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The transcription factor hairy/E(spl)-related 2 induces proliferation of neural progenitors and regulates neurogenesis and gliogenesis

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ABSTRACT

The study of molecular regulation in neural development provides information to understand how diverse neural cells are generated. It also helps to establish therapeutic strategies for the treatment of neural degenerative disorders and brain tumors. The Hairy/E(spl) family members are potential targets of Notch signaling, which is fundamental to neural cell maintenance, cell fate decisions, and compartment boundary formation. In this study, we isolated a zebrafish homolog of *Hairy/E(spl)*, *her2*, and showed that this gene is expressed in neural progenitor cells and in the developing nervous system. The expression of *her2* required Notch activation, as revealed by a Notch-defective mutant and a chemical inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). The endogenous expression of Her2 was altered by both overexpression and morpholino-knockdown approaches, and the results demonstrated that Her2 was both necessary and sufficient to promote the proliferation of neural progenitors by inhibiting the transcription of the cell cycle inhibitors *cdkn1a*, *cdkn1ba*, and *cdkn1bb*. Her2 knockdown caused premature neuronal differentiation, which indicates that Her2 is essential for inhibiting neuronal differentiation. At a later stage of neural development, Her2 could induce glial differentiation. The overexpression of Her2 constructs lacking the bHLH or WRPW domain phenocopied the effect of the morpholino knockdown, demonstrating the essential function of these two domains and further confirming the knockdown specificity. In conclusion, our data reveal that Her2 promotes progenitor proliferation and maintains progenitor characteristics by inhibiting neuronal differentiation. Together, these two mechanisms ensure the proper development of the neural progenitor cell pool.
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Introduction

In the developing central nervous system, neural progenitor cells in the ventricular zone of the neural plate proliferate extensively. The early stages of neural development are characterized by symmetric cell division and are followed by asymmetric cell division. Each asymmetric cell division generates one new progenitor cell and one neuronal precursor to give rise to different types of neurons. This is followed by the formation of glial precursors and the differentiation of glial derivatives. The generation of these diverted neural cells requires numerous gene regulatory and signaling processes. As such, understanding this during development may provide important implications for the development of repair therapies for the treatment of nervous system injuries and tumors. Recent studies demonstrated that Hairy and Enhancer-of-split [*Hairy/E(spl)*] genes play critical roles in maintaining the undifferentiated state of neural progenitors in addition to regulating neurogenesis and gliogenesis. Hairy/E(spl) proteins are transcription factors belonging to the basic helix–loop–helix (bHLH) superfamily and are structurally related to the *Drosophila* Hairy and Enhancer-of-split proteins. Seven Hairy/E(spl) homologs have been identified in mammals (HES1–7), each of which contains a conserved bHLH domain that facilitates dimer formation (HLH region) and DNA binding (basic region). The carboxyl terminus contains another highly conserved motif, the Trp-Arg-Pro-Trp (WRPW) domain, which functions as a transcriptional repression

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domain by recruiting the co-repressors Groucho/TLE/Grg (Kageyama et al., 2008).

Hairy/E(spl) proteins are major downstream targets of Notch signaling, and they suppress the expression of proneural genes, such as *Neurogenin1* (*Neurog1*) and *Achaete–scute complex homolog 1* (*ASCL1*) (Louvi and Artavanis-Tsakonas, 2006). Alternatively, they form heterodimers with ASCL1 to inhibit transcriptional activity, resulting in the inhibition of neuronal differentiation. Indeed, neural progenitor cells deficient in HES1 and HES5 expression cause these cells to prematurely differentiate into neurons and become depleted without giving rise to the later-born cell types (Ohtsuka et al., 1999). Therefore, Hairy/E(spl) homologs inhibit neurogenesis and maintain neural stem/progenitor cells until later stages to ensure that sufficient numbers of cells with a full cell-type diversity are generated (Kageyama et al., 2007). Mouse HES1 and HES5 also regulate glial differentiation (Furukawa et al., 2000; Ohtsuka et al., 2001) and the choice of astrocyte *versus* oligodendrocyte differentiation in glial precursors (Wu et al., 2003). In zebrafish, more than 15 Hairy/E(spl) homologs have been identified and named Her [zebrafish homolog of Hairy/E(spl) related] (Sieger et al., 2004). Among these, Her3, Her4.1, Her5, Her8a, Her9, and Her11 have been shown to repress proneural or neuronal genes. Expression of *her3* and *her5* is repressed by Notch activation, whereas expression of *her4*, *her6*, and *her8a* is induced by Notch signaling; on the other hand, expression of *her9* is independent of Notch activation (Bae et al., 2005; Chung et al., 2011; Geling et al., 2004; Hans et al., 2004; Ninkovic et al., 2005; Takke and Campos-Ortega, 1999).

In the present study, we demonstrated that *her2* is expressed in neural progenitor cells and neural derivatives and is regulated by Notch signaling. We altered the endogenous expression of Her2 using overexpression and morpholino-knockdown approaches and showed that the gene regulates the proliferation of neural progenitors and differentiation of neuronal and glial cells. The effect of the morpholino knockdown was phenocopied by the overexpression of dominant-negative *her2* constructs. Our data identified Her2 as a critical transcription factor in the formation of neural progenitors and neural derivatives. In addition, our data provide further insights into the roles of Hairy/E(Spl) homologs in neural development and their response to Notch signaling.

Materials and methods

Ethics statement

All experiments were performed in strict accordance to standard guidelines for zebrafish work and approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: CGU08-86 and CGU11-118).

Fish maintenance and mutants

Tü (wild type) zebrafish embryos were purchased from the Zebrafish International Resource Center (ZIRC, Oregon, USA) and were raised, maintained, and paired under standard conditions. The embryos were staged according to the number of somites, hours post fertilization (hpf), and days post fertilization (dpf) (Kimmel et al., 1995).

Sequence comparisons

Amino acid sequences were aligned and displayed using the Vector NTI (Invitrogen). The GenBank accession numbers of the compared proteins are as follows: zebrafish Her2 (XM_005155921), Her4.1 (NM_001161408), Her4.2 (NM_131090), Her12 (NM_205619), Her15.1 (NM_182875), mouse HES5 (NM_010419.4), and human HES5 (NM_001010926).

Construct generation

The open reading frame of zebrafish *her2* was PCR amplified with high-fidelity Pfu polymerase (Fermentas) and primers (5'–CGGGATCCCGGCCACCATGGCACCAACTGTCTGCAAAG-3' and 5'–GCTCTAGAGCCCAGGGTCTCCACAATCCAT-3'), which were designed according to the zebrafish genome database (www.ensembl.org; reference number ENSDARG00000038205). For the rescue experiment, a deletion construct, in which the first 75 nucleotides after the start codon were deleted, was amplified by primers (5'–CGGGATCCCGGCCACCATGGCACCAACTGTCTGCAAAG-3' and 5'–GCTCTAGAGCCCAGGGTCTCCACAATCCAT-3') to avoid the possible binding of *her2* morpholinos. $her2^{\Delta bHLH}$ was created with primers (5'–CGGGATCCCGGCCACCATGCATGCGCGGAGCTACTCGG-3' and 5'–GCTCTAGAGCCCAGGGTCTCCACAATCCAT-3'), whereas $her2^{\Delta WRPW}$ was made by primers (5'–CGGGATCCCGGCCACCATGGCACCAACTGTCTGCAAAG-3' and 5'–GCTCTAGAGCCAATCCATGCTTGGCGATTTCTG-3'). *her2* MO1 and MO2 binding sequences were inserted upstream of an enhanced green fluorescent protein reporter in the pCS2 vector to create a *5'her2-EGFP* construct to evaluate the specificity and efficiency of morpholinos.

