTGCATAAAAATGTAGTGCAATGTATGTATATCCCCATTCAACGC
CATCTATTTTACAGAGACATTTTTCAACAGCACTTTGCAAAATAAATGCCAAATGCTCA
AATGCAAACTACATGGAAATTTTACAAAAATAAAGTATGCCTGTGTTGAAACATAGAAC
ATAGAAGTTTCTCTATAATTAATTACTGTCCTCCCGCGTA-3’

Clone He 272 pGEM-T (SP6 ->)

5’-GAGCGGCCGCCCGGGCAGGTACGCGGGGACATTACGTCATCGGCAGTCAGAGTG
GGCGCAGAGCAAAAGTCGCGCGGGTGGACACTCAGAAACTTGTGCACATTCACTCT
GTTATTATCCTGCGGCGTTCAGATAAAAGGAAGGATCGAGTGGAGATTTCCCCTGAG
CAACTTTCTGCAGCAACTATAGAGGCTGATACAGACTTGCTGACATTACTGGGCGTC
CTATGAGAGTAGTTGGGTGGTATCACTCCCACCCTCACATCACTGTATGGCCTTCCC
ATGTGGATGTGCGCACTTTTTTTATGTACCTCGGCCGCGACCACGCTAATCCCGCGG
CCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTA
TTAAAG-3’

Clone He 291 pGEM-T (SP6 ->)

5’-ACATTTCAGTCATCAGGACATCACACAATGTGAATAGGTTAGGCTCAGTTTTTCTCT
GTTTATGTCCTGAGTATTTCCCTGTCCCTGTTCATTTTAATCCAGGGAAGTTTTCACGC
ACGATTTCTCTTCTCTGCGAGGATTCAGGCACTTTCTTCAAATCGATCCAATTCAGTT
CTCTGTTGCCACTTCATTTCCCAGCGTCTCTTAGAAACCAATTCTCCACCTGGTAGTC
ACCGGCCACCAGTTAATGCGGCTGTCTAGATAACCTACGAGT-3’

Clone He 307 pGEM-T (SP6 ->)

5’-ACAGCGGGGACAAAACCATTTCCCTTTAGGTTTAGTGGTCAGATCCACACAGGCAA
AATGGAACCATTCAATTGGACAATCTGGATTGTCACAGCCAATCATTTCTCCATAGGA
CACCTGGTGGCAAAGGCAGTATGTGGGTTCATTGGGATCCACTGGCATATCCAAGAC
ATCCGAAGGGGTCACAGACAAGACTGAATCAGCATACAAAGGCCCTCCTTTCATTTTT
TTTTTCTTTACAGNGTTCTCTTCANATACTCTCCGTCCTCTTGAGCCTCGTTTTTCTTTT
TGACCTCTATTCTTCTTTACTCCACGTCCTGATGGACTGTCAAACTCTGACCCTTCCA
GTTTCTCTTTAANATCAGCCTCAAAGCGAGCCAGGTCAGCATCCAGCCTTCGTATATG
TTTATCAACCATTTCGTAGGTCTGCATGGCCAGCTGCACTTTGTCATCACTGT-3’

Clone He 313 pGEM-T (SP6 ->)

5’-GGCGCGCACNANTCCCTTTCGAGNCGGCCGCCCGGGNCAGGTACNTNGNCACTA
NTACAGANTGNTAGCCTNTCAGGNGANACCACAAGAAGTGCAAANCNCTGTTACTGT
ACCGGCATGCAGCCATCATTATATACACACNTTTCACANAAAGGTGNTCNCAGCAAC
NCTTTCGCATGTCNGNCAANNGGGGCNCNGTTCNNGCNTNCATCTTGANGNNCNCN
NANGCACTGGGNANNGCCNCNCGNGCCTGNNNNGGAACNGCTCTNATNCTAACNNN
NTTGGANAANGNTTAGCNNNNTNAGCAGGATANNAATGTATANNGATCATANANTCG

AATGNAGGGGACAGAGCTGATTGAAATTAACATCGTCAGGCGACGGCACAATCATAT
CCCTCATGTCCCACTCTGAGCTTCCCACTTTGTTTATATTACAGGGTCTGTCCGATTG
GGGATTAAATGGATTCTATTTGCTTTCGCACTTTCTCCAGCGCCCGCATGTCCAGGGT
CCGCTGTCTG-3'

Clone He 330 pGEM-T (SP6 ->)

5'-TACTTACGGCGGCGCAGTCACCCCCGTGTTGGGCACCAACTCCTTCGTTGTTCCG
GAGAGCAGTAATGCGGACCCCACATTCAGCAACCCCATCCGATTCCCCTTTGGATTC
ACATGGCCTGGT-3'

Clone He 333 pGEM-T (SP6 ->)

5'-ACACTTCTATACCTGCAATGGTATTGTATTACATAATAATAATAATAATGACATCATC
TTGTAACAGATATGCTACACATATAACATAACTATTATATACCAAACCTAATAATCCTG
CACTTGCAACAGAATTATTCAAGCATTATAAGGAGTAATTGTCCCTAATTTCAAAGAAG
AATAAAAAGAATCCAAATCATAACAGTATGATACATAAATACTAATGGAATAGAGAGCT
ACTGTCCCCTCCATACCATATAATGAACTGATGAAATTGATACCAACAATTACATTGTA
ATATACATGAAATATTTAACAGAACACAATTTCCTTCTCAGAAGGCAGCATTTGTTTTT
CTGTAGGAAATATCTTACTGGATAAACAGCCCTATAATCTATTACATAGGACACTTTGA
TTACAACCAGCTTATAAATAGCAACCATTACCTTTTTTCATTAAAAGGCTTAATTAGTC
CCATTGCAATTGCCTAATGCCCAGAAGAGCTGATGCTGCATCAGAAACCATAGTCTAT
TTAATGTCTACCAGGGTCTCCAGAGAACTTTGTTGGCCTCAGGGGTTGTGCACTTGG
CAGGATTCTGGTAAAAAGGTGACACAGAAGGACACGCTGTCACATCATCATGGAAAT
TTTGCAGAAGCTTCTCTGGGAGCTCTGAGTGTTTCTGCAGGGAGAAGAAGTGTTGAG
AGTCCTGGAGACATCTAGAGTGGCCTTCCATGTAGGCTGGGCTGGATGATTGTGCAT
ATTTGGAAGCCATTTGCTGCTGCAGGAAGCTCACTGCCACCTC-3'

Clone He 371 pGEM-T (SP6 ->)

5'-ACATTAGCGTCAGACGCAAAGGCGAAACGATCCTGTATCGATATGAATATACTGTC
GCATGTTTTCTAAATGAGAATGTCATGTTGTTTCTATGAAATAATTGTCCCCGTTAGTA
AGGAATATCAGTTATGATAGATGTATTCTGTGATCAGAAATGGTGTGCTCTGTGAGCT
GAATAAATACACGATTTGTGCCAGTAAAAAAAAAAAAAAAAAAAAAAAAAGT-3'

Clone He 376 pGEM-T (SP6 ->)

5'-ACGCGGGGAGACAGGAAACAGCTGAAGCCGCAGCAGCACCGAAACAGTAACTGA
GCGCAATGGCACCTTGCACTATTAGCCAGCAGCCTGCTAACAGCATTAGAAAGATAA
GAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCATCGAGCAGC
TCAGGATGTTACTGGAGAAAGAGTTTGAGAAAAATCATCTCCCATCCAAACCAGAGAA
AGCTGACATTCTGGAAATGACAGTCAGCTTCTTGCAGCAGCACATAGCTACTAAAACT
GGTGAGATGTCAGGCAAATCCTACAAGGAGGGATACTCCAAATGTGTTGAAGATTCG
CTTCATTTCTTGTCCGTTCACAATCAAGGAAGCCTGCTGAAACATTTCCATGGTCATC
AGTTTAACGCAGGAGAGACCCATCAGCTGTGCCCTG-3'

Clone He 383 pGEM-T (SP6 ->)

5'-ACAGCAGCAAACACCCCCACAAACCTTTCAAGCCAGAAAGTCAACACAAAGGACT
CTCTTTCCCCCTACCAGTTCCTCTGTTAACCCAGCCCTTGTGTGGAGGCCCTGGCCA
TAAATATTTTATTTATTTTTTCCTCATCCCTCTTTTTGTAAATATGGACTTTCAAATTAAA
TTATATCTTTATCTTTTTTTATACTTTCATTGCTCTGTAATTTCTACATGCATAGATCTATA
AATTAAAAAGCTATAATTTCTGTTTTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAA-3'

Clone He 400 pGEM-T (SP6 ->)

5'-ACGCGGGGAGATATCACAGGACACTCTCCCAGCTCTCCGGCTCCATCACATCCAC
TTACTAAACAGGATGGCTCCTTACAGCGCTAGCAGCATGCTCAATACTGAACACTGTC
ACAAGGCACAAGGAATAAGAAAGATGAGAAAACCAGTGGTTGAGAAAATGAGGAGAG
ATCGGATTAACAGCAGCATCGAGCAGCTCAGGATGTTACTGGAGAAACAGTTTGAGA
AACATCATCTCCCATCCAAACCAGAGAAAGCTGACATTCTGGAAGTGGCAGTGAGCT
TCCTGGAGCAGCACATGGCTTCCAAATATGCACAATCATCCAGCCCAGCTCACACAG
AAGGCCACTCTATAAGTCTCCAGGACTCTCTGCATTTCGTCTCTCCAAAGAAACACTC
ACAGTTTTCAGAAAATCTTCTACACAATTTCTATGAGGATCATTCTGTGGCAGTCAGTC
CTCCTCTATCACCTTTGTATCAGACCCCCACCAAATGCACAACACCTGGGGCCAGCA
AAAGTGCTNTGGANACCCNGGTAGACATTTAATGAACGTTTTNAGCATCACCTTCANN
TCNGGNAATCCTGGAGGCNGGGGGTT-3'

Clone He 413 pGEM-T (SP6 ->)

5'-ACTTTTGGCTCCCCTATCTGTGCGCACAATACAGTTATAACTAAGCAAGCAGAACC
CTTACTAAGGATATGATACAATGTAATATACAAATGGTTTAACGCTATTCAAAAGTGTG

CTTAGTGTATTATCATATACAATTACAGCATTACTCTGTCCTTTTCTATTAAAAGAACTC
ATCTATACTGTGCAAGTCAAGCACCATTTTGTTATATAATAATATAATTATTATAAGCTT
CCTCTCAGAAGGCAGGCCCTGTTTATATGCTATGACAGCAATATCTATTCTCTATACA
GGACTTCATCCTGTAATAATATTCTCTTGGATTACAACAATATATGGTAAATAATTATTT
TCCCATGCATAGTTCGAAATGAGCTTATAGGCAGGTAGTATCAACAAGTCCATCAATT
TAGTTCCAGGGTCTCCAGAGGAGCTTGGCTGATGGTGCTGTGCNTTAGTGGGAGTCT
GGTAATAAAGTGACACAGGACGACACAGACCCTGTGCANTGGAAGATCCTCATGGAA
NTCTGCATCATTTTTCCTGAATCTCTGAGTGTTTCTGCAGGAAAGNAATTGCTNAAAG
TCCNGAAACNTTAAAGTAACNTTTNTGTAACCNGACTGGGTGAAGG-3'

Clone He 426 pGEM-T (SP6 ->)

5'-TACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGT
TGACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTC
CATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCA
GCAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAAC
CCTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGT
GCACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCA
TGAGTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCT
CTCCTTCTTTTCTCTATTACAGGCTTCATTAGCCTCTTATCCTCACGGCTGANGTAGG
AGTTCCTCATCTTGGNGCCANAAAAGCTTGAAATGGGCA-3'

Clone He 435 pGEM-T (SP6 ->)

5'-ACTGACCAGCAGCATGTCCCGGATCTCAGCAGAAACCAAAGACTTGCTGAAGTAA
CTCCATGAACTCCATGCTGAGCCAATGCAAAGACTGGCCTCCTTAATCTGTTTTTTAT
CTAACCAGATTACTGGAAAAAAACATATATAGAATTATTGAAAACAAATCTATTTGTGG
TGTAGCGGGAAGACTCAGCCAAGAAATGTAGTTTGTATGCATGCTTCTTTTATGTTCA
CAGCCATTATTTCCCCATTATTCGTATTGCGTTTTAGAGGGGCTAGTGTCTTATAAAA
GCTGTAAATACTGAATAAGGTTCCATATCATTTGGGTCTGAACCACGCAGGACAAGTT
GTTGTCGATGCATTTTTTCTACCACATTTCTACCTTTGATTTGACTTATAANACTGTAAA
GAAAACATGCATGCTTAGAAGCGAGTNTTATTCCAAACCTGTGCCTGACTACNATACA
GTTTTCCTTGNACCTNGGCCGGAACCCCCCTAATCCGGGGNCCT-3'

Clone He 440 pGEM-T (SP6 ->)

5'-CTNGCTNNGCATNCCACNCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCG
GCCGCACTAGTGATTCGAGCGGCCGCCCGGGCAGGTACCACAGAAGGTAGATGTAA
TACCATCTTGGCTCTATGTTCTCCTTACAAAGGCCAAAATCAGTCAGAACAATGTGGC
CCTGGGAATCCAACAAAATGTTTTCTGGCTTCAAGTCACGATAAACAATGTTGAGAGA
GTGCAAGTATCCTAGTGCACTGGCTATTTCAGCAGCATAAAAACGAGCTCTGGGTTC
CAAGAAACATCGTTCTCTCTGGAGGTGGTAGAACAACTCTCCACCATTGATGTAGTCC
AGATTAAAATACAGTCTGCTAGTTGTTTGGAAAGAGAAGTGGAGCCCAACCAAGAAA
GGGTGTTTCACATTTTTTAACAG-3'

Clone He 457 pGEM-T (SP6 ->)

5'-ACTGACCAGCAGCATGTCCCGGATCTCAGCAGAAACCAAAGACTTGCTGAAGTAA
CTCCATGAACTCCATGCTGAGCCAATGCAAAGACTGGCCTCCTTAATCTGTTTTTTAT
CTAACCAGATTACTGGAAAAAAACATATATAGAATTATTGAAAACAAATCTATTTGTGG
TGTAGCGGGAAGACTCAGCCAAGAAATGTAGTTTGTATGCATGCTTCTTTTATGTTCA
CAGCCATTATTTCCCCATTATTCGTATTGCGTTTTAGAGGGGCTAGTGTCTTATAAAA
GCTGTAAATACTGAATAAGGTTCCATATCATTTGGGTCTGAACCACGCAGGACAAGTT
GTTGTCGATGCATTTTTCTACCACATTTCTACCTTTGATTTGACTTATAAGACTGTAAA
GAAAACATGCATGCTTAGAAGCGAGTCTTATTCCAAACCTGTGCCTGACTACCATACA
GTTTTCCTTGT-3'

Clone He 459 pGEM-T (SP6 ->)

5'-GGCCGCACTAGNTGATNTAGCGTGGNTCGCGGCCGAGGNTACTCCTAGNTNTATN
TTGGCACAATGCATNTCTAATGCTNTGCAACCAGTGAGATAAAAATGGCATGAGCATG
AGCACCAGCAAGGAAACAGAACATCAGGCCAAACTCTGAAGGGCGGAAATATTCAAC
CTGAACATATAACAGGCAGTATACGTTTCTAGCTGAATTACAGACATTAGGCCTACGC
TCCTGATCTGGCTCCCACTTTAAAGCAAAGCCAGTGTTAAATGTATTTTAGAAATACTT
TAAATGGTCATTTAGATGTAAATCTATTAATCTGCATGACAGTATTAAAAATAAAATACT
GTAAATTCTGCAAATTCCTCTTTCCAGTGCAGAAGCAGGAATACAGT-3'

Clone He 472 pGEM-T (SP6 ->)

5'-ACATGATCCCACTCCCAGCATATCCCATCATGCATGACATCCTCAAACACATCATAT
ACCATGGATGGTATTGTCATGGGACTGCTAACATATCTTTTTGCTGGGCTGTGGAGAT
GTTCATGGTTATGAGAGGTATGAGGAGGGGACACTTCAATGAAAAGGGACGAGTCAA

TAATGACCTTGAACCAAAGAATAACCTGATAGAAAAGGAACCCCATTTCAAAATGCCT
AATCCAGACTATCTAAGAGAAAAATCAAGTAGCAAACAAAAGCTTCTGCAAGGATCTG
AAAGTGAGGAGGAGAGAAGTGGGCGCAGAACTGACCGAAAGCCAGATACCAAACAG
TGCAACCCCACATCAAGGTATCCAGAGGATGGCGCTTATCACCCAATATATATTCTCC
CAGAACCTGAGCAATGNATATTTGCCACTGAGGTCTANTCNTCCCNGAGACATGACT
TCGACTATCATCCTGCAANTCTAAGAACTGTAAGCAGACAATNTCCCCATNANNAATA
AAATAATCCCCCTCCNAANNATTGNCAATGTANCCTANNNTTATGANCCTTTTTGAAA
ATCTNGTAAATGCTGCTGCAATTA-3’

Clone He 474 pGEM-T

No insert

Clone He 479 pGEM-T (SP6 ->)

5’-ACTGAGTCTTGAAAGCCAAGAGAAGGCTTGTGTTTTGTCAGTGGAGCACAGGTGG
GACTCCAGGTGTTTGGAGATGGCTTATAGGAGGACAGCTGGTGGGAGAGGAACTCA
TTGGTCACACCATTCATGTCAGGTTTGGAGTTCATGAAATGCAAGATTGTCTCTAGGC
AATGCTGGAACCCCGATTGGTATTCTTTACCCACCGCTTCCCTATCCAGTGGGAGAA
GTTTCCTGGACTGGAGAAACTGGACCGTGCACTCCAGAATCTCAGCCTTCTCCACTT
TTGGATTCTTAAGTTTCTCACTTTTCAGGGTTTGAAATAGGAAAATTCTCAGCTTCTCC
AGGCTGTTATTTATCCTCTCCCTCCGCCTTTTCTCCACCAACGGCTTTAATAGCTTTCT
ATGGGTTTCCATGGGTTTGACTTCACTTGTTCCCTTCATGGTAAGATGATGATGATGA
TGAANAAAAAATGCTCTAGGGAAGAACTTGTCCCCTCTGT-3’

Clone He 487 pGEM-T (SP6 ->)

5’-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGNNTTTNTNC
ACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCATG
AGTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTCTC
CTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGAGT
TCCTCATCTTGGNGCCAAGAGAAGCTTGAGATGGGCAAGTCCAGGAGATGCATGTGA
TCAGTATGAAGAGAATGTAGCATGTGNCCNNGT-3’

Clone He 491 pGEM-T (SP6 ->)

5’-ACAATAGATGCTTTAAGAAGAGGTCAAAGCTTATAGATACTGAACAGAAGCGCAAA
CGTATCTATATTTTACAATATCTGTATCCTCAGTGAGGAGAAAACATATTCCTAAATTT
GGGTTAGGAAGCAAAACGGTGAAACTAGATTGCTAGTTATGACTTATATTCTACTATG
TGGTATACTATGTTCTTTAATTCCCAGCACCCTAACCATCCTAAAGGGTTGCTGGGAG
TTGTAGTTTCTTACAGTATTTGGATAATTGTTATCTCTGTGCTGTGATTTTTTTTGTGAA
TTTGTAATTCATTTCCTACAATGTATGCAGTTTTATTGCACTTATTGGTATATTATATAG
ATATATAAGTTATGCAATATTTGCAAGTTATATTTGTGCCTGAATGTTAAATGTATGCC
TTTATTTAAAATGCTGCCAATGGCATTTCAAATAAATTGATTTTTTGAAAAAAAAAAAAA
AAAAAAA-3’

Clone He 496 pGEM-T

No insert

Clone He 501 pGEM-T (SP6 ->)