RNA and morpholino injection

Capped RNA encoding the full coding sequence of *her2*, $her2^{\Delta bHLH}$, and $her2^{\Delta WRPW}$ were prepared as described previously (Chung et al., 2011). Antisense morpholino oligonucleotides were purchased from Gene Tools, LLC (Oregon, USA). Two morpholinos against *her2* were used: MO1 (TGCCATTGCTGCTGTGTTTATGTGC) and MO2 (CTGGAAAGAGAAGGTAAAAGTTTGG). Blast analysis revealed a homology of less than 20-bp identity for MO1 or MO2 to other genomic sequences, none of which corresponded to 5' UTR or exon-intron splicing site of predicted or characterized genes, suggesting that MO1 and MO2 act specifically on *her2*. A control morpholino designed to a random sequence of nucleotides not found in the zebrafish genome (5'–CCTCTTACCTCAGTTACAATTTATA-3'; Gene Tools) and a morpholino with five bases mismatch to MO2 (5'-CTaGAAAaAGAAGaTAAAAaTTTaG-3'; mismatched bases are indicated by small letters) were injected in an equal amount of MO1 as a control experiment. All injections were performed at the one- to two-cell stage, and cRNAs or morpholinos were introduced into blastomeres. For rescue experiments, embryos were first injected with the *her2* morpholino and then with the *her2* cRNA using a separate needle to ensure that the morpholino was effective.

Chemical treatment

Notch signaling was inactivated by DAPT (EMD Millipore) treatment. DAPT was dissolved in dimethyl sulfoxide to prepare a 10-mM stock solution. The stock solution was added to the embryos in order to reach a final concentration of 100 μM DAPT. The control embryos were treated with an identical amount of dimethyl sulfoxide (DMSO).

Histological analysis

Digoxigenin-UTP labeled riboprobes were synthesized according to manufacturer's instructions (Roche), and *in situ* hybridizations were performed as described previously (Cheng et al., 2012). The color reaction was carried out using NBT/BCIP substrate (Roche). For immunohistochemistry, the embryos were blocked in 5% goat serum and incubated with rabbit phosphohistone H3 antibody or rabbit monoclonal anti-active caspase-3 (1:200, Abcam). Goat anti-mouse IgG HRP or goat anti-rabbit IgG HRP

(Roche) was used to detect the primary antibodies, and DAB was used as a substrate for secondary antibody-conjugated HRP (Amresco). Embryos were mounted with Vectashield mounting medium (Vector Laboratories, Inc.).

Chromatin immunoprecipitation (ChIP) and PCR

The full coding sequence of *her2* was inserted into a pCS2-MT vector that contained six repeats of Myc-tag. Zebrafish embryos at the one-cell stage were injected with *her2-myc* cRNA for the ChIP analysis. ChIP was performed on 75% epiboly zebrafish embryos according to previously described methods (Lindeman et al., 2009) with an anti-Myc tag antibody (EMD Millipore). In general, the embryos were manually dechorionated and fixed with formaldehyde. The unreacted formaldehyde was quenched by glycine, and the chromatin was isolated and precipitated by a Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (EMD Millipore). The isolated chromatin was sonicated to an average size of about 300 bp. Protein A/G magnetic beads were then added to the chromatin solution and incubated with an antibody against Myc at 4 °C overnight (2.5 μg of antibody for each immunoprecipitation experiment). In each ChIP experiment, a portion of the chromatin solution corresponding to 1% of that used in the ChIP reaction was used as an input DNA control. The protein A/G bead-antibody/chromatin complex was pelleted with a magnetic separator, and the Protein/DNA Complexes were recovered by Elution buffer. DNA was purified with a polypropylene spin column after immunoprecipitation. Immunoprecipitated DNA and input DNA were used as templates for PCR amplification, and the primers are listed in Supplementary Table 1.

Quantitative analysis

For quantitative real-time PCR (qPCR), embryos were homogenized in TRIzol reagent (Invitrogen), and total RNA was extracted using a standard method. cDNA was synthesized from total RNA with random hexamer priming using RevertAid First Strand cDNA Synthesis Kit (Fermentas). qPCR was performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems) with SYBR green fluorescent label (Fermentas). Primers for *neurogenein1* (F: 5'–CGCACACGGATGATGAAGACTCGCG-3';

R: 5'–CGGTTCTTCTTCACGACGTGCACAGTGG-3'), *deltaA* (F: 5'–ACCGGGTGAAGCTTGTGAAC-3'; R: 5'–CGTCATGCYCGTCCAGAAGTT-3'), *elavl3* (F: 5'–ACTGAGGAGTGGTATCGCTCAAA-3'; R: 5'–AGACCCACGGAGAGATTCCA-3'), *cdkn1a* (F: 5'–GGTGTTCCTCAGCTCCTGTTT-3'; R: 5'–CGGCCCATTACCGAGTGAAC-3'), *cdkn1ba* (F: 5'–AAAAGTGCGCGTCTCCAATG-3'; R: 5'–CCGTACATCCTTGGCGAACT-3'), *cdkn1bb* (F: 5'–CGGGAATCACGACTGTAGGG-3'; R: 5'–GGGTGTCGGACTCAATGGTT-3'), and *gapdh* (F: 5'–ACCCGTGCTGCTTTCTTGAC-3'; R: 5'–GACCAGTTTGCCGCCTTCT-3') were used. Gene expression levels were normalized to *gapdh* and assessed using the comparative CT (40 cycles) according to the manufacturer's instructions (Applied Biosystems).

Statistical analysis was performed by Student's t-test using Microsoft Excel® 2007. The significance level was set at $P < 0.05$. All reactions were performed in triplicate for each sample.

Results

Characterization of zebrafish her2

A 399-bp sequence encoding a 133-residue peptide of the *her2* open reading frame was identified on the zebrafish genome database and cloned by PCR. The amino-acid sequence of Her2 shows the structural features of other Hairy/E(spl) family members, particularly a bHLH domain and a carboxy-terminal WRPW motif. These similarities imply that Her2 may act as a transcriptional repressor (Supplemental Fig. 1). The most similar paralogs of the zebrafish Hairy/E(spl) subfamily are Her4.1, Her4.2, Her 12, and Her15, which belong to the Hes5 subgroup (36%, 36%, 43%, and 42% identity to Her2, respectively) (Chung et al., 2011). Compared with other orthologs of the Hairy/E(spl) family, Her2 showed the highest degree of similarity to mouse and human HES5, with 50% identical amino acids. Within the bHLH domain, the identity increases to 71%.