5’-CCANANGGTCGACCTGTNTGGCGGGCCGACAAAGCGATTTCGAGCGGCCGCCGG
GGCGGNTACTATGCAGTATTTGCAATATTTAAATGTTATATGTTTTCAGCAATATGCAA
AATTTGCCGTATTTAACAGTATAATAACTGTTTAACTTGTAAACTGGCAATATTTGCNG
AATTTGTGTNATATTTTNAATTAATTTGCAACATTTGCNGTATTTAACAGTATAACTGTN
ATATTTCTTTGTAATATGCAATATTTGCNGTATTTAACAGGCATGCATGTTACATTTNCT
TTGTAATATGCNAANTTGGCNGAATTTAACAGTGTANCGGCCANATTTCTCTNTTTTTT
GCAANATTTGCCGTATTAAAGAGNATAATAACNNGTNAACCTGGNAAACTGCANTATT
NGCGGAATTNGGGNAATANTTTTAAATTANTTTGCACCATTTTGCAGGATTTAANAAG
ANANACTGGTATANTTTCCTTTGTAAAAGGCAACATTTTGCAGTATTTAANAGCATGNC
TTGNTATATTTCTTTTGTAANANGCAATAATTTGCTGGANTTTAACAAGTGNAACTGCC
AAANTTCCCCCGGNNAAAAGGCAAAAATTTNCCGGCGTNNCCCNCCCCCN-3’

Clone He 504 pGEM-T (SP6 ->)

5’-ACTGAGCACAGTCACAAGGCACAAGGAATAAGAAAGATAAGAAAACCAGTGGTTG
AAAAGATGAGGAGAGATCGAATTAACAGCAGCATTGAGCAGCTCAGGATGTTACTGG
AGAAAGAGTTTGAGCAACATCATCTCCCATCCAAACCAGAGAAAGCTGACATCCTGG
AGGTGGCAGTGAGCTTCCTGCAGCAGCAAATGGCTTCCAAATATGCACAATCATCCA

GCCCAGCCTACATGGAAGGCCACTCTAGATGTCTCCAGGACTCTCAACACTTCTTCT
NNTTNNAGAAACACTCAGAGCTCCNAGAGAAGCTTCTGCAAAATTTCCATGATGATGT
GACAGCGNGTCCTTCTGTGTCACCTTTTTACCAGAATCCTGCCAAGTGCACAACCCC
TGAGGCCAACAAAGTTCTCTGGAGACCCTGGTAGACATTAAATAGACTATGGTTTCTG
ATGCAGCATCAGCTCTTCTGGGCATTAGGCAATTGCAATGGGNCTAATTAAGCCTTTA
AATGAAAAAGGTAATGGTTGCTATTATAAGCTGGTTGTAATCAAAGGTGTCCTATGNA
AAANTTATAGGNCTGTTTATCCAGTAGAATTTNCTACANAAAACAAATGCTGCNTCTG
GAAGGAAATGGGTCNGNTAAAAATTCANGGATATANAANGAATTGTNGGANCAATTC
ANCGNTCATNTATGGNATGGAGGGGNAGNNGCCCCCNATNCCNTAGGATTTNGGNT
CNAACNGGNNTGATTGGGANTTTTTTTNTT-3’

Clone He 509 pGEM-T (SP6 ->)

5’-ACAAAGTCCAAGCTGACATTCACCACGACCTCCAGATGGAGCTGTTGAGCTGCGG
GGGACATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACT
GAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGA
TGATTACAATCTGAACTGCACAGCNTCAGGGCTCCTCTGCAGGTATTCTAGAAGGCG
GTTACTCGCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCGCTCAGACGCTC
TACACATGCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTGGACCGGA
GGNATGTCTCTTAGGAATCTCACTGTCATTTCCAAAATATCAGCTTTTTCCAGCTTGG
AG-3’

Clone He 531 pGEM-T (SP6 ->)

5’-ACAAGGCAGAGGCAAGATTTTTCTCCNATATCTGTGTTCNTCATGAAAATACATATA
TTATCAGTATCCAGCTGCTGGANATAGAGGCACATACCCTTCCCAAAAGAAATGAGG
GAGCCATTGTCTATCTATNGACAGGCAANAATATGCCTAACAATTAATATAANTATAAC
AGACATTCAGTCAGTGCNGT-3’

Clone He 534 pGEM-T (SP6 ->)

5’-ACAGGGCACAGCTGATGGGTCTCTCCTGCGTTAAACTGATGACCATGGAAATGTTT
CAGCAGGCTTCCTTGATTGTGAACGGACAAGAAATGAAGCGAATCTTCAACACATTTG
GAGTATCCCTCCTTGTATGATTTGCCTGACATCTCACCAGTTTTAGTAGCTATGTGCT
GCTNAAGAAGCTGACTGCCATTTCCAGAATGTCAGCTTTCTCTGGTTTGGATGGGAG
ATGATGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATGCTGCTGTTA
ATTCGATCTCTCCTCATCTTTTCAACCACTGGTTTTCTTATCTTTCTAATGCTGTTAGA
NGGCTGCTGGCTAATAGTGCAAGGTGCATTGCGCTCAGTTACTGTTTCGGTGCTGCT
GNGCTTCAGATGTNTCCTGTCTCCCCCGT-3’

Clone He 557 pGEM-T (T7 ->)

5’-ACTCTCACAAAGAACTGTGACAATGCTACAGCTCGTGGGCATTATTACCCCGTTAT
AAAACACCCCAGTGATATGTGTNTTTATTTATTGTAAATATGTCCCCTCACATGAAATC
CTGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACGAG
AGGACCCCNNTTNGGGAACCCAAGGGNTTAATCTAGTATGCGATTTNNATTATTTTAC
ATNACCCATGGACTGCAGAGTGGCNATGCTACAGNCCTGCATGTATNGCTTTTCTGN
AGCNAGGGTTNATGGNACGGGGTTATGGCACAATANGCNTGGNTGACCCTGTNGCA
GCCTTTTTTTTTGGNTNGTTGGNTGNAAATANTTTTTAAAAAANCCTAAATGGGGAAAA
AAAAAAAAAAAAAAAAAAA-3’

Clone He 560 pGEM-T (SP6 ->)

5’-ACAGGGCACAGCTGATGGGTCTCTCCTGCGTTAAACTGATGACCATGGAAATGTTT
CAGCAGGCTTCCTTGATTGTGAACGGACAAGAAATGAAGCGAATCTTCAACACATTTG
GAGTATCCCTCCTTGTAGGATTTGCCTGACTTCTCACCAGTTTTAGTAGCTATGTGCT
GCTNAAGAAGCTGACTGCCATTTCCAGAATGTCAGCTTTCTCTGGTTTGGATGGGAG
ATGATGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATGCTGCTGTTA
ATTCGATCTCTCCTCATCTTTTCAACCACTGGTTTTCTTATCTTTCTAATGCTGTTAGC
AGGCTGCTGGCTAATAGTGCAAGGTGCNATTGCCCTCAGTTACTGTTCGGTGCTGCT
GGGGCTTCAGCTGTTTCCTGTCTCCCCGCGT-3’

Clone He 588 pGEM-T (SP6 ->)

5’-ACANGTCACTGCACNGCATGGGGTACNTTGGTGNNCTTAGAANGNCATATAGAAC
GCTTGCTTCGCAGCAGTAAGCNNTNACTTANCNANNNGCNCNGGCANANACCAATAA
ACCTTTGTTCTCTCATGNNCCGGNACAANCTCTGNAAAGNGTCTTCGGTTCTTCTTCT
CACAGATACTGTCAGCAGAGNTGNGGAAGGAAGTGGCCTGCNGGACTCCTTCCCGA
AAGCCNNNNTTAAACCCTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGAC
ACATTTTGTNTNNGTGCACTGNCTTTTTCAGAATATCAGCCTTCTCAGCCTNGGGATT
TTTNAANGACTCATCATGAGTGGCCTCCATGAGANGGGNCCTGAGATGTCCCAAGCT
CTGATNAATTCTGNCTCTCCTTCTTTTCTCTATCACAGGCTNATTAGCCTCTNATCCTC

ACGGCTGAGGNAGGAGTCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAGG
CCAGGANAATGCNTGTGATCAGTATGAAGAGATGTAGATGTGNCCCNGGACCNGGC
CGNACACNCTANCCCGGGCANGGNGCGGGAGATNNANGNCGGNCNATTC-3’

Clone He 600 pGEM-T (SP6 ->)

5’-ACAAGTCACTGCACNGCNTGGGGNACATTGGTGNACTTAGAANGGCAATGACCCT
GCTTCGCAGCAGNAAGCGNANACATTAACNAANAGCNCCGGCACCANACCAACAAG
ACGCTTNGTNCTCCCATGTGCNCGGCAAAACCTCTGGAAAAGGNACTTCGGGCCTTC
NNCTCACAGATACNGCCAGCAGAGNNGAGGAAGGAAGTGGGCCTGGTGGACCCCTT
CCCGAAAGCGGNGNNTAAACCCNGACTGCAANTTTCTTTTTATCTGANGGCACTGGA
TTATGACACATTTTTNTTNTNTGCACNGGNCTTTTTCAGAATATCAGCCTTCTCAGCCT
GGGNANTTTTTAAAGACTCANCATGAGNGGCCTCCATGAGANGGGNCCTGAGANGT
NCCAAGCCCNGANTAATTCTGCCCCNCCCTCCNTTCTCCTATCACAGGCTTCATTAAN
CCNCTTANCCCTCACGGCTGAGGNAGGGNGNNCCCTCAACCTNGGGGGCCAAGAN
AAGCTTGAGANGGGGCAAAAGCCCAGGNGAANGGCATGGGGANCCGGGANGNAAG
GGGAAAGGNGGCNATGGGGCCCCGGGGNACCTNGGCCGGGGACCACCCCAAANC
CCCGGGGCCNATGGGGGGCCG-3’

Clone He 610 pGEM-T (SP6 ->)

5’-CCTCCCATATGGTCGACCTGNNGGCGGCCCGACNTAGTCGATTTCGAGCGGCCG
CGGGNCGGTNNCCCNGGGGAGNCAGGAAACAGCTGAAGCCGCAGCAGCACCGAAA
CAGTAACTGAGCGCAATGNCACCTTGACTATTAGCCAGCAGCCTGNTAACAGCTTTA
GAAAGATAAGAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCA
TCGAGCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAAAATCATCTCCCATCCA
AACCAGAGAAAGCTGACATTCTGGAAATGGCAGTCAGCTTCTTGAGCAGCCCATAGC
TCCTAAAACTGGTGAGATGTCAGGCAAATCCTACAAGGNGGGATACTCNAAATGTGT
TGANGATTCGNTTCATTTCTTGTCCGTCCACAATCAAGGAAGCCTGCTGAAACATTTC
CATGNTCATCAGTTTANCGCAGGAGAGACCCATCAGCTGTGCCCTGTACCTCGGCCG
GGACNACGCTAATCCCCGCGGCNATNGCGGCCGGGAGCATGCGACGTCGGGCCCA
ATTCGCCNTATAGTGAGCNGNAT-3’

Clone He 629 pGEM-T (T7 ->)

5’-CNGNGGGNTTTCGAGCGGCCCCCNGGGAGGGTACTCTCACAAAGAACTGTGACA
ATGCTACAGCTTCGTGGGCATTATTACCCCGTTATAAAACACCCCAGTGATATGTGGT
ATTTATTTATTGTAAATATGTCCCCTCACATGAAATCCTGACTTACTAAAGGCAATATA
TTTTGGACAAATAGTCCTGAATACAAAGGGCACGAGAGGACCCCCATTTGTGANCCC
AAGGGTTTAATCTAGTATGCNATTTNNATTATTTTACNTNACCCCNNGGACTGCAAGA
GATGNCAATGCTACAGTCTCTGCCATGTTTNACTTTTCTGCAGNCNAGGGTTTAATGA
CAAGGGCTTTGGCNACAATATGCCTGGCATGACCCTGTNGCAGCCTTTTTTANTGNC
TGTTGGAATGAAANTNNNTTTTAAATAAATCCTAAATGGGAANAAAAAAAAAAN-3’

Clone He 674 pGEM-T (SP6 ->)

5’-ACGCGGGGAGACAGGAAACAGCTGAAGCCGCAGCAGCACCGAAACAGTAACTGA
GCGCAATGGCACCTTGCACTATTAGCCAGCAGCCTGCTAACAGCATTAGAAAGATAA
GAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCATCGAGCAGC
TCAGGATGTTACTGGAGAAAGAGTTTGAGAAAAATCATCTCCCATCCAAACCAGAGAA
AGCTGACATTCTGGAAATGGCAGTCAGCTTCTTGCAGCAGCACATAGCTACTAAAACT
GGTGAGATGTCAGGCAAATCCTACAAGGAGGGATACTCCAAATGTGTTGAAGATTCG
CTTCATTTCTTGTCCGTTCACAATCAAGGAAGCCTGCTGAAACATTTCCATGGTCATC
AGTTTAACGCAGGAGAGACCCATCAGCTGTGCCCTGT-3’

Clone He 677 pGEM-T (SP6 ->)

5’-ACTTAGAGTTAACTGTTAAAAGGGTGTTCCCCCTCCTTTGGTTAAATTTCATAGAAG
AGATTGTAAACAAAGTCCCATAATTTTATGGGCAGGTATATGCCTTTTATTATAAATGG
CACTTAGTCTTCTCTTAACCTTTTAACATTCTTACTCTGAACTCTCCCGCTTTGT-3’

Clone He 703 pGEM-T (SP6 ->)

5’-ACGCGGGGAGATATCACAGGACACTCTCCCAGCTCCCCAGCTCCATCACATCCAC
TTACTAAACAGGATGGCTCCTTACAGCGCTAGCAGCATGCTCAATACTGAACACTGTC
ACAAGGCACAAGGAATAAGAAAGTTGAGAAAACCAGTGGTTGAGAAAATGAGGAGAG
ATCGGATTAACAGCAGCATCGAGCAGCTCAGGATGTTACTGGAGAAACAGTTTGAGA
AACATCATCTCCCATCCAAACCAGAGAAAGCTGACATTCTGGAGATGGCAGTGAGCT
TCTTNCNGCAGCAGTTAGTTACCAAATGTAAACCGTTATCCAGTCCTGCTTACAAGGA
AGGTTACTCAAAATGTCTCCAGGACTCTCTGCAATTCCTCTCCCTCCAGAAACACTCA
GAGCTTCAAGGAAANGTGATGCTGAATTTCCATGAGGATCCTTCTGCTGCACAGAGT
TTGAGTCGTACCCTGCCCGGGCGCCCCTCGNAAATCCCGCGCCATGGNGCCGGNA

NCATGCNACGTCGGNCCAATTCGCCCTATAGTGAGCNGATAA-3’

Clone He 712 pGEM-T (SP6 ->)

5’-ACGCGGGGATTTAAAGAGATATATATTGCTACCCTAGGCTGAACGCAGGCAGGCC
GGACTTGCAGCCCTCTGGCAATTTTGCCCTGAGGAAAACTTGCAAATTCTGCAATGG
TTTATCCATGGCACTTAAGATAAAGAATTGAAATATAAAATTGAATTTTATGGAAAAGA
GTATGACAATCATGAGTATATCAGATACTTCTCTTGAATGTTCTCAGGATTTCTACAGT
CATACTGAGTGAGCAAAGT-3’

Clone He 714 pGEM-T (SP6 ->)

5’-ACTGGTCACTGGTGCTTTTCCTAACTCAGCAATCTCTGTATCCACCAGAAAGTAAA
ATAAGCATGGCTCCCATCTGCCTTTCCTTTTGTAAGATAAATATTTGTAAATCCCATAA
TACATGAAATAATAGTGT-3’

Clone He 729 pGEM-T (SP6 ->)

5’-ACGCGGGGAAGTCCCACTTGGAATCTCATAGAGTATCTGTCTCATCTCCCTGCTCT
TCTGACCCACTCTCACACCAGCCTGATTGCCCCACTAGCTCCCACTGCCCTGCCCGG
CTCTTCACTCCTACATTCCCCGATCTCTGCGCTGNNCCNAANCCCTTNCTTCTGNATN
GGGTCCCNACCTANCNANGCNNGCCCTAGNAGGATCNGNNNNNNNNNNNNAGCCN
TGAGAAAGGTATCNGAAACCTCTGATGGAAAAGAAAAGAAGAGCGAGGATCAANTGT
CTCTCTGGAGCAGCTTATNTTNTTTCTAGAAAAGCACTATTNCCAAAATATTCGGAAG
AGAAAGCTGGAGAAGCAGATATTCTTGACTGACTGNAAAAATCTGAAACCCTTCAGA
GNTCT-3’

Clone He 733 pGEM-T (SP6 ->)

5’-TCCTAGCGCAACATCGCTCAGGTTTTATAATTTCACNATATTCGNCCCATTTAANTC
TCTCTTCTGNTGNAGGNAACATAGNATAACGTACTTCTTTGCCTGTTNGAAGAAGCTG
CCTTTTCTGCGTGCCCTCATTTTTCATCATGAGGTCANGNTNGGNTTTGANGGATGTG
ATCTGNTCAATATCCTCCTCCCATCGCTATCATCACTAGAANNCAGGNCTGCCTCTTT
GGATTCGCTCCAGTGTTTTTAGCAGCTTCTTTTTTCAGANNCTCCTTCTCCACGNNCC
TGCTCGGGNGCNTGTCTGAAANCCCGCGNCNNTGNTACCATGGNAGCNTGCTNCGN
CAGGNACCAATTCNGCTATANTGCGCCGNNTNACNCTCGAATCCCGCGCCATGCGG
CCGGGAGATGCGACGCGGGCCNATTCGCCTATAGTG-3’

Clone He 768 pGEM-T (T7 ->)

5’-GCCCGCCATGGCCCGGGGNNTTTCNGAGCGGGCCCCCCCNGANNNCCCTACCG
ACTTCAAACTCTGTGCAGGCAGAGGGANCCTCATGGAAATTCAGCATCACTTTTCCTT
GAGNCNCTGAGTGTTNCTGGGGGGNGAGGAATTGCAGAAAAGCCCTGGNGAANTTT
TGAGTAACCTTCCTTGTANGCNGGNCTGGGATAACGGTTACATTTTGGGNAACTAATT
GCTGGCTNAGGGAGGCTCACTGGCCATCTTCCAGNATGGTCAGCNTTNCCTCTGGN
TTGGGNTGGGGAGAATGATGNTTCCTCAAACTGTTTCTCCAGAACATCCTGGAGCTG
CTCGANGCTGCTTGGTTANTCCGANCTCTCCCTCATTTTTCCTCAGCCNNTGGGTTTC
CNCAANCTTTTCTTTNTTNCCTTTGGGCCTTNGGGNCAGGNGTTTCAGANTTTTGNGC
NTGGCTGCCAGGCCCNTGTAAGGGGGGGCCATCCNGGTTTAAGT-3’

Clone He 778 pGEM-T (SP6 ->)

5’-TACACTTCTATACCTGCAATGGTATTGTATTACATAATAATAATAATAATGACATCAT
CTTGTAACAGATATGCTACACATATAACATAACTATTATATACCAAACCTAATAATCCT
GCACTTGTAACAGAATTATTCAAGCATTATAAGGAGTAATTGTCCCTAATTTCAAAGAN
GAATAAAAAGAATCCAAATCATAACAGTATGATACATAAATACTAATGGAATAGGAGA
GCTACTGTCCCCTCCATACCATATAATGAACTGATGAAATTGATACCAACATTTACATT
GTAATATACATGAAATATTTAACAGAACACAATTTCCTTCTCAGAAGGCAGCATTTGTT
TTTCTGTAGGAAATATCTTACTGGATAAACAGCCCTATAATCTATTACATAGGACACTT
TGATTACAACCAGCTTATAAATAGCACCCATTACCTTTTTTCGTTAAANGGCTNATTAG
TCCCATTGCAATTGCCTAATGCCCAGAAGAGCNGGATGCTGCATCAGAAACCATAGG
TCTATTTAAAGGTCTACNAGGGGCCCCCAGAGAACTTTTGTNGGCCCTNAGGGGNTT
GGGCCACTTGGCAGGGAATNCTNGGNAAAAAAGGGTGGANNNAAAAAAGGAANCCC
CNTNTNCCCAATCNATCAATGGNAAANTTTTNGCAGGAAA-3’

Clone He 814 pGEM-T (SP6 ->)

5’-ACTTTCTCAAAATGTGTCATAATCCAGTGCCCTCTGATAGAAAGAAATTGCTGTCAG
GGTTTAAAGGAGGCTTTCGGGAAGGACTTAACCAGGCCACTTCCTTCATCAACTCTG
CAGACAGTATCTGTGAGAAGAAAAAGGAATACATGGTCCAGAGGTTGTGCCAGCATA
TGGAAGAACAAACTCAGAAACAAAGGAAGGACTCTGTTCAAGATGTCTCCTTGAGGG
TGAACCAAAGGCAGATCTTGCCAAGTCCACCAATAATCTCCAGGGTTTCAGNTTTTTT
TCCTCGGCCGCGACCACGCTAATCCCGCGGCCATGGCGGCCGGGNAGCATGCGAC
GTCGGGCCCAATTCGCCCTATAGTGAGTCGGATT-3’

Clone He 859 pGEM-T (SP6 ->)

5’-ACAGGGCACAGCTGATGGGTCTCTCCTGCGTTAAACTGATGACCATGGAAATGTTT
CAGCAGGCTTCCTTGATTGTGAACGGACAAGAAATGAAGCGAATCTTCAACACATTTG
GAGTATCCCTCCTTGTAGGATTTGCCTGACTTCTCACCAGTTTTAGTAGCTATGTGCT
GCTGAAGAAGCTGACTGCCATTTCCAGAATGTCAGCTTTCTCTGGTTTGGATGGGAG
ATGATGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATGNTNTTNTTAA
TTCGATCTCTCCTCATCTTTTCAACCACTGGTCTTCTTATCTTTCTAATGCTGTTAGTA
GGCTGCTGGCTAATAGTGCAAGGTGCCATTGCCGCTCAGTTACTGTTTCGGTGCTGC
TGCGGCTTCAGCTGTTTCCTGTCCCCGCGT-3’

Clone He 865 pGEM-T (SP6 ->)

5’-NCGATTCGAGCGGCCGCCGGGGNGGGTACCCGCTGCAGGTTCCTCAGGTGCTTT
ACTGTCATCTCTAGTATATCAGCCTTCTCTAGCTTGGAGTGCCGGGAGCTATCTTTCT
TGGGCGCATCCAGGATGAGGGTTTTGAGTTGCCCGAGGCTCTCGTTGATCCGGGCT
CTCCTTCTCTTCTCCATGATGGGCTTGGAGGACTTTCTGTGCTCGGAGGCAGTTTTG
GGTTTATCCGGGGTATTGCTCACACTGGCCGGAGTGGCAGCCACTGGGGATGAGGA
GTTCTTCTCCATCACATCAGCCGGCATCTTCTGATCTCACACCAACTGAACANCACAC
TGAACACAAATGGATTAAAAGTCCAGGTTTATATAGGATGTGAGCAAGAAGCAATTGG
CAATCAAATAAAAGAATGAACCAGTGNCACTAGAAGAGAGAGGGAGGCAAAGTCCGC
AGCAAACGTCCTGACTGATACTCCCCGGGTACCTNGCCCNNACCACGCTAATCCCG
GGNCATGGCGGCCGGNACATNCAACGTCGGGCCCANTTCGCCTATAGTGNG-3’

Clone He 877 pGEM-T (SP6 ->)

5’-CCATATNGTCGACCTNTTTGCGGGCCGANAAAGNNATTAGCGTGGTCGCGGGCC
GAGGTACTTAGAGTCAACTGTTAAAAGGGTGTTCCCCCTCCTTTGGTTAAATTTCATA
GAAGAGATTGTAAACAAAGTCCCATAATTTTATGGGCAGGTATATGCCTTTTATTATAA
ATGGCACTTAGTCTTCTCTTAACCTTTTTAACATTCTTACTCTGAACTCTCCTGCTTTG
T-3’

Clone He 907 pGEM-T (SP6 ->)

5’-ACTGAGCACAGTCACAAGGCACAAGGAATAAGAAAGATAAGAAAACCAGTGGTTG
AAAAGATGAGGAGAGATCGAATTAACAGCAGCATTGAGCAGCTCAGGATGTTACTGG
AGAAAGAGTTTGAGCAACATCATCTCCCATCCAAACCAGAGAAAGCTGACATCCTGG
AGGTGGCAGTGAGCTTCCTGCAGCAGCAAATGGCTTCCAAATATGCACAATCATCCA
GCCCAGCCTACATGGAAGGCCACTCTAGATGTCTCCAGGACTCTCAACACTTCTTCT
CNCTGCAGAAACACTCGGAGCTCCCAGAGAAGCTTCTGCAAAATTTCCATGATGATG
TGACAGCGTGTCCTTCTGTGTCACCTTTTTACCAGAATCCTGCCAAGTGCCCAACCC
CTGAGCCNACAAAGTTCTCTGGAGACCCTGGTAGACATTAAATAGACTATGGTTTCTG
ATGCAGCATCAGCTCTTCTGGGCATTAGGCAATTGCNATGGGACTAATTAAGCCTTTA
ATGNAAAAAGGAATGGTTGNTNTTATAAGCTGGNTGTAATCAAAGGGGTCCTATGTAA
AAATTATAGGGCTNTTTATCCNGTAAGANATTTCCTANANAAAACAATGCTGCNTCTG
GAAAGGAAATTGGGTCNGNNAAAANTTCANGGNNTTAACAATGNATTTTGGGNTCAA
TTTCACCGGTCCNTNANTGGATNGGGGGGNCAG-3’

Clone He 933 pGEM-T (SP6 ->)

5’-ACATNNCCTGTAANTCTCTGCNANANTATTGNTGCANNNGGCANNCACTATGANGN
TGGTTCGAACNTGACAGCGTAGCCACTCATANNACTNCNTCCNTCTTCANTCANGGA
CTNNGTAANCNCCANGACNNGANCCNTCTATNGGTACTCCTGCAGGTCCTTTCANCN
NNNCANAGAATANTNCCNNCCATAGCATNNGTACGGTACNTGCTTCANCCGTGAGAC
CTTCCNNAAAGCATCTCTTAAACNCNGACTNCATCTTCTTGTGTATCTGATCGANCNC
CAAAATGANCACANAGTTGANAAATTGCANTATCTCNTNTAGATGATGANCTCTAAGC
AGCNTTGCAGNTTTTNAGCCNCATCACGAGTAGCCTCCATNNNAGGNNCCTGAGACN
TNCAAGCTCTGANNANTCTGNCTATNNTGATTTCNCTATCAAGGCTTCATTAGCCTCT
TATCCTCACGGCTGAGGTAGGAGTTCCTCATCTTGNGNCAAGAGAGCTTGAGACGGN
NAAGTCCAGNAGAATGATGTGATCAGTATCCCNCGTACCTNGNCNCNACACGCAANN
CCGCGCCATGCGGCNGGAGCATCNACGTCGGCCCANTCGCCNAAGNA-3’

Clone He 947 pGEM-T (SP6 ->)

5’-ACGCGGGGAGACAGGAAACAGCTGAAGCCGCAGCAGCACCGAAACAGTAACTGA
GCGCAATGGCACCTTGCACTATTAGCCAGCAGCCTGCTAACAGCATTAGAAAGATAA
GAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCATCGAGCAGC
TCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCATCTCCCATCCAAACCAGAGAA
AGCTGACATTCTGGAAATGGCAGTCAGCTTCTTGCAGCAGCACATAGCTACTAAAACT
GGTGAGATGTCAGGCAAATCATACAAGGAGGGATACTCCAAATGTGTTGAAGATTCG
CTTCATTTCTTGTCCGTTCACAATCAAGGAAGCCTGCTGAAACATTTCCATGGTCATC

AGTTTANCGCAGGAGAGACCCATCAGCTGTGCCCTGT-3’

Clone He 953 pGEM-T (SP6 ->)

5’-ACTATGCAGTATTTGCAATATTTAAATGTTATATGTTTTCAGCAATATGCAAAATTTG
CCGTATTTAACAGTATAATAACTGTTTAACTTGTAAACTGCAATATTTGCAGTATTTGT
GTTATATTTTTAATTAATTTGCAACATTTGCAGTATTTAACAGTATAACTGTTATATTTCT
TTGTAATATGCAATATTTGCAGTATTTAACAGCATGCATGTTACATTTCTTTGTAATATG
CAAAATTTGCAGTATTTAACAGTGTAACTGCCATATTTCTCTGTTANATGCAATATTTG
CCGTATTTAAGAGTATAATAACTGTTAAACTTGTAAACTGCAATATTTGCAGTATTTGT
GTTATATTTTTAATTAATTTGCAACATTTGCAGTATTTAACAGTATACACTGTTATATTT
CTTTGTAATATGCAACATTTGCAGTATTTAACAGCATGCATGTTATATTTCTTTGTAATA
TGCAATATTTGCTGTATTTAACAGTGTACNTGCCATATTTCTCTGTAAAATGCAATANT
TGGCAGTATTTAACAGTNCCTGCCCGGCGGCCGNTCGAAANCCCCNGGCNNNGCGG
CCGGGNGCA-3’

Clone He 954 pGEM-T (SP6 ->)

5’-ACACGTTTATTCCTCATGACACAAATGGCTTGCACGAAACATCAGTACATTTTATTT
TCATTACTTTAAAACACTTTCATTATTTGTTGTTATGGTTCCTTTAAACAGGGAACTTAA
AATACCTGTCACAAAAATGCCCTTGAGGGAGAACAGGTGTAATTATATGCCTGATACT
TTGAAAAGCAAGAAGGAACAGCAAATGATTTAATTATATAACATTTATATTTATGTATTT
AAGTGCATTTACATTTTCTTTGAAATACTACAGTCCATTAACACTTNNTCATGAAGCAA
CTAGTAATTAAACATGAAAAGTAACTTTTCTACTTTAAAGAGAAATTCAGGGTCAATCC
ATACAGGCCTGGGGCATTCCTCTATTTCATTAATATAACAGTCTTTACCCCCTGTGCC
CTGGCATGATCTGAATATGCAGGTCCTCCAGCAGTAGGCAAGGGCTGCTGTGATTAC
CACGGCCTCCAGATTATTTCTGATGTGTGGATGAGGAGGNTTGAGGCTTGGGGCAC
NTNGGGCAATCCTGNAGCTGGANATTTCTTTAAAATGCGCATANNTTCAGTTTGCNTT
TTTTGATTTCTGTTGNANNGAATTGAGGNCCCTTCAGAACCTGNTGAGCNTGTTNAAN
NTNGGAATGGGTTTNCGGGGAAANCNNNNCNTTNTTTGGGNGNNAANTTCAGGGGG
NGNTTNANCNNNGGGNGNNTNCTTTTCCNTTAANGTTGGGNGGGNCCNTAAANNCC
TTCCAGAAAATTG-3’

Clone He 956 pGEM-T (T7 ->)

5’-ACATTAGCGTCAGACNAAAGGCGAAACGATCCTGTATCGATATGAATATACTGTCG
CATGTTTTCTAAATGAGAATGTCATGTTGTTTCTATGAAATAATTGTCCCCGTTAGTAA
GGAATATCAGTTATGATAGATGTATTCTGTGATCAGAAATGGTGTGCTCTGTGACCTG
AATAAATACACGATTCGTGCCGGGNAAAANAAAANAAAAAAAAAANAANNNNCCNNC
CCCGGGGGCCCNTTAAAANNCCNNNGGCCNCCCCNCCNGGGGGNCNNAATTGGGG
ANGNCCCCCCACCCGGNGGGNGCCANCCTTGGGGGTTCNNATAGGGCC-3’

Clone He 979 pGEM-T

No insert

Clone He 989 pGEM-T (SP6 ->)

5’-ACAGTTCCTTCATAGAGATGGATACTGCTGAAGCTGGCAACCACTATGGCTTGCTT
GTAGAGAGCTTCTGTTGCTTCCTCATCCTTGGTTTTCTCACTCCACTCAACCTTTAATG
TCCATAAAAACACAGAGTCCTTTGGTTTAAAGTGTTTAAATGCCTCTTCAAACACCTGA
TCAATGCCAGTTTGACCCAGCTTCACTAGAATTTCAAGCCTAGTCTGCCACAGTTTTG
CAGACTGGCTGTAGCGTTTGATGGCTGCTTCAGAAGCTCCCACAATACTGTCTGTCT
CTCCAAGAGCCAAAAGTTCAGAGATCCACTCTATGT-3’

Clone He 1025 pGEM-T (SP6 ->)

5’-CNNANCANGATNCCTCNTTGGGGGGNACCCCTCCCATATGGTCGACCTGTTGGCG
GNCGAAATAGCGATAATCCCGCGGCCATGGCGGGCCGGGAGCATGCGACGTCCGG
GCCCAATTCGCCCTATAGTGAGTCGTATTAA-3’

Clone He 1028 pGEM-T (SP6 ->)

5’-ACAGACTTGTTTTTTTTTAAAAGTTCAAGTTTTCTCTTGGGCCAAAAAAAGGGGTCG
ATGAATCAGGGAGATGGTGTCCTTTAGTTTGGACACGATCTTCTTGTCCTTGACCCTC
CTGTTCTGGACCAGATAGTGACCTGTCTCTCAGAAAGATTGGNGGTGGCAGATATCC
TTCTCCTCTTGNCCTTGTNAATGAATTTGTTCATTGCATATTCATTTTCNAGTTCTTTCA
GTTGGAGTTNGGGGTAGGGNACCCTCTTCTTTCTGCCGCGCNTGTACCTCGNCCGG
ACCACGCTAATCCCGCGCCATGCGGCCGGGAGCATGCGACGNCGGCCCAATTCGC
NTAAGTGAGCGGATTAA-3’

Clone He 1068 pGEM-T (T7 ->)

5’-ACNNGGGGGCGCAAGCTCTCCATAAGANTATGTGTGTTTAATATGGNGTCTCCTAT
ACCAGCGTTGCCGTNATCTTACTGGAGATGTTCTTGCATNACGTTACAGCATATGTNG
AGGTTTGCNCATCCGGAGAAGAGANAATTATTCCAAAACTTTTTCACANCNGCTNCTT

ATTTNAGNAGCACCGGNTCAAAANCATTTAATAAACAGGTTACTCNTGTTGTTTTTAAA
GATGACNATCAGGGGTNCCTGCNCNGGCGNCCGCTNGNAATCACTAGTGCGGCNCC
CGCAAGGTNNTCNATCTGNNGAGCTCNNACGCGGGGGACTNTANGCTGGCTACTCT
AAAGGGTCCCTNGATACTTGCTTTCCTCTCTTTCACNTATTGAGGANAGATGAGCTTA
AGCTGANNANNATTTTNACTGCTNGNTACCAGCCAGANGCACTTCGGTCTCTTCTCC
CATCCANGGGACCTGGNCAGTGCCNGCAAAAGATTCTTNGGGACCGGGGANAGNCA
NACTAANACCCCAGNTAAAA-3'

Clone He 1081 pGEM-T (SP6 ->)

5'-TGNCGGNGGGACAGCATTNANTANTNNAGNNTCAANTACGGCGNNTATANCGAGA
GCTGTACATATCCAGTGGCTGTCCGGCATACGCCTCTTATTAACCCTTTCACCACGGT
CTCCACAAAACTTTGCTGTTGGTCACAGGGGACTGTCCAGGGAGCAGCTGAGACTG
GTGGGTTTCTACTGAGGAACTCTGGTGACCATGGAAATGTTTGAGCAGGTTTCCTTC
ACTGTGATGATTGCGAGCCGACAAGAAATGCAGCGATTCTTCAACACATTTGGAATAT
CCCTCCTTGTAGGATTTGNCTGATATATCAGCAGATTTGGTAACTAATTGCTGCTGTA
GGAAGCTCACTGCCACCTCCAGAATGTCAGCTTTCTCTGGTTTGGATGGGGAGATGA
TGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATGCTGCTGTTAATCC
CGATCTCTCCTCATCTTTNCACCACTGGTTTTCTCATCTTTCTATTGGTGCTAGCAGG
CTGTGGGCTAATGTTGCANGGNGCCATTNTGCTCAGNTACTGTCAGGNAAAGCTGTG
CTGCTGCACTACAAGCNTTNCTGGGNCCCCNGNACTTNCCNGGCGGCCGTCGAAAC
CC-3'

Clone He 1085 pGEM-T (SP6 ->)

5'-ACAGTCCCCTGTGACCAACAGCAAAGTTTTGTGGAGACCGTGGTGAAAGGGTTAA
TAAGAGGCGTATGCCGGACAGTAACTGGACTGACACTTTGGATTGCCAACTGGAAAC
ACTTTATTAGTGAATCTGTACGCCTCGTCTCCGCGTTCACTGTGCTTCTCGCTTCCAG
GCCCGGCAACACAATGGATGCGTAAGGCCTAGCGTCACAAGAACTTTCACACTCGAA
GCCCTATCGCCTCTTGAGATGAACCAACAGCCCGCGTTGAAAAAAATAAAAATCCGG
TCTTGCCTCCCGCTCCGTTCTCTTCCGCCTCCTAATTACTACTGATTCTTCTAATGGC
GGCGTGACCGCTTCCTCTTCGCAGTTCTAATGGCGGACGGAGTGATCATCTCCCAGA
GAATGGGGTTTCCNAGCAGGGGCAGCGT-3'

Clone He 1093 pGEM-T (SP6 ->)

5'-ATNNAGNANGATNCCATCTTGGTGGGGAGCTCTCCCATATGGTCGACCTGNGGCG
GGCGANATAGNCGATATCCCGCGGCCATGGCGGGCCGGGAGCATGCGACGTCCGG
GCCCAATTCGCCCTATAGTGAGTCGTATTA-3'

Clone He 1153 pGEM-T (SP6 ->)

5'-ACGACTCAAATTCTGTGCAGCAGAAGGAACCTCATGGAAATTCAGCATCACTTTTC
CTTGAAGCTCTGAGTGTTTCTGGAGGGAGAGGAATTGCAGAGAGTCCTGGAGACATT
TTGAGTAACCTTCCTTGTAAGCAGGACTGGATAACGGTTTACATTTGGTTGTCATGTG
CTGCTNAGGAAGCTCACTGCCATCTCCAGAATGTCAGCTTTCTCTGGTTTGGTTGGG
AGATGATGTTTCTCAAACTGTTTCTCCAGTAACATCATGAGCTGCTCGATGCTGCTGT
TAATCCGATCTCTCCTCATTTTCTCAACCACTGGTTTTCTCAACTTTCTTATTCCTTGT
GCCTTGTGACAGTGTTCAGTATTGAGCACGCTGCTAGCGCTGTAAGGAGCCATCCTG
TTTAGTAAGTGGATGTGATGGAGCTGGGGAGCTGGGAGAGTGTCCTGTGATATCTCC
CCGCGT-3'

Clone He 1175 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGCCTCCAAGAGAAGGGTCCTGAGATGTTCCAGACTCTGATTAATTCGGTCTC
TCCTTCTTTTCTCTATTACCGGCTTCATTAGCCTCTTATCTTCACGGCTCANGTAGTTC
TTCATCTTGCTGCNAGAGAAGCTTGAGAGGGGNAANTCCAGGAGAATGCATGCGCTC
AGTGNGAANAAAAGTAGCTTGTGTCCCCGCGTACCTTCCCNGGCGGCCCCNCGAAA
TCCCGGGGCCNTGGCGCCCGGGACCNTNCAANTCGGGNCCNATTC-3'

Clone He 1192 pGEM-T (SP6 ->)

5'-AAGCCCTCCCATATGGTCGACCTGCAGGCGGCCCGACATAGNGATTTCGAGCGG
CCGCCGGGCAGGNACCCAGAGGGGANAAGTTCTTCCCTAGAGCATCTTCTTCTTCAT
CATCATCATCATCTTACCTTGANGGGACANGTGAAGTCAAACCCATGGAAACCCATA
GAAAGCTATTAAAGCCGTTGGTGGAGNAAAAGCCGGAGGGAGAGGATAAATAACAG