her2 is expressed in the developing nervous system

The expression of *her2* was analyzed by whole-mount *in situ* hybridization. Transcripts first appeared in the developing nervous system at the 75% epiboly stage in the neuroectoderm (Fig. 1B). The expression of *her2* in the neuroectoderm was further analyzed by counterstaining with the neural progenitor marker *sox2*. This demonstrated their overlapping expression (Fig. 1C D) and indicated that *her2* was expressed in neural progenitors. At the bud stage, a strong expression of *her2* was detected in the primordium of brain and proneuronal domains representing neuronal precursors and the midbrain-hindbrain boundary (Fig. 1E and Supplemental Fig. 2). From the midsegmentation stages, the cells with different levels of expression of *her2* spanned the entire central nervous system (Fig. 1F I). At the 8-somite stage (13hpf), strong *her2* expression was retained in the dorsal telencephalon, ventral midbrain, hindbrain, and spinal cord (Fig. 2F G). In the hindbrain, *her2* expression was observed in all of the rhombomeres at different levels (Fig. 1F G). At the 18-somite stage (18 hpf), *her2* was expressed in the entire forebrain, with relatively weak expression retained in the midbrain and segmented expression in the hindbrain (Fig. 1H I). At 24 hpf, strong *her2* signals were detected in many structures in the telencephalon (see figure legend for details), and the expression in the hindbrain was now widespread without a clear segmented pattern. Strong signals were also observed in the developing cerebellum at this stage (Fig. 1J K). By the late pharyngula stages (48 hpf), the expression of *her2* was weak in the spinal cord and was mainly restricted to the brain, notably in the tectum and dorsal hindbrain (Fig. 1L M). In addition to the nervous system, the expression of *her2* was detected in the presomitic mesoderm from the bud stage to the 8-somite stage (Fig. 1E, G), suggesting a role in somitogenesis. The dynamic expression of *her2* in neural tissues suggests its importance in central nervous system development.

her2 is regulated by the delta–notch signaling pathway

In the developing nervous system, *Hairy/E(Spl)* homologs can suppress or promote neurogenesis acting in either a Notch-dependent or Notch-independent manner (Fischer and Gessler, 2007; Kageyama et al., 2007). To examine the response of *her2* to Notch signaling, we analyzed its expression in *mind bomb* mutant embryos (mib^{ta52b}) that have a strong deficiency in the Notch pathway (Jiang et al., 1996) due to a mutation of a ubiquitin ligase required for Delta ligand activity (Itoh et al., 2003). Compared with wild-type embryos, *her2* was significantly downregulated in mib^{ta52b} embryos (Fig. 2A), suggesting that the expression of *her2* requires the activation of Delta–Notch signaling. To further examine whether the loss of *her2* expression in late stages was not a consequential phenotype caused by defective Notch signaling at earlier stages, we used the γ-secretase inhibitor, DAPT, which has been shown to efficiently block Notch signaling at different time points (Geling et al., 2002). Embryos were treated with DAPT at the shield stage (6 hpf) and harvested at 75% epiboly (8 hpf), at 8 hpf and harvested at the bud stage (10 hpf), or at 10 hpf and harvested at 24 hpf. The embryos analyzed at 75% and the bud-stage exhibited a downregulation of *her2* expression in a level similar to the result obtained from mib^{ta52b} mutant (Fig. 2B). In contrast, DAPT treatment at 10 hpf with embryos harvested at 24 hpf only slightly reduced

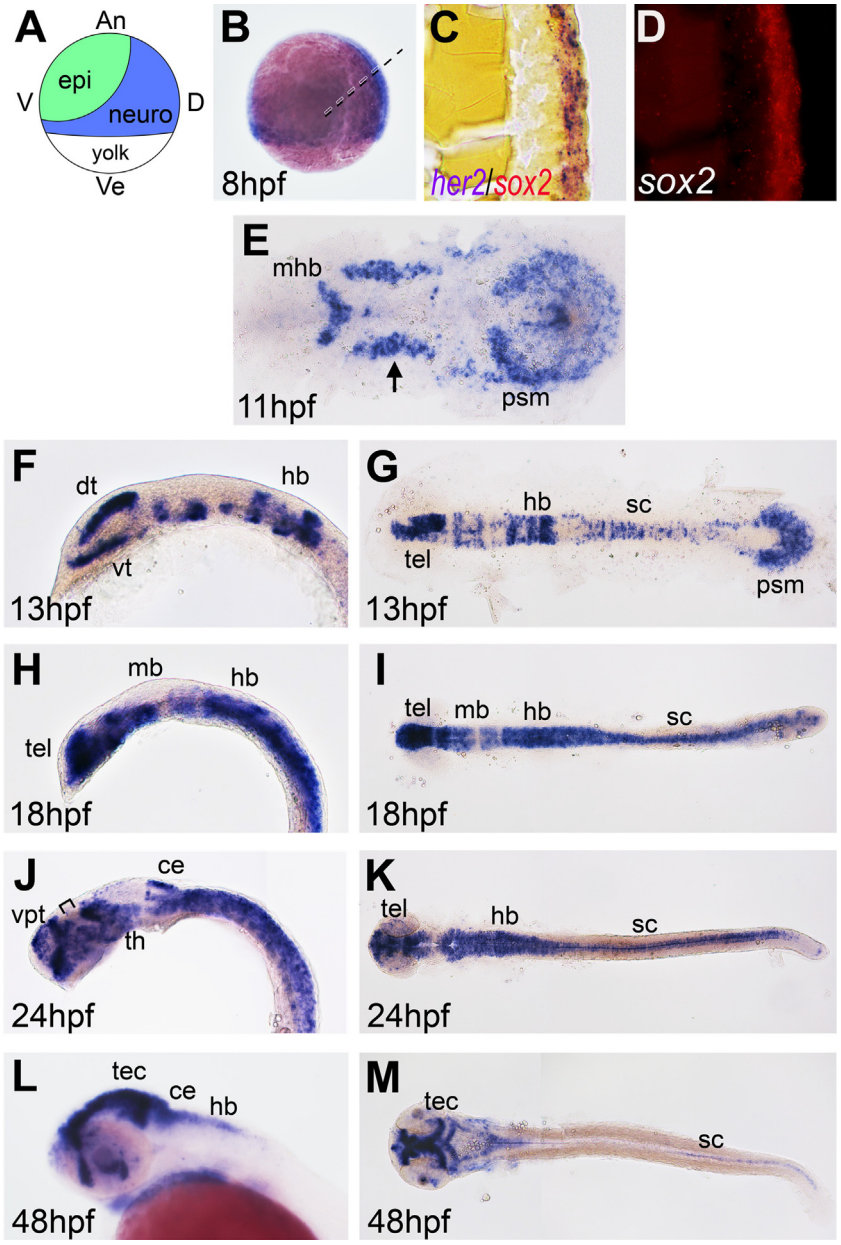

Fig. 1. The expression of *her2* in developing zebrafish embryos. (A) Schematic illustration of a gastrula stage embryo in lateral view. Prospective epidermis (epi) is green; prospective nervous system (neuro) is blue. an, animal pole; D, dorsal; V, ventral; ve, vegetal pole. (B-M) *In situ* hybridization analysis of the expression of *her2* in the developing nervous system. (C-D) A coronal section of the *her2*-stained neural ectodermal region (as marked in B) with the double *in situ* hybridization for *her2* (purple) and *sox2* (red) and that shows overlapping expression. The expression of *her2* was detected by NBT/BCIP and *sox2* was detected by Fast Red. The stages of the embryos are shown in the bottom left corner of each panel. (B, E, G, I, K, and M) Dorsal views, with anterior to the left. (F, H, J, and L) Lateral view, anterior to the left. Arrows in E indicate *her2* expressing cells in the proneuronal domains. The square bracket in J indicates the zona limitans intrathalamica (zli). Abbreviations: ce, cerebellum; dt, dorsal telencephalon; hb, hindbrain; mb, midbrain; mhb, midbrain-hindbrain boundary; psm, presomitic mesoderm; sc, spinal cord; tec, tectum; tel, telencephalon; th, thalamus; vt, ventral telencephalon; vpt, ventral-posterior telencephalon.

her2 expression compared to that in the *mib*^{ta52b} mutants (Fig. 2B). This result demonstrated that Notch signaling was essential for *her2* expression during early neural development but that the regulation had lesser effects at later stages (see Discussion).