CCTGGAGANGCTGAGANTTTTCCTATTTCAAACCCTGAAAAGTGAGAANCTTAGNANT
CCAAAGGTGGAGAAGGCTGAGGATTCTGGNGNCACGGTCCAGTTTCTCCAGTCCAG
GAACTTCTCNANTGTTAGGGNAGCGGTGNTAAAGATACCAATCGGGTTCAGCATTGC
CTGAGACGACCTGCATTNATGACTCAAACCTNTATGATGNGTGCAAGGAGCTGTCTC
CACAGATGTCCTCTGNAGTACCTCTGNGCTGNGTCCCACTGGCTCATTGCAACACGT
CTCGCCNNACACGTATCCNCGCATGNGCCN-3'

Clone He 1193 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGTCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTC
TCCTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGANGTANGG
AGTTCCTCATCTTGNTGCCAAGAGAAGCTTGAGATGGGCAAAGTCCAGGAGAATGCA
TGTGATCAGTATNAAANAGAATGTNGCATGNGNCCCCCNNNNNCTTNCCCGGGCGG
CCNCTCNNAAANCCCNNGNCCNTGG-3'

Clone He 1201 pGEM-T (SP6 ->)

5'-ACATGTATGGAATGTGCTCAAATGGTGCATCAGTAAAATAAAAGTCCAGGTCCAAA
CTCTTATATTTACACTGTATATACATGCACAGATACGTTGTATCTCAAGAGCTTTGGGT
TCTGCTAAATAGTTCTGCAGATTTTCATGCCATAGACAAAATGTCCCTTCCATAGCATT
CAAGGTGCCTGAAAACCCTGGGGAACCCATAATTCTAGCCTGGTGGCTCTTCTTGCA
CCCTTCAAAGATTAAAAGTCATCATACCCAGGGTCTCCAGGTCTTGAAGCTTTGGTAA
GTGAAATGACTGTCAACCAGAGTCTTTGTAGGGCTGATGGAGATGCTGTTGGACACT
AAATGCGGTGNTGAGTCTTGGTAGCTAAGGGAAGGT-3'

Clone He 1226 pGEM-T (SP6 ->)

5'-ACTCTCACAAAGACTGTGACAATGCTACAGCTCGTGGGCATTATTACCCCGTTATA
AAACACCCCAGTGATATGTGTATTTATTTATTGTAAATATGTCCCCTCACATGAAATCC
TGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACGAGA
GNGACCCACATTGTGAACCCAAAGGNTTTAATCTAGTATGCGATTTGATTATTTTACAT
TANCCCATGGACTGCCAGAGATGNCAAATGCTACAGTCTCTGCCATGTATTGACTTTT
CTGGCAGTCCNGGGTTTAATGACACAGGGCTATGTGCAACANTAATGCCTGNNATGA
CACTGTTGCACTTTTTTTTATTGACTTGTTNNAATGAAAATTATATTTTTAAAAAAAATC
NTAAATGNAAAAAAAAAAAAAAAAAAAAAAGTCCTTGGGNCNAACCCNTNNNNCCGG
GCCTTGGGGGCGGGANATNNNAATN-3'

Clone He 1229 pGEM-T (T7 ->)

5'-ACTCTCACAAAGACTGTGACAATGCTACAGCTCGTGGGCATTATTACCCCGTTATA
AAACACCCCAGTGATATGTGTNTTTATTTATTGTAAATATGTCCCCTCACATGAAATCC
TGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACGAGA
GTAACCCACATTGTGAACCCAAAGGGTTNANCCTAGTATGCGATTTGATTATTTTTAC
ATTAACCCATGGACTGCCANAGATGGCAATGCTACAGTCTCTGCATGTNTTGACTTTT
CTGGCAGCCAGGGTTTANTGAACAGGGCTATGGCACAATAATGCNGGGNATGANAC
TGTNGCAGCACTTTTTTATNNCTTTTTGAATGAAANTATTTTTTAAATAAANCCTAANG
GAAAAAAAAAAAAAAAA-3'

Clone He 1235 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CCGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTC
TCCTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGA
GTTCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAAGTCCAGGAGAATGCAT
GTGATCAGTATCCCCGCGT-3'

Clone He 1238 pGEM-T (SP6 ->)

5'-ACAAAGTCCAAGCAGACATTCACCACGGCCTCCAGATGGAGCNGTTGAGCTGCGG
GGGACATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACT
GAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGA

TGATTACAATCTGAACTGCACAGCTCAGGGCCCCTCTGCAGGTATTCTAGAAGGCGG
TTACTCGCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCCGCTCAGACGNTT
TNTTTTTGCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTGGACCGGA
GGAATGTCTCTTAGGAATCTCACTGTCATTTCCAAAATATCAGCTTTTTCCAGCTNGG
AGT-3'

Clone He 1247 pGEM-T (SP6 ->)

5'-ACTGGAACAAAGCCCCAAGAACCAGACCAGCCATACATCTCCATCCAGTTCATTCA
GAACGCCCTGTATGGGGCAAGCACATCAGCAGACACCCCCACAAACCTTTCAAGCCA
ACACCATCAGAAAGTCAACGCAAAGGACTCTCTTTCCCCCTATCAGTTCCTCTGTTAA
CCCAGCCCTTGTGTGGAGGCCCTGGCCTTAAATATTATATTTATTATTCCCCATCCCT
CTTTTTGTAAATATGGACTTCAAAATTAAATTATATCTTTATCTTTTGTATACTTTCATCG
CTCTGTAATTTCTACATGCATAGGATCTATAAATTAAAATTCTACAAAAAAAAAAAAAAA
AAAGGTCCCTNGGCCGGGAACNNCCTAANTCCCGGGCCNNTGGNGGNCGGGGGNC
ATNNCAACGNCGGGCCCAANTTCCCCC-3'

Clone He 1262 pGEM-T (SP6 ->)

5'-ACAGATTCACTAATAAAGTGTTTCCAGTTGGCAATCCAAAGTGTCAGTCCAGTTACT
GTCCGGCATACGCCTCTTATTAACCCTTTCACCACGGTCTCCACAAAACTTTGCTGTT
GGTCACAGGGGACTGT-3'

Clone He 1274 pGEM-T (SP6 ->)

5'-TACTCTCACAAAGACTGTGACAATGCTACAGCTCGTGGGCATTATTACCCCGTTAT
AAAACACCCCAGTGATATGTGTATTTATTTATTGTAAATATGTCCCCTCACATGAAATC
CTGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACGAG
AGNGACCCACATTGTGAACCCAAAGGGTTTAATCTAGTATGCNGATTTGATTTATTTT
ACATTAACCCATGGACTGCCAGAGATGNCCAATGCTACAGTCTCTGCCATGTATTGA
CTTTTCTGGCAGTCCNGGGNTTTAATGACACAGGGCTATGTGCAACANTAATGCCTG
NNATGANCACTGTTGCACTTTTTTTATNNCTTGTTTGTAATGAAANTATNTTTTAAAAAA
ATCCTAAATGAAAAAAAAAAAAAAAAAAAAGACT-3'

Clone He 1299 pGEM-T (SP6 ->)

5'-ACGCGGGGAGAGACTCCCTGATTCACACACGGACAGACAGGGCTGGAAGGGACT
TAGACAGGGACGCGTGTAGCTTCAGTTCCCATCCCGGCACCGATACACACTGGATTC
CTTATAAACTGACACCTCTGGGACAGAGAGAGCAACACACTCGCCATCTGCTGCTTT
TTCTATTCTGATTTTCTTTCCTTTGGGTTCATGAGCAGAGAGACAAATAGAGACATAAC
TGGAATTATTGCCTGGACTCGGGATTGACAGAATAAACCATATCTCTAAACATTTGGA
TTGTGTCAGGGGCAGATTTTTTGCTTTCCCAATGGACCTGTTAACGAACTGATTCATT
CCGTGTGGATTGCGACAAGTGAAAGTCGAGTTTCAGAGTCGAACTGTTCGCAGCCTT
TACCTGGGGCTAATCCTCACCTTTCATAGGTTTTTTTGT-3'

Clone He 1300 pGEM-T (SP6 ->)

5'-ACTTCAGATGATGCAAATATTGCACTACTTGGGATATACTTCAAACCTAGAGGCTAT
ACTTGTATATTATTCCACAGGAATATACAGATTTGCTCATAAAGTGTTTCCAGCTGGCA
GTCCAAAGTGTCAGTTCACTGACTGTCTGACAAACTCTTCTTATTAACCCTTTCACCAT
GGTCTCCACAAAACTTTGCTGTTGGTGACAGGGTTGGTGAAAGGGGACTGTCCAGG
GAGCAGCTGAGACTGGTGGGTTTCTACTGAGGAACACTGATGACCATGGNAATGTTT
GAGCAGGTTTCCTTCACTGTGATGATTGCGAGCCGACAAGAAATGCAGCGACTCTTC
AACACATTTGGAATATCCCTCCTTGTAGGATTTGCCTGATATATCAGCAGATTTGGTA
ACTAATTGCTGCTGTAGGAAGCTCACTGCCACCTCCAGAATGTCAGCTTTCTCTGGTT
TGGATGGGAGATGATGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGAT
GCTGCTGTNAATCCGATCTCTCCTCATCTTTTCACCACTGGTTTCTCATCTTTCTATTG
GNNCTAGCGGGCTGGGGCCTAATGCTGAANGGGGCATTGTGCTCAGNAACTGNAGG
GAGAGCTGNCTGTTG-3'

Clone He 1302 pGEM-T (SP6 ->)

5'-ACATTTGCTATAGGAGGGTTGAATCCTTATATAACATTCCATAATATCTGAACATAC
ATTTCTTCACAGAATAGTGTATAAATAAGCTTCTTCTTGAAAACAATAATAAATTAAGAT
TGGCACAGTCCAAAGGAGAGAGGGAATGGGGCGTCAGNAGAGAACTCGCTAAAGGC
CGGAGTGTATTTGCATGTGATAGCGAAATTCTTCCATTTCCAGGAAGGTTTTCCCGCA
CAGGNAGCAGACGTAGCCCTTTTCCTTGGTGTGAGTNCNACNNANGTNNNTTTTTGN
GGGCGGCATGTGACGCAAAGGCTTTTCCACANTGTTNNCATTTGANGGGTTTCTCCC
CCGAG-3'

Clone He 1303 pGEM-T (SP6 ->)

5'-ACGCGGGGACACAGCAAAGCCTTGTAGTGGCAGCAGCACAGCTCTCCCTGACAG
TAACTGAGCACAATGGCACCTTGCAGCATTAGCCCACAGCCCGCTAGCACCAATAGA

AAGATGAGAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGGATTAACAGCAGCATC
GAGCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCATCTCCCATCCAAA
CCAGAGAAAGCTGACATTCTGGAGGTGGCAGTGAGCTTCCTACAGCAGCAATTAGTT
ACCAAATCTGCTGATATATCAGGGCAAATCCTACAAGGAGGGATATTCCAAATGTGTT
GAAGAATCGCTGCATTTCTTGTCGGCTCGCAATCATCACAGTGAAGGAAACCTGCTC
AAACATTTCCATGGTCATCAGAGTTCCTCAGTAGAAACCCACCAGTCTCAGCTGCTCC
CTGGACAGTCCCCTTTGACCAACCCTATCACCAACAGCAAAGTTTTGTGGAGACCAT
GGTGAAAGGGTTAATAAGAAGAGTTTGTCAGACAGTCAGTGAACTGACACTTTGGAC
TGCAGCTGNAACACTTTATGAGCAAATCTGTATTTCCTGGGAAAAAATACAAGTATAG
CCCTAGNTTGANGNTNTCCAAGTAGTGAATATTNCANATCTGAGACTNGCCNGACCA
NCNATCCGGGCATGN-3’

Clone He 1316 pGEM-T (SP6 ->)

5’-ACGCGGGGACACAGCAAAGCCTTGTAGTGGCAGCAGCACAGCTCTCCCTGACAG
TAACTGAGCACAATGGCACCTTGCAGCATTAGCCCACAGCCCGCTAGCACCAATAGA
AAGATGAGAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGGATTAACAGCAGCATC
GAGCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCATCTCCCATCCAAA
CCAGAGAAAGCTGACATTCTGGAGGTGGCAGTGAGCTTCCTACAGCAGCAATTAGTT
ACCAAATCTGCTGATATATCAGGCAAATCCTACAAGGAGGGATATTCCAAATGTGTTG
AAGAATCGCTGCATTTCTTGTCGGCTCGCAATCATCACAGTGAAGGAAACCTGCTCA
AACATTTCCATGGTCATCAGAGTTCCTCAGTAGAAACCCACCAGTCTCAGCTGCTCCC
TGGACAGTCCCCTTTGACCAACCCTATCACCAACAGCAAAGTTTTTGTGGAGACCAT
GGTGAAAGGGTTAATAAGAAGAGTTTGTCAGACAGTCAGTGAACTGACACTTTGGAC
TGCCAGCTGGAACCACTTTTTGAGCAAATCTGTATNTTCCTGTGGAATAATATACAAG
TTTAGCCCCTAGGTTTGANGTTTTCCCAAGTAGTGCAATNTTTGNTCATCTGAAGNAC
CNGGCCGGAACCACGCTAATCCCGGGNCATGGGGGCCGGNAGCA-3’

Clone He 1332 pGEM-T (SP6 ->)

5’-CATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTTG
ACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCCA
TGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAGC
AGAGTTGAGGAAGGAAGTGGTCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACCC
TGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGATAGTGCA
CTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCATGA
GTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTCTC
CTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGAGT
TCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAAGTCCAGGAGAATGCATGT
GATCAGTATGAAGAGAATGTAGCATGTGTCCCGCGT-3’

Clone He 1346 pGEM-T (SP6 ->)

5’-ACGGCACTGACTGAATGTCTGTTATATTTATATTATTTGTTAGGCATATTATTGCCT
GTCTATAGATAGAAATGGCTCCCTCATTTCTTTTGGGAAGGGATTGCCTCTATTTCCA
GCAGCTGGAATGATAATATATGTATTTTCATGATGAACACAGATGTAGGGAGAAAAAT
CTTCCTCTGCCTTGTACATTAGCGTCAGACGCAAAGGCGAAACGATCCTGTATCGAT
ATGAATATACTGTCGCATGTTTTCTAAATGAGAATGTCATGTTGTTTCTATGAAATAAT
TGTCCCCGTTAGTAAAGAATATCAGTTATGATAGATGTATTCTGTGATCAGAAATGGT
GTGCTCTGTGAGCTGAATAAATACACGATTTGTGCCAATGAAAAAAAAAAAAAAAAAA
AA-3’

Clone He 1349 pGEM-T (SP6 ->)

5’-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGCCTCCAAGAGAAGGGTCCTGAGATGTTCCAGACTCTGATTAATTCGGTCTC
TCCTTCTTTTCTCTATTACCGGCTTCATTAGCCTCTTATCTTCACGGCTCAGGTAGTTC
TTCATCTTGCTGCAAGAGAAGCTTGAGAAGGGGAAAGTCCAGGAGAATGCATGCNCT
CAGTGTGGAGAGAAAGTAGCTTGTGTCCCCGCG-3’

Clone He 1373 pGEM-T (SP6 ->)

5’-ACAGAAGAATGAAAAGCAAAGNAGTAAGAACATTTAGGCGTGATATGCTAAGCTAA
ATCCATTCATAGTAAAGAATTAATTGATTTTATCTGCCCATATAGTAGTCATAAATAGC
AGCAGCAATATACATAGGATTTTTCAAAAAGTGTTCATAAATATAAGATACATATGTCA

ATAAATAAGAGGGGGGAATATTTTAAATAATAAAGGGGAAAATGTCTGCTTTACAGTT
CTTAGAATTGCAGTGATGATAGTCTGAAGTCATGTTTCTGGGGAAGATTAGACCTCAG
TGGCAAATATACATTGCTCAGGTTCTGGGAGAATATATATTGGGTGATAAGCGCCATC
CTCTGGATACCTTGATGTGGGGTTGCACTGTTTGGTATCTGGCTTTCGGTCAGTTCTG
CGCCCACTTCTCTCCTCCTCACTTTCAGATCCTTGCAGAAGCTTTTGTTTGCTATTTGA
TTTTTCTCTTAGATAGTCTGGATTAGGCATTTTGAAATGGGGTTCCTTTTCTATCAGGN
TATTCTTTNGGTTCAAGGTCATTATTGACTCATCCCNTTTTCATTGAAGTGTCCCCTCC
TCATACCTCTCATAACCATGAACACTCCACAGCCCAGCAAAAAANAATGTTAGCAGTC
CCAGACAAACCACCATGGTAAGAGTGNTTGGAGGANGNNAGCNCNATGGGGNAGCC
GGGGGTGGGANCANTGTACCTNCCCGGGCNGNCCCNNCGAAATCCCCNGGCCATG
GCNGCCGG-3’

Clone He 1375 pGEM-T (SP6 ->)

5’-ACGCGGGGACGCATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGACT
TTGCCCATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTGA
GGATAAGAGGTTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGANTTAATCA
GAGCTTGGAACATCTCAGGACCCTTCTCCTGGAGGCCACTCATGATGAGTCTTTAAA
AAATCCCGAGGCTGAGAAGGCTGATATTCTGAAAAGACAGTGCACTTTCTCANANTG
TGTCATAATCCAGTGCCTTCAGATAAAAAGAAATTGCAGTCAGGGTTTAAAGGAGGCT
TTCGGGAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTGTG
AGANGAAGAAGGAATACATGGTCCAGAGNTTGTGCCAGCACATGGAAGANCAGACT
CAGAACACTGGAGGACTCAGTTCAAGATGTCTCCTTGAGGTCAACCAGCGTCAAATC
TTGCAAGTGCCCAATAATCTCNNGGGTTGAGGGGATGTNCCTCGNCCCGACCCCCT
AATCCCNGGCCATGGCGCCGGGAGATGNNACGTCGGGCCNATTCGCCNATNGC-3’

Clone He 1403 pGEM-T (SP6 ->)

5’-ACACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCAT
CATGAGTGGCCTCCAAGAGAAGGGTCCTGAGATGTTCCAGACTCTGATTAATTCGGT
CTCTCCTTCTTTTCTCTATTACTGGCTTCATTAGCCTCTTATCTTCACGGCTCAGGTAG
TTCTTCATCTTGCTGCCAAGAGAAGCTTGAGAAGGGGAAAGTCCAGGAGAATGCATG
CGCTCAGTGTGGAGAGAAAGTAGCTTGTCCCGCGTA-3’

Clone He 1425 pGEM-T (T7 ->)

5’-ACAAAGTCCAAGCTGACATTCACCACGGCCTCCAGATGGAGCTGTTGAGCTGCGG
GGGACATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACT
GAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGA
TGATTACAATCTGAACTGCACAGCTCAGGGCTCCTCTGCAGGGTATTCTAGAAGGCG
GTTACTCGCTTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCGCTCAGACCCTC
TACACATGCTCTGTAGCCTTCCTTGTAGCGATCTGCTNGATTTTGTGCCTGGACCGG
AGGATNTCTCTTAGGAATCTCACTGTCATTTNCAAAATATCAGCTTTTTCCAGCTTGGA
GTACTTCGGCCGCGACCACCCNNNNGGCCCGNNNNNNNNNNNCNNNGGNGGGGTT
TTCNNCCCCNNNCN-3’

Clone He 1432 pGEM-T (SP6 ->)

5’-ACTTCTGACTCCCCTATGTGAGCCATAATACATTTATAACCAAGCAAGCAAAATCCT
TACTAAGGATACAGACACAATGTAATATACAAAAAGTTTAAAACTTTGCTTTGTGT-3’

Clone He 1435 pGEM-T (SP6 ->)

5’-ACTTAGAGTTAACTGTTAAAAGGGTGTTCCCCCTCCTTTGGTTAAATTTCATAGAAG
AGATTGTAAACAAAGTCCCATAATTTTATGGGCAGGTATATGCCTTTTATTATAAATGG
CACTTAGTCTTCTCTTAACCTTTTAACATTCTTACTCTGAACTCTCCCGCTTTGT-3’

Clone He 1444 pGEM-T (SP6 ->)

5’-ACAAAGTCCAAGCTGACATTCACCACGGCCTCCAGATGGAGCTGTTGAGCTGCGG
GGGACATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACT
GAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGA
TGATTACAATCTGAACTGCACAGCTCAGGGCTCCTCTGCAGGTATTCTAGAAGGCGG
TTACTCGCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCGCTCAGACGCTCT
ACACATGCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTGGACCGGA
GGAATGTCTCTTAGGAATCTCACTGTCATTTCCAAAATATCAGCTTTTTCCAGCTTGGA
GT-3’

Clone He 1449 pGEM-T (SP6 ->)

5’-ACGCGGGGAACACATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGAC
TTTGCCCATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTG
AGGATAAGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGAATTAATC
AGAGCTTGGAACATCTCAGGACCCTTCTCATGGAGGCCACTCATGATGAGTCTTTAA

AAAATCCCAAGGCTGAGAAGGCTGATATTCTGAAAAAGACAGTGCACTTTCTCAAAAT
GTGTCATAATCCAGTGCCTTCAGATAAAAAGAAATTGCAGTCAGGGTTTAAAGGAGG
CTTTCGGGAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTG
TGAGAAGAAGAAGAATACATGGTCCAGAGTTTGNNCAGCACATGGAAGAACAGACTC
AGAAACACTGGAGGNCTCAGTTCAAGATGTCTCCTTGANGTCAACCAGCGTCAAATC
TTGCCANGTGACCAATAATCTCCNGGTTGCAGGNGATGTACCTGCCCGGCGGCCNC
TCGAAATCCCGGGCCATGGCGGCCGGGAGCATNCGACGTCN-3’

Clone He 1451 pGEM-T (SP6 ->)

5’-CTCTTTTTTTTTTTTTTTTTTTTGNCAAANNAAATTTTANGGATTTTAATTTTATGGNN
NTNNCCNNGGGNAATTTNCNGNNCGNGNAAGNTTTCCAAAAATAAANGTTNNATTTN
ATTTTNNAGGCCCNTTTTNNNAAAANGGGGGGGGGGAAAAAAAAAANANAATTTTAA
GGNCCGGGCCCCCNNNNAAGGGCCGGGTTAANNANAGAACCNGGAGGGGGAAAAA
ANANCCNTTNNNGTGNCNTTTNNNAGGGNNTGGCNTTNAAAGTTTNGGGGGGNTTN
NNCCGNANGGCCTNCCCCAAAACGGGGNNTNCTNAAAAACCTGAAGGNGGGNGGC
CNGGCCGGTTNCCCGGGNCTTGGTNCAAGNNCCTCCCGGGNGGCCCCTNNAAAAC
CCNGCNCCATGGGGGCGGGGAGCATNNAAANTCCGGCCCNAATTCNCCNTA-3’

Clone He 1485 pGEM-T (SP6 ->)

5’-TCTTTTTTTTTTTTTTTTTTTTGGCAAANNAAATTTTANGNANTTTAANTTNANGGNN
CTNGNCNNGGGNAATTTNCNGNNCGNGNAAGGTTTCCAAAANNAAANGTTNNATTTN
ATTTTGNAGGCCCATTTTNCNAAAAGGGGGGGGGGGNAAAAAAAANTNNAATTTTAA
GGNCCGGGCCCCCNNNNAAGGGCCGGGTTAANNANNGAACCGGGNGGGGGAAAAN
ANANCCNTTGNCGTGNCNTTNNNNAGGGGGTGGCCTGNAANGNTTNGGGGGGTTTT
NTCCNNAGGGCCTCCCCCAAANCGGGGNGNCCNAAAAAACCNGAAANGGGGCNGC
CNGNCCGGNTCCCGGGNCCTTTGTNCAANANCCCNCCCGGGNGNCCCNNNAAANC
CCCNGCCNTGGGGCCNGGGGGNNTGCANCNCNGGCCCNAATTCNCNAANNGGGGC
CGGN-3’

Clone He 1500 pGEM-T (SP6 ->)

5’-CGCGGGGAGACAGGAAACAGCTGAAGCCGCAGCAGCACCGAAACAGTAACTGAG
CGCAATGGCACCTTGCACTATTAGCCAGCAGCCTGCTAACAGCATTAGAAAGATAAG
AAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCATCGAGCAGCT
CAGGATGTTACTGGAGAAAGAGTTTGAGAAAAATCATCTCCCATCCAAACCAGAGAAA
GCTGACATTCTGGAAATGGCAGTCAGCTTCTTGCAGCAGCACATAGCTACTAAAACT
NNTGAGATGTCAGGCAAATCCTACAAGGAGGGATACTCCAAATGTGTTGAAGATTCG
CTTCATTTCTTGTCCGTTCACAATCAAGGAAGCCTGCTGAAACATTTCCATGGTCATC
AGTTTAACGCAGGAGAGACCCATCAGCTGTGCCCTGT-3’

Clone He 1510 pGEM-T (SP6 ->)

5’-ACAAAAAAACCTATGAAAGGTGAGGATTAGCCCCAGGTAAAGGCTGCGAACAGTT
CGACTCTGAAACTCGACTTTCACTTGTCGCAATCCACACGGAATGAATCAGTTCGTTA
ACAGGTCCATTGGGAAAGCAAAAAATCTGCCCCTGACACAATCCAAATGTTTAGAGAT
ATGGTTTATTCTGTCAATCCCGAGTCCAGGCAATAATTCCAGTTATGTCTCTATTTGTC
TCTCTGCTCATGAACCCAAAGGAAAGAAAATCAGAATAGAAAAAGCAGCAGATGGCG
AGTGTGTTGCTCTCTCTGTCCCAGAGGTGTCAGTTTATAAGGAATCCGGTGTGTATC
GGTGCCGGGGTGGGACTGAAGCTACACGCGTCCCTGTCTAAGTCCCTTCCAGCCCT
GTCTGTCCGTGTGTGAATCAGGGAGTCTCTCCCCGCGT-3’

Clone He 1532 pGEM-T (T7 ->)

5’-ACTAGAACAAAGCACCGAGAACCAGACCAGCCGACCTCCATCCAGTTCATTCAGA
ACACCCTGTATGGGGCAAGCACATCAGCAGACACCCCCACAAACCTTTCAAGCCAAC
ACCATCAGAAAGTCAACGCAAAGGACTCTCTTTCCCCCTACCAGTTCCTCTGTTAACC
CAGCCCTTGTGTGGAGGCCCTGGCCTTAAATATTATATTTATTATTCCCCATCCCTCT
TTTTGTAAATATGGACTTCAAAATTAAATTATATCTTTATCTTTTGTATACTTTCATCGCT
CTGTAATTTCTACATGCATAGATCTATAAATTAAAATTCTATAAATTCTGTTTTGCAAAA
AAAAAAAAAAAAAAAGGGNCCCTTGGGGGGGNACCCCNAAAAAAANTNGGGNGGCC
CCTCGNGGNCGANNATTTGGGNANGNCCCCCACCGGGNGGGNGGCAANNNTGGGG
NTTNTAANGGGGCCCCAAAAAAA-3’

Clone He 1538 pGEM-T (SP6 ->)

5’-ACTTTATCCCCTATAGAACAAACATCTAATAAAAACATGTCTTACAGCATGAGCTCA
GGTCTGTTGGCATTACAGTCTTTACTTGGGTGTTGCTGGTTATTCTTCATTGTGATAA
CTGTTTTTTTTTTTAATTAGGAGGTAAGACTTTGAAAAGAAACAAGAATCACTGACATT
ATACATGAAGGGAAATTTACATTCCGATGTAAGATGCTGTAAATCATAAGATGATGAA
ACAAAAAATACAGTTTGGCCAGAGT-3’

Clone He 1541 pGEM-T (SP6 ->)

5’-ACCAAGGTGACATAGGAGGACTGACTGTCATAGAATGATCCTCATAGATATTGTGT
AGAAAATTCTCTGGAAACTCAGACTGTATCTTTGGGGAGAAGAATTTCAGAGAGTCCT
GGAGACATCTAGAGTGGCCTTCCATGTGGGCTGGGCTGGATGATTGTGCATATTTGG
AAGCCATTAGCTGCTGAGGGAAGCTCACTGCCACCTCCAGGATGTCAGCTTTCTCTG
GTTTGGATGGGAGATGATGTTGCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTC
GATGCTGCTGTTAATCCGATCTCTTCTCATTTTCTCAACCACTGGTTTTCTTATCTTTC
TTATTCCTTGTGCCTTGTGACTGTGCTCAGTATTGAGCATGCTGCTAGCGCTGTAAGG
AGCCATCCTGTTTAGTAAGTGGATATGATGGAGCTGGNAGAGTGCCCTGTGATATCT
CCCCNGCGT-3’

Clone He 1547 pGEM-T (SP6 ->)

5’-ACGTTCAACATCTATTTTTAAAGCCTGATTCCTTTCTACTTCCTGTAAAGTAGAGGT
CAAACGTGAGCGAATGGTCTCCAGTTCAGTTTCTAGCCTATCCTTGCCTTGTTTTTCA
TGCTCTAACTTTGAACACAACATGGTATTCTCTGTCTTCAGGGCATGAAGTTGACTAT
TATACTGTATAACAGTTTGTGTTAAAGTTTCTTCATTCATTTTAAGATCTCTTCTAAGAT
CAGCAATCTTTTCCTTCAAGTTTTCATTTTCATCCATGAATTTGCTCTCTTCCTGTTGAT
TCTGAGATCGAACATGATTGATTTCTAATTTAAGT-3’

Clone He 1555 pGEM-T (SP6 ->)

5’-ACAGATTCACTAATAAAGTGTTTCCAGTTGGCAATCCAAAGTGTCAGTCCAGTTACT
GTCCGGCATACGCCTCTTATTAACCCTTTCACCACGGTCTCCACAAAACTTTGCTGTT
GGTCACAGGGGACTGT-3’

Clone He 1563 pGEM-T (SP6 ->)

5’-CATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTTG
ACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCCA
TGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAGC
AGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACCC
TGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTGCA
CTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCATGA
GTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTCTC
CTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGANGTAGGAGT
TCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAAGTCCAGGAGAATGCATGT
GATCAGTATGAAGAGAATGTAGCATGTCCCCGCGT-3’

Clone He 1577 pGEM-T (SP6 ->)

5’-ACTCTTTATCCTCAGCTTCTCCATCCAGTGGGAGANGTTTTCTGGACTGGAGAAAC
TGNACTGTGCNCTCCAGANTCTCAGCNTTNTCCACCTTCGGATTCTTTAGTTTCTCAC
TTTTCAGGGTNTGAGATAGGAAAATTCTCAGCTTCTCCAGGNTGNNATTTATCCTCTC
CCGTCGCCTTTTCTCCACCAACGGCTTTGAAAGCTTTCTATGAGCTNCCATGGNTTTG
ACTTCACTTGCTCCCTTCANGGACGATGATGATGATCGATGANNATGAAGNAGATGC
TGCAGGANAGAAGTTNTCCANAGGAATNTNCCCGCGT-3’

Clone He 1608 pGEM-T (SP6 ->)

5’-ACGGGGACACATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGACTTT
GCCCATCTCAAGCTTCTCTTGGCACCAAGATGGGGAACTCCTACCTCAGCCGTGAGG
ATAAGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGANTTAATCAGA
GCTNGNANCATCTAGGACCTTCTCTGAGGCACTCATGATGAGCNTAAAATCCAAGCT
AAACGCAGGCTNCTAGAANGAANTCAAANAANAAAAGANCTTNTCAATGNTCAAAT-3’

Clone He 1632 pGEM-T (T7 ->)

5’-TACTCTCACAAAGAACTGTGACAATGCTACAGCNTCGTGGGCATTATTACCCCGTT
ATAAAACACCCCAGTGATATGTGTATTTATTTATTGTAAATATGTCCCCTCACATGAAA
TCCTGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACG
AGAGGACCCCACATTGTGANCCCAAAGGGTTAAATCTAGNATGCGATTTNGATTATTT
TACATTANCCCATGGACTGCAAGAGATGGCNAATGCTACAGTCTCTGCCATGTNTNG
NNTTTTCTGNAGTCCAGGGGTTTATTGACACAGGCTACGTGCAANAATANTGCCTGG
NATGANACTGTNNCAGCCTTTTTTTTTTGNCTTGTTGGANGAAAAAAAAAAAAAAAAA
AAAA-3’

Clone He 1654 pGEM-T (SP6 ->)

5’-ACAGATTCACTAATAAAGTGTTTCCAGTTGGCAATCCAAAGTGTCAGTCCAGTTACT
GTCCGGCATACGCCTCTTATTAACCCTTTCACCACGGTCTCCACAAAACTTTGCTGTT
GGTCACAGGGGACTGTCTAGGGAGCAGCTGAGACTGGTGGGTTTCTACTGAGGAAC
ACTGATGACCATGGAAATGTTTGAGCAGGTTTCCTTCACTGTGATGATTGCGAGCCG
ACAAGAAATGCAGCGACTCTTCAACACATTTGGAATATCCCTCCTTGTAGGATTTGCC

TGATATATCAGCAGATTTGGTAACTAATTGCTGCTGTAGGAAGCTCACTGCCACCTCC
AGAATGTCAGCTTTCTCTGGTTTGGATGGGAGATGATGTTTCTCAAACTCTTTCTCCA
GTAACATCCTGAGCTGCTCGATGCTGCTGTTAATCCGATCTCTCCTCATCTTTTCACC
ACTGGTTTTCTCATCTTTCTATTGGTGCTAGCGGGCTTGGGGGCTAATGCTGNANGG
TGCATTGTGCTNAGTAACTGTCAGGGAGNGCTGTGNTGCTNCCANTACAAGGNTTNC
TGNNTCCCCCGT-3'

Clone He 1677 pGEM-T (SP6 ->)

5'-ACAAAGGTGACATAGGAGGACTGACTGTCATAGAATGATCCTCATAGATATTGTGT
AGAAAATTCTCTGGAAACTCAGACTGTATCTTTGGGGAGAAGAATTTCAGAGAGTCCT
GGAGACATCTAGAGTGGCCTTCCATGTGGGCTGGGCTGGATGATTGTGCATATTTGG
AAGCCATTAGCTGCTGAGGAAGCTCACTGCCACCTCCAGGATGTCAGCTTTCTCTGG
TTTGGATGGGAGATGATGTTGCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCG
ATGCTGCTGTTAATCCGATCTCTTCTCATTTTCTCAACCACTGGTTTTCTAATCTTTCTT
ATTCCTTGTGCCTTGTGACAGTGTTCAGTATTGAGCATGCTGCTAGGCAGTGTATGGA
GCCATCCTGTCTGGTAAGGGGGATGTGATGGAGCTGGAGAGCTGAGAGATAGTGTC
CTGTGATATCTNCCCCGCGTACCCTCGCCCGGGACACGC-3'

Clone He 1682 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTAGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGANAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGTCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTCT
CCTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGAG
TTCCTCACCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAAGTCCAGGAGAATGCATT
GTGATCAGTATGAAGAGAATGTAGCATGTGTCCCCGCGT-3'

Clone He 1693 pGEM-T (SP6 ->)

5'-ACGCGGGGNCACAGCAAAGCCTTGTAGTGGCAGCAGNCAGCTCTCCCTGNCAGT
AACTGAGCACAATGGCACCTTGCAGCATTAGCCCACAGCCCGCTAGCACCAATAGAA
AGATGAGAAAACCAGTGGTTGAAAAGATGAGGAGAGATCGGATTAACAGCAGCATCG
AGCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCTTCTCCCATCCAAAC
CAGAGAANGCTGACATTCTGGAGGTGGCAGTGAGCTTCCTACAGCAGCAATTAGTTA
CTAANTCTGCTGATATATCNGGCAAATCCTACANGGNGGGATATTCCAAATGTGTTNG
ANGAATCGCTGCATTTCTTGTCGNCTCGNAATCATCACAGTGANGGAAACCTGCTCA
AACATTTNCATGNNCATCAGAGTTCCTCAGTAGAAACCCACCAGTCTCAGCTGCTCC
CTGGACAGTCCCCTGTGACCAACAGCAAATTTTTGTGGAGACCGGGGTGAAAGGGTT
AATAAGAGGCGTATGCGGNNAGTAACTGGNCTGACACTTGGGNTTGCAACTGGAAN
CCCTTATTAGTGATCTGTNCCTNGNCNGNACCCCNTAATCCGGGGCCATGGNGNCG
GGNGCAT-3'

Clone He 1714 pGEM-T (SP6 ->)

5'-ACTCTCACAAAGACTGTGACAATGCTACAGCTTCGTGGGCATTATTACCCCGTTAT
AAAACACCCCAGTGATATGTGTATTTATTTATTGTAAATATGTCCCCTCACATGAAATC
CTGACTTACTAAAGGCAATATATTTTGGACAAATAGTCCTGAATACAAAGGGCACGAG
AGTGACCCCANNTTGTGANCCCAAAGGNTTAAATCTAGTATGCGATTTNNATTATTTT
ACATNACCCCATGGNCTGCAGAAATGNCAATGCTACAGTCTCTGCATGTTTGACTTTC
TGCAGNCAGGGGTTANTGACCAGGCTTTGGCNCAATAATGCCGGNATGANCCTGTN
CAGCACTTTTTATGGCTTTTNGAATGAAATNATTTTTAAATAAACCTAANGNGAAAAAA
AAAAAAAAAAAAAA-3'

Clone He 1736 pGEM-T (SP6 ->)

5'-CGCGGGGAGACAGGAAACAGCTGAAGCCGCAGCAGCACCGAAACAGTAACTGAG
CGCAATGGCACCTTGCACTATTAGCCAGCAGCCTGNTAACAGCTTTAGAAAGATAAG
AAAACCAGTGGTTGAAAAGATGAGGAGAGATCGAATTAACAGCAGCATCGAGCAGCT
CAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCNTCTCCCATCCAAACCAGAGAA
NGCTGACATTCTGGAAATGGCAGTCAGCTTCTTGCAGCANCACATAGCTNCTAAANN
TGNNGAGATGTCAGGCAAATCATACANGGNGGGATACTCCAAATGTGTTGANGATTC
GCTTCATTTCTTGTCCGTTCACAATCANGGAAGCCTGCTGAAACATTTCATGGTCATC
AGTTNACGCAGGAGAGACCCATCAGCTGTGCCTG-3'

Clone He 1755 pGEM-T (SP6 ->)

5'-ACAAAGTCCAAGCTGACATTCACCACGGCCTCCAGATGGAGCTGTTGAGCTGCGG

GGGACATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACT
GAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGA
TGATTACAATCTGAACTGCACAGCTCAGGGCTCCTCTGCAGGTATTCTAGAAGGCGG
TTACTCGCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCGCTCAGACGCTCT
ACACATGCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTACCTGGACCGGAG
GNATGTCTCTTAGGAATCTCACTGTCATTTCCAAAATATCAGCTTTTTCCAGCTTGGA
GT-3’

Clone He 1765 pGEM-T (SP6 ->)

5’-ACATGATCCCACCCCAGCTTCCCATCGTGCTGACTCCTCAAACACTCTTACCATGG
TGGTTTGTCTGGGACTGCTAACATTCTTTTTGCTGGGCTGTGGAGTGTTCATGGTTAT
GAGAGGTATGAGGAGGGGACACTTCATGAAAAGGGACGAGTCAATAATGACCTTGAA
CCAAAGAATAACCTGATAGAAAAGGAACCCCATTTCAAAATGCCTAATCCAGACTATC
TAAGAGAAAAATCAAGTAGCAAACAAAAGCTTCTGCAAGGATCTGAAAGTGAGGAGG
AGAGAAGTGGGCGCAGAACTGACCGAAAGCCAGATACCAAACAGTGCAACCCCACA
TCAAGGTATCCAGAGGATGGCGCTTATCACCCAATATATATTCTCCCAGAACCTGAGC
AATGTATATTTGCCACTGAGGTCTAATCTTCCCCAGAAACATGACTTCAGACTATCAT
CACTGCAATTCTAAGAACTGTAAAGCAGACATTTTCCCCTTTATTATTTAAAATATTCC
CCCCTCTTATTTATTGACATATGTATCTTATATTTATGAACACTTTTTGAAAATCTATGT
ATATTGCTGNTGNANTTATGACTCNATATGGGCNGATAAANNAATTANTTCTTACATG
AATGGNTTAGNTAGCNATCNNACCTAATGTTTTTC-3’