Her2 knockdown causes developmental abnormalities

To delineate the role of Her2 during embryonic development, the morpholino-knockdown approach was used to decrease the expression of Her2. To block protein production, two 25-bp antisense morpholinos (MO1 and MO2) were synthesized to target different regions located adjacent to the translation start site of *her2* mRNA. The specificity of morpholinos was confirmed by rescue experiments in which the morpholinos were co-injected with cRNA for *her2*, as described for each experiment below. To confirm the efficacy of the morpholino-knockdown approach, each of the two *her2* morpholinos was co-injected with cRNA of a reporter construct that contained the *her2* MO1 and MO2 binding sequences upstream of an enhanced green fluorescent protein reporter (5′*her2-EGFP*). Effective knockdown, as revealed by the loss of the EGFP protein, was observed upon co-injection with either of the two *her2* morpholinos, whereas no reduction in EGFP protein expression was observed upon co-injection with a control morpholino (Fig. 3A).

Embryos injected with MO1 or MO2 were analyzed for morphological defects at 24 hpf and two days post-fertilization (dpf). Injection of 4.3 ng of MO1 or 2.16 ng of MO2 resulted in identical

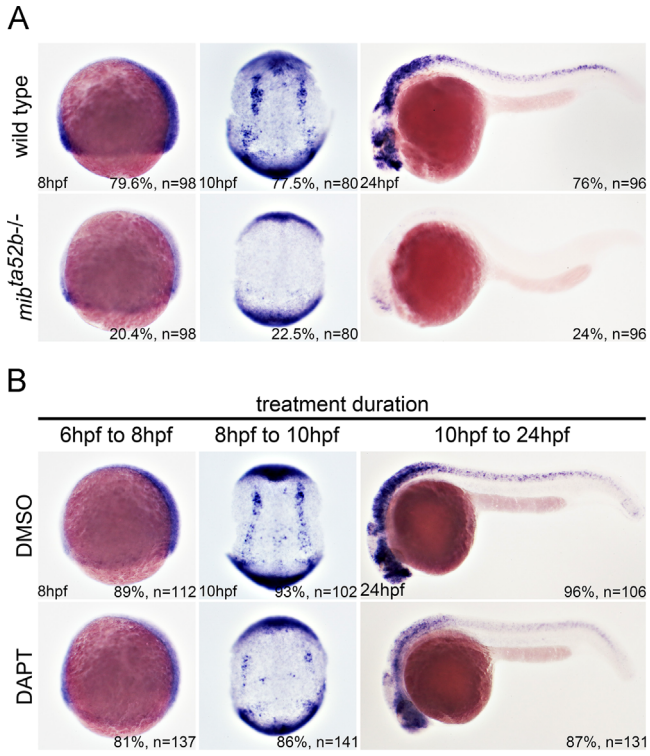

Fig. 2. The expression of *her2* is lost in Notch-deficient embryos. (A) The expression of *her2* was analyzed in *mib*^{ta52b} homozygous mutants and wild-type siblings by *in situ* hybridization. The stages are shown in the bottom left corner. Left and right panels are lateral views, and the middle panels are dorsal views. The arrows indicate *her2* expression in the neural progenitor cells. (B) DAPT treatment was performed at different time points, and the embryos were harvested at different stages as indicated, showing that DAPT treatment caused a downregulation of *her2* expression.

phenotypes; therefore, only embryos injected with 2.16 ng of MO2 are shown (see Supplementary Fig. 2 for the phenotypes of both morpholinos). Her2 knockdown resulted in severe defects, including brain malformations, such as an irregularly folded structure and enlarged third and fourth brain ventricles, a bent trunk, and edema in the pericardial chamber and fourth ventricle. Notably, the morphology of the midbrain-hindbrain boundary was not affected (Fig. 3B). The phenotypes caused by morpholino injection were rescued by concomitant injection of *her2* mRNA, indicating that the morpholino-induced defect was specifically due to the loss of Her2 function (Fig. 3B).

Her2 induces the proliferation of neural progenitors

We first analyzed the role of Her2 in neural progenitor cells. A previous study has demonstrated that *Sox2* is expressed in neural stem/progenitor cells where it maintains the stemness identity and inhibits neurogenesis (Lefebvre et al., 2007). We found that the expression of *sox2* was significantly downregulated following Her2 knockdown (Fig. 4A). To confirm this, we used *sox3* as an additional neural progenitor marker (Okuda et al., 2006; Shih et al., 2010) and found that the expression of *sox3* also decreased in Her2 morphants (Fig. 4A). These results were confirmed by quantitative real-time PCR (qPCR), which revealed a 0.5- and 0.6-fold decrease in *sox2* and *sox3*, respectively (Fig. 4C). We further analyzed the proliferating progenitors using a phosphohistone H3 antibody and *sox2* counterstaining. The result revealed significantly decreased neural progenitor proliferation (Fig. 4E, G). Co-injection with *her2* cRNA attenuated the morpholino effects (Fig. 4), indicating that the neural defects in Her2 morphants were due to the specific inhibition of Her2 function. These results demonstrated that Her2 is required for the proliferation of neural progenitors.

The loss of *sox2*- or *sox3*-positive neural progenitors could be explained by either decreased proliferation or induced apoptosis. Therefore, we examined cell apoptosis using immunohistochemistry to detect the presence of activated caspase-3. No significant differences were observed between *her2* morphants and controls (data not shown), suggesting that Her2 does not participate in neural progenitor apoptosis.

Previous studies have shown that morpholinos can cause off-target apoptosis mediated by p53 activation (Robu et al., 2007). To rule out this possibility, all the *her2* MOs were co-injected with a *tp53* MO. The results revealed no significant differences between the phenotypes of embryos co-injected with *her2* and *tp53* MOs and those injected with *her2* MO alone (Supplemental Fig. 3). Because the phenotypes resulting from *her2* morpholino injection were also rescued by concomitant injection of *her2* cRNA (as noted above), the phenotypes of the *her2* morphants did not result from p53 activation but rather resulted from the specific inhibition of Her2 function.