Clone He 1766 pGEM-T (SP6 ->)

5’-ACAAAGGTGACATAGGAGGACTGACTGTCATAGAATGATCCTCATAGATATTGTGT
AGAAAATTCTCTGGAAACTCAGACTGTATCTTTGGGGAGAAGAATTTCAGAGAGTCCT
GGAGACATCTAGAGTGGCCTTCCATGTGGGCTGGGCTGGATGATTGTGCATATTTGG
AAGCCATTAGCTGCTGAGGAAGCTCACTGCCACCTCCAGGATGTCAGCTTTCTCTGG
TTTGGATGGGAGATGATGTTGCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCG
ATGCTGCTGTTAATCCGATCTCTTCTCATTTTCTCAACCACTGGTTTTCTAATCTTTCTT
ATTCCTTGTGCCTTGTGACAGTGTTCAGTATTGAGCATGCTGCTAGCAGTGTATGGAG
CCATCCTGTCTGGTAAGGGGATGTGATGGAGCTGGAGAGCTGAGAGATAGTGTCCT
GTGATATCTCCCCGCGT-3’

Clone He 1774 pGEM-T (T7 ->)

5’-ACGCGGGGAGATATCACAGGACACTCTCCCAGCTCTCCGGCTCCATCACATCCAC
TTACTAAACAGGATGGCTCCTTACAGCGCTAGCAGCATGCTCAATACTGAACACTGTC
ACAAGGCACAAGGAATAAGAAAGATGAGAAAACCAGTGGTTGAGAAAATGAGGNGAG
ATCGGATTAACAACAGCATCGAGCAGCTCAGGATGTTACTGGAGAAACAGTTTGAGA
AACATCATCTCCCATCCAAACCAGAGAAAGCTGACATTCTGGAAGTGGCAGTGAGCT
TCCTGGAGCAGCACATGGCTTCCAAATATGCACAATCATCCAGCCCAGCTCACACAG
AAGGCCACTCTATAAGTCTCCAGGACTCTCTGCATTTCGTCTCTCCAAAGAAACACTC
ACAGTTTTCAGAAAATCTTCTACACAATTTCTATGAGGATCATTCTGTGGCAGTCAGTC
CTCCTCTATNACCTTTGTATCAGACCCCCACCAAATGCACAACACCTGGGGCCAGCA
AAGTGNTTNGGGNCCCTGGTAGACTTTAATGAACGTTCTGANATCACNTTNACTCNG
GACATCNTGNGGCTGGGGGTTANTTGA-3’

Clone He 1783 pGEM-T (SP6 ->)

5’-ACAAAAGAGATTTCTCTAGACATAAGAGCTATTAATCAGGTCGGAGCGCTGGCNCC
GGAATCTCCCTCGTCTCATTCCGTTTCAGTGTTAAAGCAAAGGACAGGCTGAAATCCA
CAGTGAGAAGTCATGGCAAAATATATATATTTATAGGATCTAGGCAACGAAAGAGAAA
TCGAATAAAAGTCATACCTTCGTCATTCTCGTCAATAAATAAAAAAATAAATAAACATA
ATTAATCATAAAATTAACATTTTGGATAATAAACGCATACAGAACAGATATTATAGCTG
GGGGGACAAAACACGTTGACAAAAAGAGATTGTTGGTCCCTATAGTGGGGGGGGGT
GACAAGGCAGTCGCTACAGCTGACTCCAGAGTTATTACAGACAATAATGACTTCAAG
GCATTTGTGGCTACGACTAAAGTAAAGTCTCCTCCTCCCAAATATCCACAGCTGGGCT
GAGAGGAGAGAGAGAGCTGAGCAGCCCCCNGCCACTATATAAAGCTAATGG-3’

Clone He 1785 pGEM-T (SP6 ->)

5’-ACTGTTAAATACTGCAAATATTGCATTTTACAGAGAAATATGGCAGTTACACTGTTA
AATACAGCAAATATTGCATATTACAAAGAAATATAACATGCATGCTGTTAAATACTGCA
AATGTTGCATATTACAAAGAAATATAACAGTTATACTGTTAAATACTGCAAATGTTGCA
AATTAATTAAAAATATAACACAAATACTGCAAATATCGCAGTTTACAAGTTTAACAGTTA
TTATACTCTTAAATACGGCAAATATTGCATTTTACAGAGAAATATGGCAGTTACACTGT
TAAATACTGCAAATTTTGCATATTACAAAGAAATGTAACATGCATGCTGTTAAATACTG
CAAATATTGCATATTACAAAGAAATATANCAGTTATACTGTTAAATACTGCAAAATGTT

GCAAATTAATTAAAAATATACACAAATACTGCAAATATTGCAGTTTACAAGTTAAACAG
TTATTATACTGTNAAATACGGCAAATTTTGATATTGCTGAAACATATAACATTTAAATTT
TGCAAATACTGCATAGT-3'

Clone He 1828 pGEM-T (SP6 ->)

5'-NNNCATTGATTCCAAAATGAAATACATTGAATCTCTTACATATATTCTTTACTATAAT
GCAAAAATACTGATGCTATTTTTATGAAAAACAAACATACTGTATGTAGGAAATATTCC
ACATTTCATTGANCCTTACATTGGTTAGCACATGCAACAATATTTTCACAATATTTTTG
GNCTAACCAAGATCTAGTCGGTTACCCNTTATAGTATGACATAAAGTTTTTATATCCAA
AGCTTAAGATATCTTCTTCTACTGAATTATAGATATAGCTATCCATCACTACANCTGTT
ACATAGTGATTCTCTGCATGCAGAATCAAACTAGCCTGAGACATAAGTGCTTTTTGGC
ACGNGGAAGGNTTATAACCTACATGT-3'

Clone He 1829 pGEM-T (SP6 ->)

5'-ACNTAAGNNATNANNNTNACTGGGGGGGAACCNNTCCCNCCCGGTCGACCTNTN
GGCGGCCCGAAANACCCGATTTCGAGCGGCCGCGGGCCAGGNNCCATGTTCAACA
CTTATATTTGTCAGTTTAAATATACAACTCTTGATTATATACAATATGCACAATAATGGT
CTGGAATGTCAAAAATATAACATAAATAAAAATGTTTGTTAAACTAGGCTTTACAGACT
TGAACCACTAAATGTGGGGCATTTTGGTCAAGAAAAAACAAAAACATTTCTAAAAATA
GAAATCTCAATAGCAGCAATGNTTTTCTTAAAGCATTTTTAACAAAGGTCATTGTGAAC
ACATTTCAATATGTTTAATTAGTACCTCGNCTNCGACCACGCTAATCCCGGGCCATGC
GGCGGGAGCATGCNACGNCGGGCCAATTCGCCTATAGTGAGCGGA-3'

Clone He 1881 pGEM-T (SP6 ->)

5'-ACAGATTCACTAATAAAGTGTTTCCAGTTGGCAATCCAAAGTGTCAGTCCAGTTACT
GTCCGGCATACGCCTCTTATTAACCCTTTCACCACGGTCTCCACAAAACTTTGCTGTT
GGTCACAGGGGACTGTA-3'

Clone He 1898 pGEM-T (SP6 ->)

5'-ACACGCTGTGNNCATAAAATCTGCANTCTGCTGNAAGTGCACAGATAAACCTGTGA
GGTATTTATTGCCTTTCTTGANTTGNTCCTGGATCAAAGTTTCAAGTTCATCACAAAAT
GCNGGACTTTGTAGTATCGTGNAGACCNTTTTCTTTTGCNCCATCATGCTTGAAGTCT
TGAGGAAGGTCTGGAGCNNTGTTCCTCTCTCNCAGATACTCAGGATTGTTTTNNTNAA
CCCTGNCAAANTATTTCNCCTTNTNANNANNATTGCNTGGACTCTGNTGAAGTCANTN
TCCCTACANTNACAGTNTANGTTGCCNGAGACTTTTCTANTCCCGNTCCATTAGNNTC
ANTCAANANNAGACATCCGTCCNANNTCACCTTNNAGCGNNGGCCATAACCACNCTA
ATCCCGCGGCCATGGNGNCCGGGAG-3'

Clone He 1911 pGEM-T (SP6 ->)

5'-ACGCGGGGGAGAAAGACTGGTGCTCTCAAAGTGTTTGTTCCTCTTGGATTATACAC
GGCGCCCGATTTTCTCACACACCCAACCATGACTGTAAAGACAGAAACGGCGGCNG
GGTNCAAGCACACTGACTTATTCTAAGATGAGGGGAATGGTGGGCTTTGCTTATCGC
TTTTATGAAACAACGAAGAATGGGACTGAATGATTTCATCCAGAAAATTGCAACCAAC
TCTTCCTATGCCTGCAAACCCTCTGAAGTGCAGTCAATCCTGAATATCTCACCCCCCC
AAGACCAGAATTACTGAATGAAAATTCCTCGCCTCCCCCTAGCCCTTCTCAACAGATC
AACCTTGGTCCTTCATCCAACCCTCATGCAAAACCATCCGACTTCCAGTTTCTGAAAT
CATTGGCAAGGGTAGTTTTGGAAAAGTTCTCCTGGCANGACACCAAGCAGATGAGAA
NTCTATGCNGNGAAAGTCNTNAGAAAAAGCCTTCTCAAAAGNAAGGGAGAAAACATA
NTGTCAGANGCATTGNCCTCGCCGGNCCCCCAATCCCNGGCATGGCGCNGGNCA-3'

Clone He 1916 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG
CAGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTG
CACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCAT
GAGTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATCCTGTCTC
TCCTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGA
GTTCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGCAAAGTCCAGGAGAATGCATG
TGATCAGTATNCANGAGAATNTTGCATGNCCGGCGNACNTANNCGGGNGGNCCTTG
GAAACCNGGGNCCNTGGGGGCCGGNANNATTCAAN-3'

Clone He 1928 pGEM-T (SP6 ->)

5'-ACATCTCCTGCAACCCTGGAGATTATTGGTGCACTTGGTAAGATTTGACGCTGGTT
GACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCC
ATGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAG

CAGAGTTGAGGAAGNAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACC
CTGACTGCAATTTCTTTTTATCTGANGGCACTGGATTATGACACACTTTGAGAAAAGT
GCACTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCA
TGAGTGGCCTCCATGAGAAGGGTCCTGAGATGTNCCAAGCTCTGATAAATTCTGCCT
CTCCTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCCCGGCTGAGGNAGG
AGTNCCTCATCTTGGGGCNAAGANAAGCTTGAGATGGNCAAAGNCCANGNAAAATNC
TTNTNATCNGNATCCCCGNGN-3’

Clone He 1940 pGEM-T (SP6 ->)

5’-TACTNNGNNATGACTNCCAACGCGTTGGGNAGCTCTCCCATATGGTCGACCTGCA
GGCGGCCGACCTAGTGATTTCGAGCGGCCGCCCGGCAGGNACTGAGCACAGTCAC
AAGGCACAAGGAATAAGAAAGATAAGAAAACCAGTGGTTGAAAAGATGAGGAGAGAT
CGAATTAACAGCAGCATTGAGCAGCTCAGGATGTTACTCGGAGAAAGAGTTTGAGCA
ACATCATCTCCCATCCAAACCAGAGAAAGCTGACATCCTGGAGGTGGCAGTGAGCTT
CCTGCAGCAGCANATGGCTTCCAAATATGCACAATCATCCAGCCCGGCCTACATGGA
AGGCCACTCTAGATGTCTCCAGGACTCTCAACGCTTCTTCTCCCTGCAGAAACACTC
AGAGCTCCCAGAGAAGCTTCTGCNAAATTTCCATGATGATGTGACAGCGTGTCCTTT
CTGTGTCACCTTTTTACCAGAATCCTGCCAAGTGCACAACCCCTGAGGCCAACAAAG
TTCTCTGGAGACCCTGGTAGACATTAAATAGACTATGGTTCTGATGCAGCATCAGCTC
TTCTGGGCATTAGGCAATTGCAATGGGACTAATTAAGCCTTTNATGAAAAAAGGNAAT
GGTTGCTATTTATAAGCTGGGTGTANTCAAAGGNGNCCTATGTAAAANAATATAAGGN
NTGTTTTTCCAGTAAGAATTTTCCTANAGAAAAAAAATGCTGGCNTCTTGNAAGGAAA
NTGGGGTNCNGGTAAAAATTTTNAGGNTNTTAAAANGGAATTNTGGGGNTCAATTTCA
NNAGTTCATTTNTGGGANGGGNGGGGNANNGNNGNCCCCTNTCCCTTAGGATTTTN
GTTCCAACCGGTT-3’

Clone He 1945 pGEM-T (SP6 ->)

5’ACGTGTTTTGTCAATGGAGCACAGTTGGGACTCCAGGCGTCAGAAGGTGACTTACA
GGAGGACATCTGGTGGGAGAGCAGCTCCTTGGTCACACCATTCATATCAGGTTTGGA
GTTCATGAAATGCAAGGTCGTCTCCAGGCAATGCTGGAACCCCGATTGGTATTCTTTA
TCCACCGCTTCCCTATCCAGTGGGAGAAGTTTCCTGGACTGGAGAAACTGGACCGTG
CACTCCAGAATCTCAGCCTTCTCCACTTTTGGATTCTTAAGTTTCTCACTTTTCAGGGT
TTGAAATAGGAAAATTCTCAGCTTCTCCAGGCTGTTATTTATCCTCTCCCTCCGCCTTT
TCTCCACCAACGGCTTTAATAGCTTTCTATGGGTTTCCATGGGTTTGACTTCACTTGTT
CCCTTCATGGTAAGATGCCCCGT-3’

Clone He 1971 pGEM-T (SP6 ->)

5’AATAANNNATNATTCCCAATGCGTTAGGGAAGCTCTCCCATATGGTCGACCTGCAG
GCGGCCGANCTAGAATATTTCGAGCAGTGCGCGGGCCGGCAGCATGNAANGTCAGA
GGCCGCAATTCACCATATAGCTGAGATCANGATATACATGTATGAGCTGCGGGGGAC
ATGGGGCAGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAACTGAGAT
GTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAGGATGATTA
CAATCTGAACTGCACAGCTCAGGGCTCCTCTGCAGGTATTCTAGAAGGCGGTTACTC
GCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCNCTCAGACGCTCTACACAT
GCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTGGACCGGAGGAATG
TCTCTTAGNAATCTCACTGTCATTTCCAAAATATCAGCTTTTTC-3’

Clone He 1983 pGEM-T (SP6 ->)

5’-TACGCGGGGACATGCTACATTCTCTTCATACTGATCACATACATTCTCCTGGACTTT
GCCCATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTGAGG
ATAAGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGAATTAATCAGA
GCTTGGAACATCTCAGGACCCTTCTCATGGAGGCCACTCATGATGAGTCTTTAAAAAA
ATCCCAAGGCTGAGAAGGCTGATATTCTGAAAAAGACAGTGCACTTTCTCAAAATGTG
TCATAATCCAGTGCCTTCAGATAAAAAGAAATTGCAGTCAGGGTTTAAAGGAGGCTTT
CGGGAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTGTGAG
AAGAAGAAGGAATACGTGGTCCAGAGGTTGTGCCAGCACATGGAAGACAGACTCAG
AACACTGGAAGGACTCAGTTCAAGATGTCTCCTTGANGTCACCAGCGTCAAATCTTG
CAAGTGCCCAATAATCTCCAGGGTTGCAGGAGATGT-3’

Clone He 2016 pGEM-T (SP6 ->)

5’-ACCAGATCCCCACCAAATGCACAACTCCTGGGACTGGCAAAGTTCTCTGGAGACC
CTGGTAGACATTTGATGAACTTTCTGAGCATCACCTTCAACTCAAGGATATCCTGCAG
ACTGGTGGCTTACCTGATGGTTTATTATGACTATGTAATAGGAATAACTGGTTACTGT
AATGGTGCAACACCAAAGCTATTTTGTAGCTGTATGAATTATATCCAGGCAATGTCTG
GTGATCTGGTTTATACCAGATTGTTCAGTTAAATGATATTTGTAGCTGTCAGTAAGGA

CAGTGAGTAACTTTTCTATGTGAAATTAATTGTCATTATTATATAATGTTATGCATATGG
CATTGACCATGAAAGTAACACAGATGCTTTAAGGTTAGAGCTTATAGACACTGAACTG
AAGTGAAAAAGTATCTATATATTACAATATCTGTATCCTCAGTGAGGAGAAAATGTATT
CCTATATTGTCAAACTANATTGTTAGNTTTGACNTATATGNNTCCACTATGGGGAAAC
GTTNTTCTTTTATTNCCAGCNCCCTANCAGCCTAGGGGTTTCTGGGNAGTTGNAGTTC
ANAANAGTGGTNGGAAANTGGTA-3'

Clone He 2018 pGEM-T (SP6 ->)

5'-ACATTAACTGTTATATTTCTTTGTAAAATGCAATATTTGCTATATTTAACAGTATATTA
ACTGTTGTATTTCTTAATGATATGCAATATTTGCAGTATTTTGTTATATTTTTTAAGTAAT
ATGCAATATTTGCAGTATTTAACAATAGAATAACTGTTCTATTTCTTAGTAATATGCAAT
ATTTGTAGTTTTTGATAAATTTTTCAAGTAATATGCAATATTTGCAGTATTTTGTTATAG
TTTTAAGTAATATGCAATATTTGCAGCATTTTGTTAAATTTCTCAAGTAGTATGCAATAT
TTGCAGTATTTAACAGTATAACAATTGTTATATTTCTTATTATTATGCAACATTTGCAGT
ATTTAACAGTATAACTGTTGCATCTCTTTGCAGAATGCGATATTTGCAGTATTTAACAG
TATATTACCTGTTACAGTTCTTAGTAATATGCAANATTTGCAGCATTTTGTTAAATTTCT
CAAGNAAATGCAATATTTGCAGTATTTACNAGTANAACAAATNGTAAATTTCNTAATAN
TANGCANCGTTTGCAGGATTTAACAGNANANCTGTNGCATCNCNTTNCAGANGNGAN
NTTTTCAGTATTAACAGGNAATNNCCTGTNNAGGTNNAAGGAAAANNCAAANTTNCAA
AAATTTGANGGAACGTTCCNAACNA-3'

Clone He 2025 pGEM-T (SP6 ->)

5'-ACTATACTTGTGTNNGTTGNNTACTAACAGCCGAGCAAATNTGGTATCCATTGTAG
CATTTGTCTATGCCTTTTAGAATGCTGNCCCCCATTCAGTATTTGAGCAGAGNNAAGG
AATAAAGNTAAANGTGACCACGAGGAGCTGCNTATANGATAATCCCTGTATGCTTTTA
AAGGCCCNACACCCAAGCTTNATGGCTTTAGNTCTTCATTACTTTTAACTCCCTGTTTT
ATGTTTCTGNTGATTACCTNTTGCTGCCTGCCGATTCCCAATCACACCCCAATCTCTG
TACCTCNGGCGCTTCCACGCTNATCCNGCGGCATAGGTGTCNGNTAGCATGCGATG
TCAGGCCCAACTCGCCNNTAGNNTNNCGNTTNTCNCNNAAAAAANNGANGTTTTNCN
NGAGCGGNATTTTNCNAGNNCCCNNNTTNGNNTCNNAAAAGGNNCCNGGGNGGNCC
TTGGNTTNNNAAACNNANANTTANCCNTNNT-3'

Clone He 2042 pGEM-T (SP6 ->)

5'-ACGCGGGGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAA
TTTGTGATGCTATTGCCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAA
CAACAATTGCATTCATTTTATGTTTCAGGGTTCAGGGGGAGGTGTGGGGAGGTTTTTT
AATTCGCGGCCAGAAAAAATAANNNANGGNAANNNANNNNAAAAAAAAAAAAAAAAA
NNTCCNTGGCCCNCAACCCCCNAAATCCCCNGGCCNTTGGGGNCCGGGGGNAATG
NAAANTNGGGCCCCAATTTGGCCCCTNAAAAGGGN-3'

Clone He 2061 pGEM-T (SP6 ->)

5'-TACAATAGATGCTTTAAGAAGAGGTCAAAGCTTATAGATACTGAACAGAAGCGCAA
ACGTATCTATATTTTACAATATCTGTATCCTCAGTGAGGAGAAAACATATTCCTAAATT
TGGGTTAGGAAGCAAAACGGTGAAACTAGATTGCTAGTTATGACTTATATTCTACTAT
GTGGTATACTATGTTCTTTAATTCCCAGCACCCTAACCATCCCAAAGGGTTGCTGGGA
GTTGTAGTTTCTTACAGTATTTGGATAATTGTTATCTCTGTGCTGTGAAGAACATTTGT
GAATTTGTAATTCATTTCCTACAATGTATGCAGTTTTATTGCACTTATTGGTATATTATA
TAGATATATAAGTTATGCAATATTTGCAAGTTATATTTGTGCCTGAATGTTAAATGTAT
GCCTCTATTNAAATGCTGCAATGGCNTTTCAATAANTNNTTTTTTGAAAGCCNAAAAAA
AAAAAAAAAAAAAANT-3'

Clone He 2066 pGEM-T (SP6 ->)

5'-ACAAAGTCCAAGCTGACATTNACCACTGGCTCGTCCAGCATGGTAGCTTNTTGAGC
TGCANAGNGGTACANGGNNCAGGTCTGTGGCTGGAGGTTTGTTTNTGGAGAGGTNT
TCCAAACTGAGATGTTNTGGNGACTNACTTGGAGCACANTCNTAGNACGTTGCATGC
TTGGTAAGGATGATTACAATCTGAACTGANAGCTCAGNGCTNCTCTGAGGTATTCTAG
AAGNCGGTTACTCGCCTTCTCCGGTNAGCACATGAGATTTACCANGGATTGCGCTCA
GACGCTCTACACATGNTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTG
GACCGNAGGNATGCCTCTTAGGAATCTCACTGTCATTTCCAAAATATCAGCTTTTTCC
AGCTTGTAGT-3'

Clone He 2074 pGEM-T (SP6 ->)

5'-TACTTTGCTGGCCCCAGGTGTTGTGCATTTGGTGGGGGTCTGATACAAAGGTGAC
ATAGGAGGACTGACTGTCATAGAATGATCCTCATAGATATTGTGTAGAAAATTCTCTG
GAAACTCAGACTGTATCTTTGGGGAGAAGAATTTCAGAGAGTCCTGGAGACATCTAG
AGTGGCCTTCCATGTGGGCTGGGCTGGATGATTGTGCATATTTGGAAGCCATTAGCT

GCTGAGGAAGCTCACTGCCACCTCCAGGATGTCAGCTTTCTCTGGTTTGGATGGGAG
ATGATGTTGCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATACTGCTGTTA
ATCCGATCTCTTCTCATTTTCTCACCCACTGGTTTTCTTATCTTTCTNATCCCTGTGCC
TTGTGACTGTGCTCAG-3'

Clone He 2077 pGEM-T (SP6 ->)

5'-ACTTCAGATGATGCAAATATTGCACTACTTGGGATATACTTCAAACCTAGAGGCTAT
ACTTGTATATTATTCCACAGGAATATACAGATTTGCTCATAAAGTGTTTCCAGCTGGCA
GTCCAAAGTGTCAGTTCACTGACTGTCTGACAAACTCTTCTTATTAACCCTTTCACCAT
GGTCTCCACAAAACTTTGCTGTTGGTGACAGGGTTGGTCAAAGGGGACTGTCCAGG
GAGCAACTGAGACTGGTGGGTTTCTACTGAGGAACTCTGATGACCATGGAAATATTT
GAGCAGGTTTCCTTCACTGTGATGATTGCGAGCCGACAAGAAATGCAGCGATTCTTC
AACACATTTGGAATATCCCTCCTTGTAGGATTTTGCCTGATATATCAGCAGATTTGGTA
ACTAANTTGCTGCTGTAGGGAGCTCACTGCCACCTCCAGAATGTCAGCTTTCTCTGG
TTTGGATGGGAGATGATGCTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCG
ATGCTGCTGTNAANCCGATCTCTCCTCATCTTTTCACCACTGGTTTCTCATCTTTCTAT
NGNGGNAGCGGCTGTGGNCAATNNGCANGNGC-3'

Clone He 2080 pGEM-T (T7 ->)

5'-ACTATAGATGCTTTAAGAAGAGGTCAAAGCTTATAGATACTGAACAGAAGCGCAAA
CGTATCTATATTTTACAATATCTGTATCCTCAGTGAGGAGAAAACATATTCCTAAATTT
GGGTTAGGGAAGCAAAACGGTGAAACTAGGATTGCTAGTTATGACTTATATTCTACTA
TGTGGTATACTATGTTCTTTAATTCCCAGCACCCTAACCATCCTAAAGGGTTGCTGGN
AGTTGTAGTTTCTTACAGTATTTGGATAATTGTTATCTCTGTGCTGTGAAGAACATTTG
TGAATTTGTAATTCATTTCCTACAATGNNTGCAGGTTTATTGCACTTATTGGNATATTA
TATAGGATATATAAGTTATGCAATATTTGGCANGTTATATTTNTGGCCTGAATGGTAAA
TGGTATGCCTTTATTTAAAAGGCGGCNATGGCNTTNCAANAAATGGGTTTTTGGAANG
ATGTAAAAAAAAAAAAAAAAAA-3'

Clone He 2093 pGEM-T (SP6 ->)

5'-ACATTCCTGTGGAATAATATAAATGTATAGCCTGTAGGTTTCATTTATATCCCGAAG
TAGTGCAATATTTGCAACATTTAACGTA-3'

Clone He 2103 pGEM-T (SP6 ->)

5'-GGGGNTTTCGGAGCGGCCGNCCNGGGAGGNTACTTAGGAAACAAAGCACCGAGA
ACCAGACCAGCCGACCTCCATCCAGTTCATTCAGAACACCCTGTATGGGGCAGGCAC
ATTAGCAGACACCCCCACAAACCTTTCAAGCCAACACCATCAGAAAGTCAACGCAAA
GGACTCTCTTTCCCCCTATCAGTTCCTCTGTTAACCCAGCCCTTGTGTGGGNGGCCC
TGGCNTTAAATATTATATTTTATTATTTCCCCATCCNTCTTTTTGTAAATATGGACTTCA
AAATTAAATATATCTTATCTTTNGATACTTTCATCGCTCTGTANTTCTACATGCATAGAT
CTATAANTNAAATTCTATAAATTCGGAAAAAAAAAAAAAAAAAAAA-3'

Clone He 2119 pGEM-T (SP6 ->)

5'-TACAAAGTCCAAGCTGACATTCACCACGGCCTCCAGATGGAGCTGTTGAGCTGCG
GGGGACATGGGGCGGGTCTGTGGCTGGAGGGTTGGTTCTGGAGAGGAGATCCAAA
CTGAGATGTTCTGGGACTCACTTGGAGCACAATCCTAGGACGTTGGGGCTTGGGAG
GATGATTACAATCTGAACTGCACAGCTCAGGGCTCCTCTGCAGGTACTCTAGAAGGC
GGTTACTCGCCTCTCCGGTCAGCACATGAGATTTACCAAGGATTGCGCTCAGACGCT
CTACACATGCTCTGTAGCCTTCCTTGTAGCGATCTGCTTGATTTTGTGCCTGGACCGG
AGGGATGTCTCTTAGGAAATCTCACTGTCATTTCAAAAAATCAGCTTTTTCNAGCTTG
GAGT-3'

Clone He 2139 pGEM-T (SP6 ->)

5'-ACTAATATACACAGTTAAAACAATTATACTCTTTTCTATCAATGCAAACACCACTTAT
TATATAACAATATAATTATTATATGCTTCCTTCAGGCAGGCACAGCCCATATGCTATGA
CAGCAATATCTGTCCTCTAGCATAGACTTCATCCTGTAATATTCTTGGATTCCAACAAT
TTACAGTAATTATTTATTTTTCCCATACATAGCTGTAAATGAGCTAATGGGCAGGCAGC
CTGAGCCATCAGTTTAGTTCCAGGGTCTCCAAAGGAGCTTGGCTGGTGGTGCCGGT
GCCTTTGGTAGGTGTCTGGTAAAAAGGTGACCCAGT-3'

Clone He 2144 pGEM-T (SP6 ->)

5'-CGCGGGGATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGACTTTGCC
CATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTGAGGATA
AGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGAATTAATCAGAGCT
TGGAACATCTCAGGACCCTTCTCATGGAGGCCACTCATGATGAGTCTTTAAAAAATCC
CAAGGCTGAGAAGGCTGATATTCTGAAAAAGACAGTGCACTTTCTCAAAATGTGTCAT
AATCCAGTGCCCTTCAGATAAAAAGAATTTGCAGTCAGGGTTTAAAGGAGGCTTTCG

GGAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTGTGAGAA
GAAGAAGGAATACATGGTCCAGAGGTGGGNCAGCACATGGAAGACAGACTCAGAAN
ACTGGAAGGACTCAGTTCAAGATGCCCCTTGAGGTNACCAGCGTNAAATCTTGCNAG
NGCNCAATATTCTCAGGNTGCNGGNATG-3'

Clone He 2157 pGEM-T (SP6 ->)

5'-ACGCGGGGACACAGCAAAGCCTTGTAGTGGCAGCAGCACAGCTCTCCCTGACAG
TTACTGAGCACAATGGCACCTTGCAGCATTAGCCCACAGCCCGCTAGCACCAATAGA
AAGATGAGAAACACCAGTGGTTGAAAAGATGAGGAGAGATCGGATTAACAGCAGCAT
CGAGCAGCTCAGGATGTTACTGGAGAAAGAGTTTGAGAAACATCATCTCCCATCCAA
ACCAGAGAAAGCTGACATTCTGGAGGTGGCAGTGAGCTTCCTACAGCGGCAATTAGT
TACCAAATCTGCTGATATATCAGGCAAATCCTACAAGGNGGGATATTCCAAATGTGTT
GAAGAGTCGCTGCATTTTTTTGTCGGCTCNAANTCATCACAGTGAAGGAAACCTGCT
CAAANATTTCCATGGTCATCAGNGTTCCTCAGTAGAANCCACCAGTNTNAGCTGNTC
CTGGNNAGTCNTTTTNACCTTNAAANANAANTTTTGGNCCNTTTNAGGTTTTAAANNN
T-3'

Clone He 2162 pGEM-T (SP6 ->)

5'-ACTAATATACACAGTTAAAACAATTATACTCTTTTCTATCAATGCAAACACCACTTAT
TATATAACAATATAATTATTATATGCTTCCTTCAGGCAGGCACAGCCCATATGCTATGA
CAGCAATATCTGTCCTCTAGCATAGACTTCATCCTGTAATATTCTTGGATTCCAACAAT
TTACAGTAATTATTTATTTTTCCCATACATAGCTGTAAATGAGCTAATGGGCAGGCAGC
CTGAGCCATCAGTTTAGTTCCAGGGTCTCCAAAGGAGCTTGGCTGGTGGTGCCGGT
GCCTTTGGTAGGTGTCTGGTAAAAAGGTGACCCAGT-3'

Clone He 2169 pGEM-T (SP6 ->)

5'-ACAAACGATTTACAGCTCCAATATAAACAATGCCAGTGTCACTTTCAACAACAAGAT
GGTTTAAATCTGTGTCACTAAGGAAAGAAAGAAATGTTTGTGCTTGTGGCATCAATAT
GATGCAGATAAATAGGCTGGCAACTTGAATCCATAAAGCCATTGTTGGAGAGTGCCT
CTTAATTATTATGAGTCACAATTGGCACCTAGAAGGTCAACTTTCCCCTTCCTCTTTCT
ATCCAGGAAGTTGCGAGCTGTGTGTAAATCAGCACAACATTTCTTTTATTGGGTCAGT
CCCAGTGTAGGTTAGCTAGTCCTTATATGACAGCAACTGTAACTTTTGACAAATTCTTT
GGCAGTCCACTAACTGCAGGAATATCAAGATGCCTCCAAGTTTCTGCACTTTAATGCC
AAATCGAAGTCTCCAGCTTGGCTCTGCTCCCCGCGGCTTTCCCAGACTCAATGGAGA
CGCACAGAGAAATGAAGTGCCGNTCCNCGCCCGGAC-3'

Clone He 2175 pGEM-T (SP6 ->)

5'-ACAAGGCAGAGGAAGATTTTTCTCCTATATCTGTGTTCATCATGAAAATACATATAT
TATCATTCCAGCTGCTGGAAATAGAGGCAATCCCTTCCCAAAAGAAATGAGGGAGCC
ATTTCTATCTATAGACAGGCAATAATATGCCTAACAATTAATATAAATATAACAGACATT
CAGTCAGTGCTGTA-3'

Clone He 2190 pGEM-T (SP6 ->)

5'-ACGCGGGGATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGACTTTGC
CCATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTGAGGAT
AAGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGAATTAATCAGAGC
TTGGAACATCTCAGGACCCTTCTCATGGAGGCCACTCATGATGAGTCTTTAAAAAATC
CCAAGGCTGAGAAGGCTGATATTCTGAAAAAGACAGTGCACTTTCTCAAAATGTGTCA
TAATCCAGTGCCTTCAGATAAAAAGAAATTGCAGTCAGGGTTTAAAGGAGGCTTTCGG
GAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTGTGAGAAG
AAGAAGGAATACATGGTCCAGAGGTNGTGCCAGCACATGGAAGACAGACTCAGAAA
CACTGGAGGACTCAGTTCAAGATGCCTCCTTGAGGTNACCAGCGTCAATCTTGCAAG
TGNCCAATAATCTCCAGGGTTGCAGGAGATGTACNTGCCNGGCGGCCCNTCGAAAT
CCCCNNGCC-3'

Clone He 2206 pGEM-T (T7 ->)

5'-ACNCGGGGCCAGCACAAGAAACCAGAAGAAACAGAGAGCTTAGCACAGAACAGA
GTGCAATAAATGGTTTCTACCCACGTTGCCCTGGACTGCAGCATGGAGAACCCAAAA
GCAAAAACTTCCCTTAGGCAAATAAGAAAGCCGGTGGTGGAGAAGATGAGACGGGA
CCGGATAAACAGCAGCATCAAGCAGCTCCGAATGTTGCTGGAGAAGGAGTTTCAGAG
GCACCAGCNAAACTCTAAACTGGAAAAAGCAGACATCCTAGAGATGACAGTCAACTA
CCTGANGGATCGTCAGCTCCAGATGANTGCAGATGCNTTTGCNCGGGAAAAGCCCA
TTCAANGATTATAAACANGGCTACAGCNGGTGTCTAGAAGAGACTCTTCAATTCTTGT
CTCAAACAGAATGCAAAAACAGGCAACTGGAACTGATGNGCATTTAATAGAATGTTCC
AGCTGACAGTGATTNGCCCCAGGGGGCCCNAAGCCTCAAAACTCCTCATNCCAANAA
TCAGAAATAATCTGGNGGCNGGGGTAATCNCAGGCAGCCNTTGCNACTGGTGGGGG

ACCTGNATATTGGGANNCATGCCAGGGNNCNAAGGGNTAANGGCTNTTATTTTAANT
NAANAGGGGAATGNCCCCNGGGCCNGTTTGGNTTTGACCCTNAATTTTTTTTTAAGG
GAGAA-3'

Clone He 2209 pGEM-T (SP6 ->)

5'-CAGGTTCCTAAAAAGGCATACATTCACATTGCAGAAACATGGTATTGGAATGCCGT
CTTTCTTCCAACATTTTACACTGTAAACAGGAAAAATTTGTTCAAAATTTCCAAGTCCA
TCAAGAAAGGCACAAAATGGCAACAACATGAAGCAATTATTTAGGTGCAACTACAAGG
TATATATATATTTTGGTAGGTTTGCAATTTGGTTAAGCAGTTTGTGGGGTTCTTTTGGC
CTTTCATAAACAAAGTTACAACTCTTCATTTTCAAATGCAAAAAAAGTA-3'

Clone He 2224 pGEM-T (T7 ->)

5'-ACNNGGGGAAACTTTCGCTTGGGATTCTAACTAGTCTTTAGCTACTGTGGATGTAA
TACTACTCTCCTTCTTCCGGACCTTTTGGTGGATATTTGAGAGGTCTCTCGCACCCAC
ATTTTTGTGGCTTACCCGGAAAATCATGACTCTGGAAGAAGTTCACGGACAAGAAACC
GTTGTTGAAAGCACTGACAGAATGCAAAGTGCTGGCAAATCCCTGCATGAGCTACTG
GTCTCTGCCCAGAGACAGGGAATGTCTGGACCGTTGGAGTCTATGAGTCTGCCAAAG
TTATGAATGTAGATCCAGACAGTGTTACATTCTGCATTTNAGCTGCTGATGAATACGA
CGAAGGGGATATAGCCCTTCAGATCNACTTNACTCTGATTCAAGCTTTCTGCTGTGAA
AATGGACATCGATATCGTGAGGCTGANCGACACAGAAAAGTCGCTCAAATACTAGGC
TTAACTGATGAGTCTGCAGAACCNAGGNCCTGCACTGATCCTTATTACGATCCAAATG
AGGTGCTGGNAAGANCAGCTTTGGANAACTNGNATTTTCNGGNGGAAAGCCGNAAG
GCTATGACTGGGTCCCATTTCCCCCGNCGNGGGAAATCTTTCCCNACGTAGGGTNG
GNAAACCNTGNATANTTTCATGGGGTTGGNCCCTGGATGNACCCCNCCNTCCGAAAT
CGGGGNATACCCCGGTTGGGGNCC-3'

Clone He 2239 pGEM-T (SP6 ->)

5'-ACCCTGCGGGGTAGATGCCTGTAGTTCCAAACCCTGCAAAAATGGTGGAACCTGC
TACACCCACTTTACTGGCAATGTATGCCAGTGCCCCACGGGTTTTATGGGACCAGTT
GTGAATTCAGAGTACATGATCCCACCCCAGCTTCCCATCGTGCTGACTCCTCAAACA
CTCTTACCATGGTGGTTTGTCTGGGACTGCTAACATTCTTTTTGCTGGGCTGTGGAGT
GTTCATGGTTATGAGGGGTATGAGGAGGGGAACTTCAATGAAAAGGGACNNGTCANT
AATGACCTTGANCCAAAGAATAACCTGATAGAAAGGAACCCCATTTCAAATGCCTAAT
CCAGACTATCTAAGAGAAAATCANGTNGCAAACAAAGCTCTGCANGNTCTGAAAGTA
AGGAGGAGAGAAGTGGGCCCAGAACTGACCGAAAGCCAGATCCAAACAGTGCANCC
CCCATCANGGTATCCAGAGNATGGCGCTTTNACCAATANATATCCTCCCAGACCTGA
GCAATGTATATTNNCACTGAGTCTAATCTTCCCANAACATGACTTAGACTATNATCNCT
GCATTNTAAGAACTNNANGCANAATTTTNCCCCTTANAATTAAAAANNTNCCCCCNCT
TATTNTTNGNAANTGNTTCTTATNTTTTTGAACNCNTTTTGGAAAATCNTTTNNTTTNG
CGNCCNNTTTTTTGNCCNCCANTTGGNCNGANAAAATCAATTATTTTT-3'

Clone He 2252 pGEM-T (T7 ->)

No insert

Clone He 2255 pGEM-T (T7 ->)

5'-ACAATAGAATGCTTTAAGAAAGAAGGTCAAAGCTTATAGATACTGAACAGAAAGCG
CAAACGTATCTATATTTTACAATATCTGTATCCTCAGTGAGGAGAAAACATATTCCTAA
ATTTGGGTTAGGGAAGCAAAACGGTGAACCTAGAATTGCTAGTTATGACTTATATTCT
ACTATGTGGTATACTATGTTCTTTAATTCCCAGCACCCTAACCATCCTAAAGGGTTGC
TGGGAGTTGTAGTTTCTTACAGTATTTGGATAATTGTTATCTCTGTGCTGTGANGACAT
TTGTGAATTTGTAATTCATTTCCTACAATGTATGCAGTTTTATTNGCACTTATTGGTATA
TTATATAGGATATATAAGTTATGCAATATTTTGCAAGTTATATTTGTGCCTGAATGTTAA
ATGTATGCCTTTTATTTAAATGCTGCAATGGATTTCAATAAATTGATTTTTTGNAAGNTC
AAAAAAAAAAAAAAAAAAAA-3'