We next examined whether Her2 is sufficient to induce neural progenitor proliferation. The overexpression of *her2* cRNA in zebrafish embryos resulted in increased expression of *sox2* and *sox3* (Fig. 4B, D). Counterstaining of *sox2* with phosphohistone H3 revealed an increased number of proliferating neural progenitors in embryos injected with *her2* (Fig. 4F, H). This observation was further confirmed by counting the proliferating cells in the *sox2*-positive and *sox2*-negative populations, which showed that disrupted *her2* expression did not cause significant effects on the proliferation of non-neural ectodermal cells (Fig. 4G, H). This result suggested that Her2 is sufficient to induce the proliferation of neural progenitors.

Previous studies have shown that mouse HES1 represses the transcription of the cell cycle inhibitors *Cdkn1a* (encoding p21^{cip1}) (Castella et al., 2000) and *Cdkn1b* (encoding p27^{Kip1}) (Murata et al., 2005). Therefore, we analyzed the expression of these cell cycle regulators to examine the mechanism by which Her2 induced neural progenitor proliferation. Two Cdkn1b paralogs, named *cdkn1ba* (previously named *cdkn1bl*) and *cdkn1bb* (previously named *cdkn1b*), exist in the zebrafish genome. The results of qPCR analyses demonstrated that the expression of *cdkn1a*, *cdkn1ba*, and *cdkn1bb* were downregulated by *her2* cRNA injections. In contrast, injection of *her2* morpholino caused upregulation of *cdkn1a*, *cdkn1ba*, and *cdkn1bb* expression (Fig. 5A). This result indicated that Her2 regulates cell proliferation by inhibiting the transcription of these cell cycle inhibitors and consequently promotes cell cycle progression.

We performed a ChIP analysis in order to confirm the direct binding of Her2 to the promoters of *cdkn1a*, *cdkn1ba*, and *cdkn1bb*. We tagged Her2 with a Myc-tag and used an antibody against Myc in the ChIP assays on extracts from 75% epiboly embryos. Following the injections of *her2-myc* cRNA into fertilized eggs, we detected high Myc levels (data not shown), and the chromatin fragments that were isolated from these embryos were immunoprecipitated with an antibody against the Myc-tag. Previous studies have shown that mouse HES1 directly binds to specific DNA sequences called N-boxes, which are also known as class C sites, in the promoter regions of target genes (Sasai et al., 1992; Takebayashi et al., 1994). Through direct sequence comparison, we identified several N-boxes in the promoters of *cdkn1a*, *cdkn1ba*, and *cdkn1bb*, and designed PCR primers accordingly (Fig. 5B) to investigate if the fragments from the ChIP were selectively amplified. The results revealed that Her2 bound to numerous N-boxes in the promoters of *cdkn1a*, *cdkn1ba*, and *cdkn1bb* (Fig. 5C). Taken together, the results demonstrated that Her2 repressed the transcription of *cdkn1a*, *cdkn1ba*, and *cdkn1bb* by directly binding to the N-boxes of the promoter regions.

We further examined whether Her2 regulated neural patterning. Although altering Her2 expression affected the proliferation of

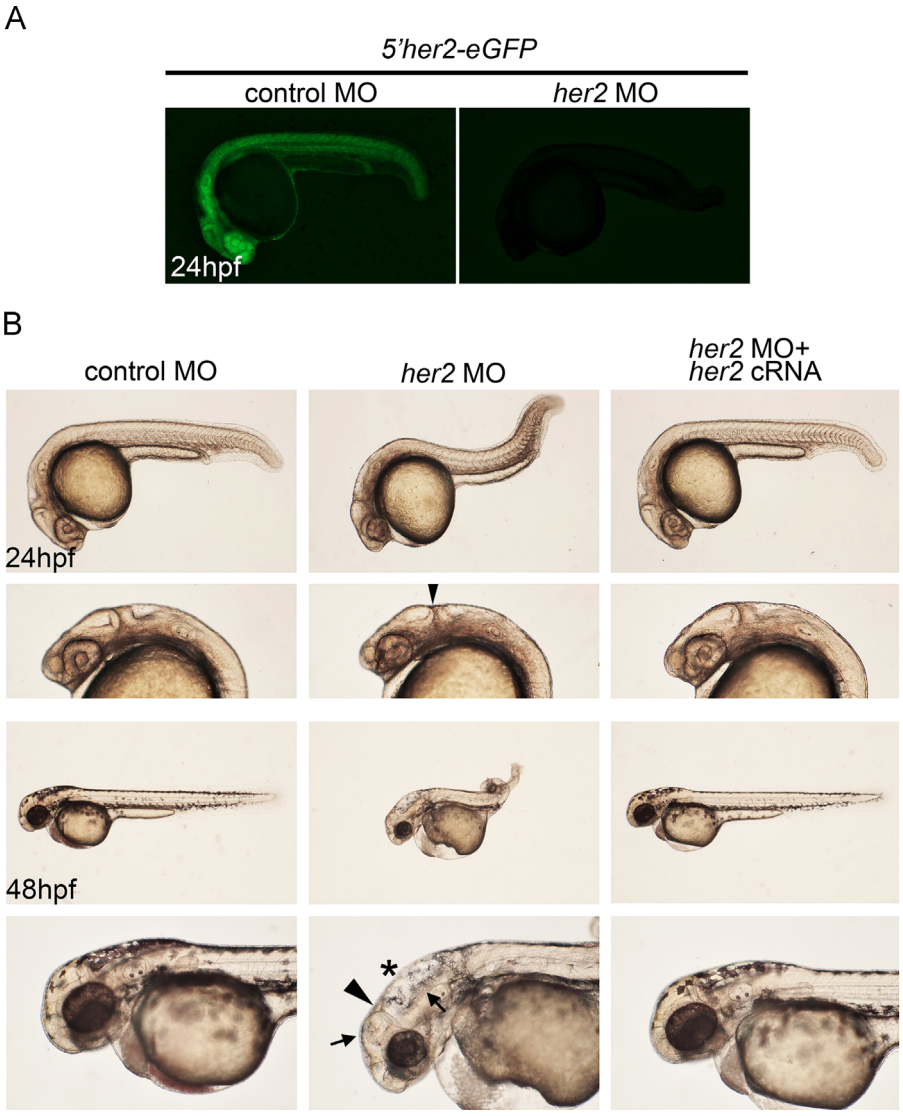

Fig. 3. Knockdown of Her2 causes developmental abnormalities. (A) Embryos co-injected with *5′her2-EGFP* cRNA and control morpholino showed strong expression of EGFP (left panel). In contrast, the EGFP signal was abolished in embryos co-injected with *5′her2-EGFP* cRNA and *her2* MO1 (right panel). (B) Representative images of morpholino-injected embryos. The asterisks denote edema in the fourth ventricle. Brain malformation is marked by arrows. These morphological phenotypes can be rescued by concomitant injection with 2 ng *her2* cRNA. The bottom panels are the enlarged brain regions of the upper panels. Note that the structure of the midbrain-hindbrain boundary was unaffected by the *her2* morpholino (arrowheads).

sox2-positive cells, altering Her2 expression did not show a significant disruption in the size of *sox2*-expressing domains (Fig. 4I, J). We examined the patterning markers *otx2* and *zic2b* (Okuda et al., 2010) and found that *otx2* and *zic2b* expression was downregulated by *her2* MO and upregulated by *her2* cRNA injections; notably, the size of the *otx2* or *zic2b* expression domain was not significantly altered (Supplementary Fig. 5). These results, taken together with the results that Her2 induced *sox2* expression as shown above, suggested that Her2 regulated the formation of neural progenitors without affecting neural patterning. We confirmed that Her2 did not regulate neural *versus* epidermal fate decisions by analyzing the surface-ectodermal marker *tp63* and showed that altering *her2* expression had no significant effects on the expression of *tp63* (Supplementary Fig. 5).