Clone He 2274 pGEM-T (T7 ->)

5'-ACATTAGCGTCAGAACGCAAAGGCGAAACGATCCTGTATCGATATGAATATACTGT
CGCATGTTTTCTAAATGAGAATGTCATGTTGTTTCTATGAAATAATTGTCCCCGTTAGT
AAAGAATATCAGTTATGATAGATGTATTCTGTGATCAGAAATGGTGTTGCTCTGTGAC
CTGAATAAATACACGATTTGTGCCAAAAAAAAAAAAAAAAAAAAAGNNCCNTCCCGGG
NGGCCCNTCNAAATCCCTNGNGCCGCCCCCTCGGGGTCCCCCAANTNGGGGGANC
CCCCAACNNGGNGGGAGGCAANCCTNGGGNTT-3'

Clone He 2281 pGEM-T (SP6 ->)

5'-ACCGTCTAGTTATACTTCTGTGCCCTCTCTGTTAAAGAGAGTATTGTTTGTATTGAT
ACTGTCGTAGAAATGAGGAGTGAATTGGTTGAACACAGAACTGCTGTAGCCAAAAGG
ATTTGACGATACAGTAGATGACCAGTCAGACCCCCCATGGTTTGGCATATTTGAAAGA

GGAGTGTAGGGAACTGAAACCAACCATGTTCTGTCCCATCTTATCAGAGGTTTCATTA
CTGAGTCCAAGTGGAACCGACCTACTGACTGGGATTGCACTATTAACATATGCAGTC
ATGCTGGAGAGAAAGTTATTAAGGCAAGGGGCTGTAGATGGAGGAGGTGACATCTTT
TCAGGGGAGTCGTCTGTTCCTGGTGATGAATTTCCCAACATGTCCTGATGCTCTCCAT
TCTTAGGACTCTCACTTAGTGGACTCCCATCAGGTTTATCAGAAGATAGCTGTCCATT
TGGNCTGACGTCGGACTTTCTTTCCTCTTTCTGCGGAAGTTTCCATTGTCAAACATTT
TTTCAAAGTTNGGNNCNAGGNCCAAAAATTGCCCTTTCCTGGNTCATCTTCGNNCTTG
GA-3'

Clone He 2288 pGEM-T (SP6 ->)

5'-ACGCGGGGAACACATGCTACATTCTCTTCATACTGATCACATGCATTCTCCTGGAC
TTTGCCCATCTCAAGCTTCTCTTGGCACCAAGATGAGGAACTCCTACCTCAGCCGTG
AGGATAAGAGGCTAATGAAGCCTGTGATAGAGAAAAGAAGGAGAGACAGAATTAATC
AGAGCTTGGAACATCTCAGGACCCTTCTCATGGAGGCCACTCATGATGAGTCTTTAA
AAAAATCCCAAGGCTGAGAAGGCTGATATTCTGAAAAAGACAGTGCACTTTCTCAAAA
TGTGTCATAATCCAGTGCCTTCAGATAAAAAGAAATTGCAGTCAGGGTTTAAAGGAGG
CTTTCGGGAAGGACTCAACCAGGCCACTTCCTTCCTCAACTCTGCTGACAGTATCTG
TGAGAAGAAGAAGGAATACATGGTCCAGAGNTTGGNCCAGCACATGGAANAACAGA
CTCAGAAACACTGGAAGGACTCAGTTCANGATGTCTCCTTGANGTCAACNAGCGTCA
ANTCTTGCAAGTGCCCNATANTCTCAGGGTNGCGGAGATT-3'

Clone He 2311 pGEM-T (SP6 ->)

5'-ACTTCAGATGATGCAAATATTGCACTACTTGGGATATACTTCAAACCTAGAGGCTAT
ACTTGTATATTATTCCACAGGAATATACAGATTTGCTCATAAAGTGTTTCCAGCTGGCA
GTCCAAAGTGTCAGCTCACTGACTGTCTGACAAACTCTTCTTATTAACCCTTTCACCA
TGGTCTCCACAAAACTTTGCTGTTGGTGACAGGGTTGGTGAAAGGGGACTGTCCAGG
GAGCAGCTGAGACTGGTGGGTTTCTACTGAGGAACACTGATGACCATGGAAATGTTT
GAGCAGGTTTCCTTCACTGTGATGATTGCGAGCCGACAAGAAATGCAGCGACTCTTC
AACACATTTGGAATATCCCTCCTTGTAGGATTTGCCTGATATATCAGCAGATTTGGTA
ACTAATTGCTGCTGTAGGAAGCTCACTGCCACCTCCAGAATGTCAGCTTTCTCTGGTT
TGGATGGGAGATGATGTTTCTCAAACTCTTTCTNCCAGTGACATCCTGNGCTGCTCG
ATGCTGCTGTTAATCCGATCTCTCCTCATCTTTTCANCACTGGTTTNCCATCTTTCTNT
TGGNGCANCGGGCTGGGGCNAATGCTGCAGGCGCNTTTGNCNCAGNACTNNAGGN
NAG-3'

Clone He 2320 pGEM-T (SP6 ->)

5'-ACAGGGCACAGCTGATGGGTCTCTCCTGCGTTAAACTGATGACCATGGAAATGTTT
CAGCAGGCTTCCTTGATTGTGAACGGACAAGAAATGAAGCGAATCTTCAACACATTTG
GAGTATCCCTCCTTGTATGATTTGCCTGACATCTCACCAGTTTTAGTAGCTATGTGCT
GCTNAAGAANGCTGACTNCANTCAAAANTNTNTTTNTGTCNTGNTGGTGATNAAATAN
NTATCTAACTANCTTCTAANANAACNCGGGGNNNGNCGNGTNTTTN-3'

Clone He 2340 pGEM-T (SP6 ->)

5'-CTTCAAGCTGGAAAAAGCTGATATTTTGGAAATGACAGTGAGATTCCTAAGAGACA
TCCCTCCGGTCCAGGCACAAAATCAAGCAGATCGCTACAAGGAAGGCTACAGAGCAT
GTGTAGAGCGTCTGAGCGCAATCCTTGGTAAATCTCATGTGCTGACCGGAGAGGCG
AGTAACCGCCTTCTAGAATACCTGCAGAGGAGCCCTGAGCTGTGAGGTTCAGATTGT
AATCATCCTCCCAAGCCCCAACGTCCTAGGATTGTGCTCCAAGTGAGTCCCAGAACA
TCTCANGTTGGATCTCCTCTCCAGAACCAACCCTCCAGCCACAGACCTGCCCCATGT
CCCCCGCAGCTCAACAGCTCCATCTGGAGGCCGGGGTGAATGTCAGCTTGGACTTT
GT-3'

Clone He 2350 pGEM-T (SP6 ->)

5'-AGGGGACACTTCNATGAAAAGGGACGAGTCAATAATGACCTTGAACCAAAGAATAA
CCTGATAGAAAAGGAACCCCATTTCAAAATGTCTAATCCAGACTATCTAAGAGAAAAA
TCAAGTAGCAAACAAAAGCTTCTGCAAGGATCTGAAAGTGAGGAGGAGAGAAGTGGG
CGCAGAACTGACCGAAAGCCAGATACCAAACAGTGCAACCCCACATCAAGGTATCCA
GAGGATGGCGCTTATCACCCAATATATATTCTCCCAGAACCTGAGCAATGTATATTTG
CCGCTGAGGTCTAATCTTCTCCAGAAACATGACTTCAGACTATCATCACTGCAATTCT
AAGAACTGTAAAGCAGACATTTTCCCCTTTATTATTNAAAATATTCTCCCCTCTTATTTA
TTGACATATGNATCTTANATTATGAAACTTTTTNNAAAATCTATGTAATTGCTGCTNNAT
TTATGACTCCTATAGGGCGGTAAATCAATAATTCTTACTNTGATGATTTAGCTNNGCAA
TCCNCCAAATGTCTACTGCTTGTTTNATCTNTGACTNGGCNGACACCTATCCGGGCAT
GGGCGGAGCTGAGCGGCCATTGC-3'

Clone He 2369 pGEM-T (T7 ->)

5’-NCGACCTGNAGCCNTGGNTCCGCGNNNGTATGTCTATACGTCGCCACANTCTGTA
GACANTGCTACAGCTTCGGTGGGGCATTATTACCCCGTTATAAAACACCCCAGTGAT
ATGTGGTNTTTATTTATTGTAAATATGTCCCCNCNCATGAAATCCTGACTTACTAANGG
CAATATATTTTGGACAAATAGTCCTGAGTACAAAGGGCACGAGAGTGNCCCCANTTN
GNGANCCCANGGGGTTAANTCCTGGTATGCGGGTTTGGCNNTANTTTNACNTTANCC
CCTGGNCCTGGCCAGGNGNTGGCCAATNGCCNACNGGCCNCTGNCANGGGNTTGG
GCCTTTTCNTGGGCAGGNCCAGGGGGGTTTAATTGGCCCANAGGGCCTNTNGGNCN
NCNAATNAAAGGCCCGGGGCCNTGGGACCCCNGNTGNCCNGCCCCCTTTTTTTTTTT
T-3’

Clone He 2370 pGEM-T (SP6 ->)

5’-ACTCCAAGCTGGAAAAAGCTGATATTTTGGAAATGACAGTGAGATTCCTAAGAGAC
ATCCCTCCGGTCCAGGCACAAAATCAAGCAGATCGCTACAAGGAAGGCTACAGAGCA
TGTGTAGAGCGTCTGAGCGCAATCCTTGGTAAATCTCATGTGTTGACCGGAGAGGCG
AGTAACCGCCTTCTAGAATACCTGCAGAGGAGCCCTGAGCTGTGCAGTTCAGATTGT
AATCATCCTCCCAAGCCCCAACGTCCTAGGATTGTGCTCCAAGTGAGTCCCAGAACA
TCTCAGTTTGGATCTCCTCTCCAGAACCAACCCTCCAGCCACAG-3’

Clone He 2378 pGEM-T (SP6 ->)

5’-ACTTCAGATGATGCAAATATTGCACTACTTGGGATATACTTCAAACCTAGAGGCTAT
ACTTGTATATTATTCCACAGGAATATACAGATTTGCTCATAAAGTGTTTCCAGCTGGCA
GTCCAAAGTGTCAGTTCACTGACTGTCTGACAAACTCTTCTTATTAACCCTTTCACCAT
GGTCTCCACAAAACTTTGCTGTTGGTGACAGGGTTGGTCAAAGGGGACTGTCCAGG
GAGCAGCTGAGACTGGTGGGTTTCTACTGAGGAACTCTGATGACCATGGAAATGTTT
GAGCAGGTTTCCTTCACTGTGATGATTGCGAGCCGACAAGAAATGCAGCGATTCTTC
AACACATTTGGAATATCCCTCCTTGTAGGATTTGCCTGATATATCAGCAGATTTGGTA
ACTAATTGCTGCTGTAGGAAGCTCACTGCCACCTCCAGAATGTCAGCTTTCTCTGGTT
TGGATGGGNAGATGATGTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGA
TGCTGCNGGTAATCCGATCTCTCCTCATCTTTTCACCACTGGTTTTCTCATCCTTCTAT
TGGGGCTAGCGGCTGTGGGCNAAATGCNGGNANGTGCN-3’

Clone He 2379 pGEM-T (SP6 ->)

5’-ACGCGGGGTCAGTCACTGAGAGAAAAAACAAGGAGGAGTGCCTTGTGGTTACCTG
CATCAACAAGAAAAACCAAAGAAGACATGGACACAGAGTATGCAGTCAATACATCTAA
AACAACAGACACTTTATTGGATAAAGCAAAGGAGGAGACAACGACTGAAAATGTAAAA
TGTAAACAGGGAACAAATAATACCAGTTGGTCCGAATTCCCATTTTCCGATTTGCAGA
CTGGCTGGATATTCTCTTAATGTTCTTTGGAGTGCTGGGAGCAATGGGATGTGGTTC
CTGTTTCCCACTCATGAACGTTGTGTTTGGAGAAATGGCTAACAGCTTCCTGTGTCAC
AACTCATCTCTTCAGAATTCCTCACTGTGTGCAGAGTTTAANCCATAGANGACAGATA
CAAGTTTTCTCACTGTATTATGCAGGACTGGGTTTTGGAGCTTTAGTCTGTGGCTATC
TCCAGGNGCCTTCTGGGTTTAACTGCTNCAGACAGACGAGAAAATGAGAAAGGCTTT
CTTTCCTCTGTGTTGCTCAAGAANTNGTTGGTTTGNATCNCAAATTCTGGNAGCTAAC
AATCNACTTACTGAAGANTCATNAAANAATAANGGAAAGGGNAAAAGTTGGCATTGTT
TNNAAANANCAAACCTNTTNTTTGGCATTTTATCGGNTATCAAGGGGGGAANCTTCCT
TTTGGTTTG-3’

Clone He 2383 pGEM-T (SP6 ->)

5’-CATCTCCTGCAACTCTGGAGATTATTGGTGCACTTGGCAAGATTTGACGCTGGTTG
ACCTTCAAGGAGACATCTTGAACTGAGTCCTTCCAGTGTTTCTGAGTCTGTTCTTCCA
TGTGCTGGCACAACCTCTGGACCATGTATTCCTTCTTCTTCTCACAGATACTGTCAGC
AGAGTTGAGGAAGGAAGTGGCCTGGTTGAGTCCTTCCCGAAAGCCTCCTTTAAACCC
TGACTGCAATTTCTTTTTATCTGAAGGCACTGGATTATGACACATTTTGAGAAAGTGCA
CTGTCTTTTTCAGAATATCAGCCTTCTCAGCCTTGGGATTTTTTAAAGACTCATCATGA
GTGGCCTCCATGAGAAGGGTCCTGAGATGTTCCAAGCTCTGATTAATTCTGTCTCTC
CTTCTTTTCTCTATCACAGGCTTCATTAGCCTCTTATCCTCACGGCTGAGGTAGGAGT
TCCTCATCTTGGTGCCAAGAGAAGCTTGAGATGGGCAAAGTCNAGGAGAATGCATGT
GATCAGTATGAAGAGAATGTAGCATGTGTCCCCCN-3’

Clone He 2390 pGEM-T (SP6 ->)

5’-ACCNNTTATTANNNTANNTANAGTANTNTNNATATCTAGTANTCAANTCGACANTAC
ATAGNTNGANCACNNCGNNNNCNTCCTNTGAGGCTTGCTACTGTGTGCATCCAAANT
ATGNAATGAACACTACCNTTAGCGCTANAAGCGCAGTCGAAGGACATAAGGAAGTGA
CTAATTGGCNAGTCCTGGTGTAGCAGCAGANATAGTAACTAAGTAGTANCCTGCNCA
TCCNGANCCTTGCGACATCTCACGGCCNTTCNTNANGGAGNNCACTCANNATNGGG
CNNTATNNAATCNAAGGCNGAGCAGTCTGATATTCTGAAAAAGACAGTGCACTTTCTC

AAAATGTGNCATAATCCAGTGCCTTCAGATAAAAAGAATTGCAGTCAGGTTTAAAGGA
GCTTTCGGGAGGACTCAACCAGGCACTTCCTTCTCAACTCTGCTGAAGTATCTGTGA
GAAGAGAGGAATACATGGTCCAGAGGTTGTGCCAGCACATGGAANACAGACTCAGA
ACACTGGAGGNCTCAGTTCAAGATGTCTCCTTGAAGNCACCAGCGCAAATCTTGCAA
GTGCACCAATAATCTCANGGTTGCNGGNNATGTGCNTNCCCGGCNGCCGCTCGAAA
TCCCCGGCCATNGCGGCCGGNNNCATNCAANNTCNGGCCCNATTTNGCCNNNNGG
GGGNCNNNATTAANNNNN-3'

Clone He 2391 pGEM-T (SP6 ->)

5'-ACGCGGGGAGAAAGACTGGTGCTCTCAAAGTGTTTGTTCCTCTTGGATTATACACG
GCGCCCGATTTTCTCACACACCCAACCATGACTGTAAAGACAGAAACGGCGGCGGG
TNCAAGCACACTGACTTATTCTAAGATGAGGGGAATGGTGGGCTTTGCTTATCGCTTT
TATGAAACAACGAAGAATGGGACTGAATGATTTCATCCAGAAAATTGCAACCAACTCT
TCCTATGCCTGNAACCCTCTGAAGTGCAGTCAATCCTGAATATCTCACCCCCCCAAG
AACCAGAATTACTGAATGAAAATTCCTCGCCTCCCCCTAGCCCTTCTCAACAGATCAA
CCTTGGTCCTTCATCCAACCCTCATGCAAAACCATCCGACTTCCAGTTTCTGAAATCA
TTGGCAAGGGTAGTTTTGGAAAAGTTCTCCTGACAAGACACCNAGCAGATGAGAAAT
TCTATGCAGTGAAAGTGCTTCAGAAAAAGCATTCTCAAAAGNAAGAGGGGAACACAT
ANTGTCAGAGCCCATTGT-3'

Clone He 2392 pGEM-T (SP6 ->)

5'-ACAGGGCACAGCTGATGGGTCTCTCCTGCGTTAAACTGATGACCATGGAAATGTTT
CAGCAGGCTTCCTTGATTGTGAACGGACAAGANATGAGGCGAATCTTCAACACATTT
GGAGTATCCCTCCTTGTAGGATTTGCCTGACATCTCACCAGTTTTAGTAGCTATGTGC
TGCTNAAGAAGCTGACTGCCATTTCCAGAATGTCAGCTTTCTCTGGTTTGGATGGGA
GATGATTTTTCTCAAACTCTTTCTCCAGTAACATCCTGAGCTGCTCGATGCTGCTGTT
AATTCGATCTCTCCTCATCTTTTCAACCACTGGTTTTCTTATCTTTCTAATGCTGTTAGA
GGCTGCTGGCTATAGTGCAAGGTGCATTGCGCTCAGTTACTGTTCGGTGCTGCTGNG
CTTCAGNTGTTTCNTGTCTCCG-3'

Clone He 2393 pGEM-T (SP6 ->)

5'-ACACGCTGTGTATTCCCACACATTGCGGCATATTTGGGCCTTTCAGAGTCTGGATA
TTATGTGTAACACTACATTGCTAGTGATTTATTTTTATATTACCATAAAAAGTGCATCTT
TAAGATTTCCTACAATGTATGCAATATTTGCAGTATTTAACAGTATAATAACTGTTGCA
TTTTTAGTA-3'

Clone R 507 pT7T3D-PacI
BX849476
Clone R 1996 pBluescriptSK-
BX843055
Clone R 3306 pCMV-SPORT6
BX848643
Clone R 3988 pCMV-SPORT6
BX846316
Clone R 4300 pCMV-SPORT6
BX853014
Clone R 4644 pCMV-SPORT6
BX852254
Clone R 6322 pCMV-SPORT6
AA530237
Clone R 7008 pCMV-SPORT6
BI450200
Clone R 8228 pCMV-SPORT6
AI678656
Clone R 8584 pCMV-SPORT6
BX850877
Clone R 8617 pCMV-SPORT6
BC072958
Clone R 9314 pCMV-SPORT6
BX849201
Clone R 9328 pCMV-SPORT6
BQ400749
Clone R 9413 pCMV-SPORT6
BX843433
Clone R 10387 pCMV-SPORT6
BX844850
Clone R 10388 pCMV-SPORT6
BX842977
Clone R 14741 pBSRN3
BX849142
Clone R 14803 pBSRN3
BX844844
Clone R 14818 pBSRN3
BX855125
Clone R 14836 pBSRN3
BX847637
Clone R 14955 pT7T3D-PacI
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Publications:

Karabinos, A., Schulze, E., Klisch, T., Wang, J., and Weber, K. (2002). Expression profiles of the essential intermediate filament (IF) protein A2 and the IF protein C2 in the nematode Caenorhabditis elegans. *Mech Dev* 117, 311-4.

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