Her2 is required for the inhibition of neuronal differentiation

After the formation of neural progenitors, the next step in neurogenesis is the specification of neuronal precursors within the neurogenic region, which is regulated by proneural genes. The expression of *her2* in the proneuronal domain prompted us to examine the role of Her2 in neuronal precursors. We found that the expression of the proneural markers *neurog1*, *deltaA*, *ascl1a*, and *ascl1b* was dramatically downregulated in the morpholino-injected embryos. We used *in situ* hybridization and qPCR and determined that this phenotype was rescued by the concomitant injection of *her2* cRNA (Fig. 6A, B).

The decreased expression of these neuronal precursors could be a result of the Her2 knockdown-mediated loss of *sox2*- or *sox3*-positive neural progenitors (as described above). However, because many Hairy/E(Spl) proteins act as transcriptional repressors of genes responsible for neurogenesis, the loss of proneural gene expression could also be due to premature differentiation of neurons caused by the loss of Her2-mediated inhibition of neuronal differentiation. Accordingly, we analyzed the effect of Her2 knockdown using a post-mitotic neuronal marker, *elavl3* (encoding HuC) (Takamiya and Campos-Ortega, 2006). *elavl3* transcripts were not detectable at the bud stage in wild-type embryos and were detected only in the 3-somite stage, reflecting the beginning stage of neuronal differentiation. In contrast, a significant amount

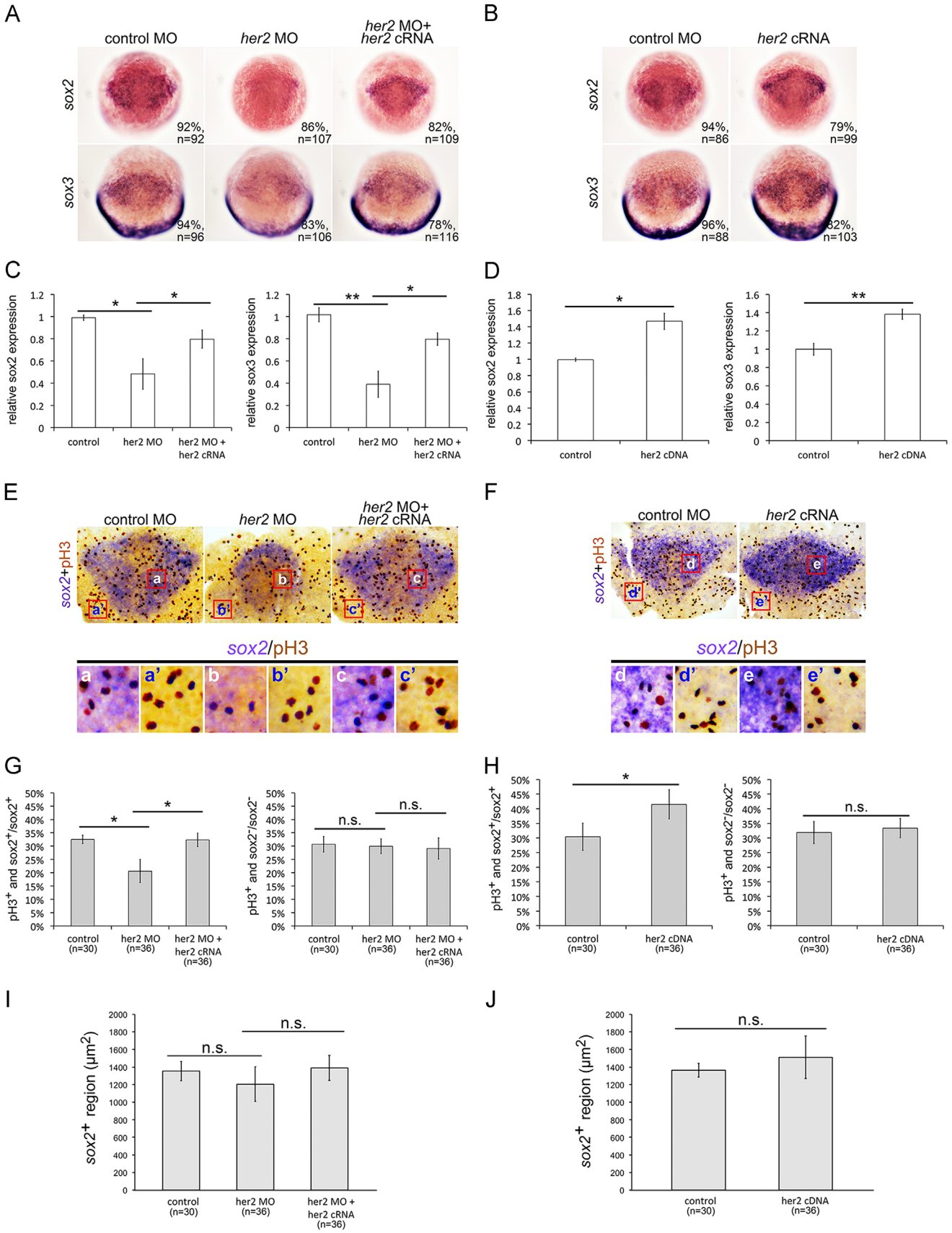

Fig. 4. Her2 regulates neural progenitor proliferation. (A) The expression of neural progenitor markers *sox2* and *sox3* was downregulated in Her2-knockdown embryos and was rescued by co-injection with *her2* cRNA. (B) *her2* induced the expression of *sox2* and *sox3*. (C and D) The results of *in situ* hybridization in A and B were quantitatively confirmed by qPCR analysis, respectively. (E, F) Proliferation of neural progenitors was detected by *in situ* hybridization using a *sox2* riboprobe (purple) and counterstaining with phosphohistone H3 antibody (brown). The bottom panels are representative of the enlarged regions of the upper panels, as indicated. (G, H) The proportions of the phosphohistone H3- and *sox2*-positive cells among the total *sox2*-positive cells were quantified (left panels in G and H), showing that Her2 knockdown resulted in the decreased proliferation of *sox2*-positve neurons (left panel in G) and that *her2* cRNA induced the proliferation of *sox2*-positive neurons (left panel in H). The right panels in G and H show the quantification of the proportions of phosphohistone H3-positive and *sox2*-negative cells in *sox2*-negative cells that were counted in the adjacent surface ectoderm, which showed no significant deviation in Her2-distorted embryos. (I, J) The *sox2*-positive region was quantified by ImageJ. All panels show embryos at 75% epiboly. pH3, phosphohistone H3; *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

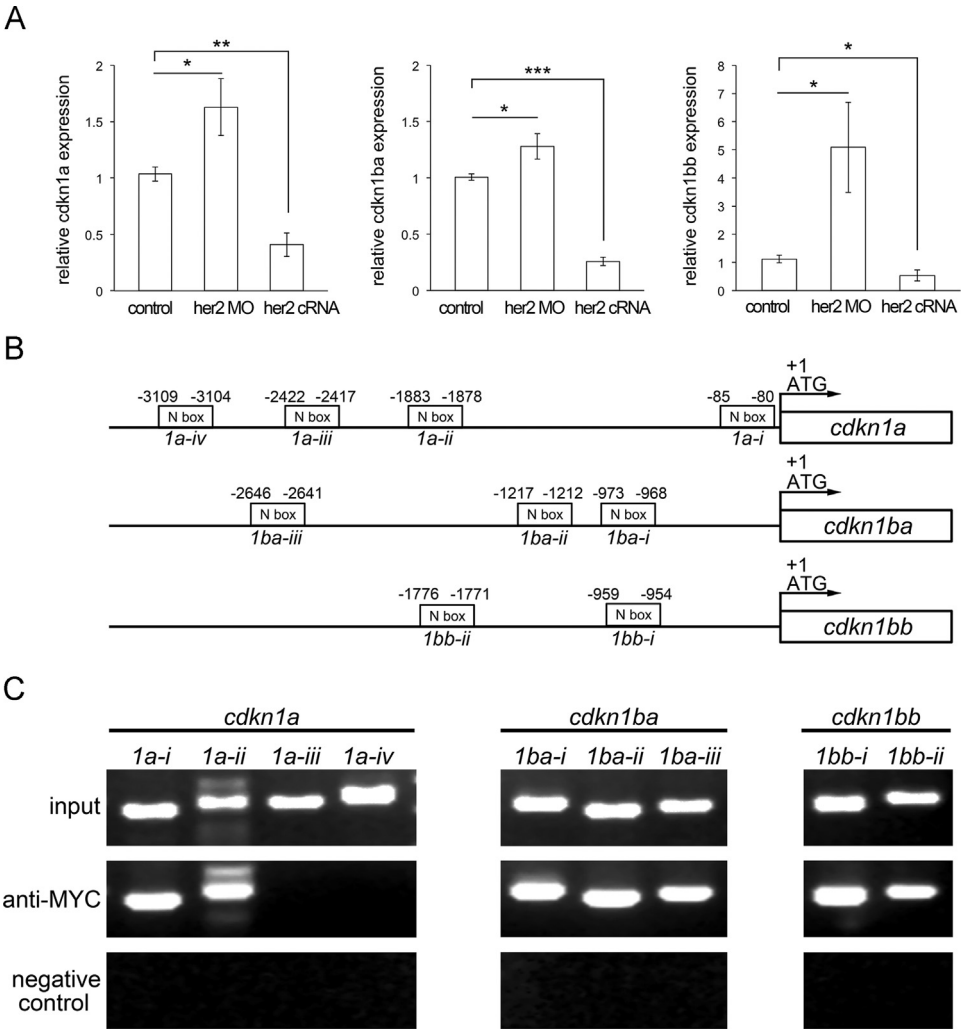

Fig. 5. Her2 inhibits the transcription of *cdkn1a, cdkn1ba, and cdkn1bb* by direct binding to the promoter region. (A) qPCR quantification showing that injection of *her2* cRNA downregulates the expression of *cdkn1a*, *cdkn1ba*, and *cdkn1bb*, whereas injection of *her2* morpholino upregulates the expression of *cdkn1a*, *cdkn1ba,* and *cdkn1bb*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) A schematic representation of the promoter region of *cdkn1a*, *cdkn1ba*, and *cdkn1bb*, and fragments containing potential N-boxes are named and indicated. These fragments were selected for PCR amplification. (C) Chromatin immunoprecipitation (ChiP)–PCR analysis was performed on 75% epiboly embryos. Many fragments (1a-i and 1a-ii of *cdkn1a*; 1ba-i, 1ba-ii, and 1ba-iii of *cdkn1ba*; and 1bb-I and 1bb-ii of *cdkn1bb*) that were amplified by PCR suggested direct binding of Her2 to these regions.

of *elavl3* transcripts was observed in Her2-knockdown embryos at the bud stage, concurrent with reduced *neurog1*-positive neuronal precursors (Fig. 6A, B). This indicates that neurons prematurely differentiated from *deltaA*- or *neurog1*-positive precursors into *elavl3*-positive differentiating neurons. The effect of *her2* morpholino could be attenuated by co-injection with *her2* cRNA but not *tp53* morpholino (Fig. 6A, B and Supplemental Fig. 3). Intriguingly, the overexpression of *her2* cRNA alone did not alter the expression of proneural markers (Fig. 6C, D), indicating that Her2 alone was not sufficient to inhibit neurogenesis. Taken together, these results indicated that Her2 is required for the inhibition of neuronal differentiation. Furthermore, the loss of neural progenitors and premature differentiation account for the decreased *deltaA*- or *neurog1*-positive neuronal precursors.

Her2 induces glial differentiation

Gliogenesis proceeds after neurogenesis. It has been shown that mouse Hes1 and Hes5 not only repress the expression of proneural genes during neurogenesis but also promote the differentiation of many glial subtypes (Louvi and Artavanis-Tsakonas, 2006). Consistent with this, we found that the early glial marker, *slc1a3a* (*Glast* in mammals) (Chung et al., 2011; Shibata et al., 1997; Storck et al., 1992), was reduced in Her2-knockdown embryos (Fig. 7), suggesting that Her2 is required for gliogenesis. To further confirm that the reduction of *slc1a3a* in Her2 morphants was not due to increased glial differentiation, we analyzed the expression of a mature radial glial cell marker, *gfap*. We found that the expression of *gfap* was downregulated in Her2-knockdown embryos (Fig. 7), supporting our notion that Her2 is required for gliogenesis. Widespread overexpression of *her2* cRNA upregulated the expression of *slc1a3a* and *gfap* (Fig. 7), suggesting that *her2* was effective in inducing gliogenesis.

Abrogation of the bHLH or WRPW domain in her2 causes a dominant-negative effect

To gain insight into the structural requirements for Her2 function, we created two deletion variants: one lacking the region containing the bHLH DNA binding domain up to the amino terminus of Her2 ($her2^{\Delta bHLH}$) and the other lacking the carboxy-terminal end, including the WRPW domain ($her2^{\Delta WRPW}$) (Fig. 8A). Injection of either $her2^{\Delta bHLH}$ or $her2^{\Delta WRPW}$ resulted in reduced levels of neural progenitors and neuronal and glial derivatives (Fig. 8B, C). The phenotypes caused by injection of $her2^{\Delta bHLH}$ or

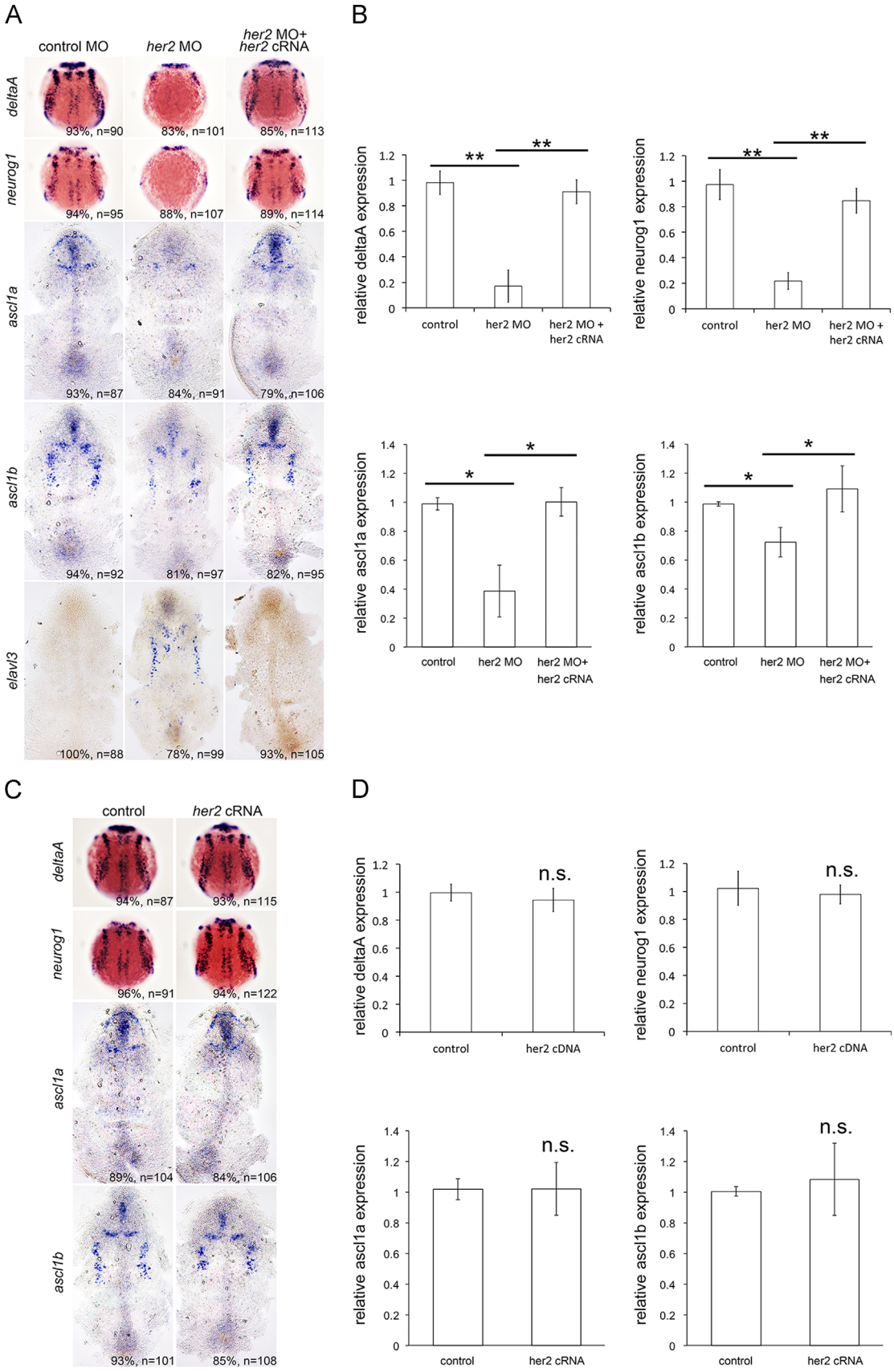

Fig. 6. Knockdown of Her2 induces premature differentiation of neuronal precursors. (A) Injection of *her2* morpholino downregulated the expression of *neurog1*, *deltaA*, *ascl1a*, and *ascl1b* and upregulated the expression of *elavl3* at the bud stage. The phenotypes caused by morpholino injection could be rescued by concomitant injection with *her2* cRNA. (B) qPCR analysis confirmed the results obtained by *in situ* hybridization. (C) Compared with the controls, injection of *her2* cRNA did not cause a significant alteration in proneural markers (*neurog1*, *deltaA*, *ascl1a*, and *ascl1b*). (D) qPCR quantification of the results in C. **, $p < 0.01$; n.s., not significant.

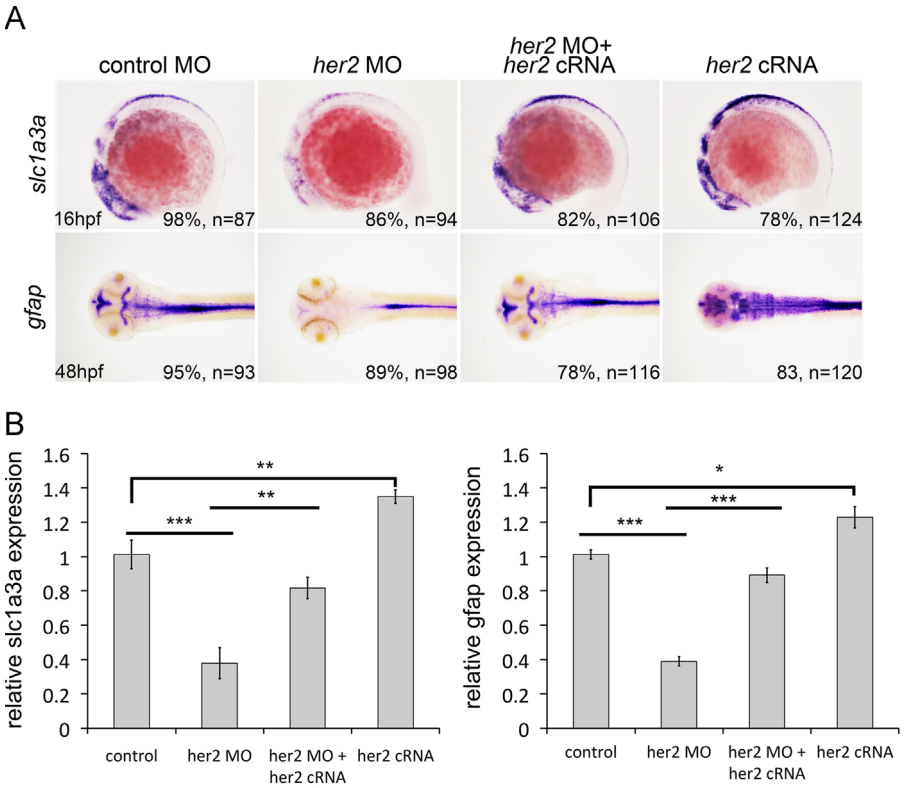

Fig. 7. Her2 induces glial differentiation. (A) *In situ* hybridization showing the expression of *slc1a3a* and *gfap* was reduced in Her2-knockdown embryos, whereas injection of *her2* cRNA upregulated their expression. The stages of the embryos are shown in the bottom-left corner. (B) qPCR analysis confirmed the results of *in situ* hybridization shown in A. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

her2$^{\Delta WRPW}$ were identical to those observed in Her2-knockdown embryos. These results indicated that both bHLH and WRPW domains are required for Her2 function and that the deletion of either domain is sufficient to cause a dominant-negative effect. These results also confirmed the specificity of the phenotypes conferred by *her2* morpholino injection.

Discussion

Development of the nervous system with the appropriate numbers of neurons and glia requires a proper balance of proliferation and differentiation to generate neural progenitors and intermediate precursors. However, the underlying regulatory mechanisms largely remain to be identified, particularly because only a few transcription factors have been shown to be involved in the proliferation and differentiation of neural stem/progenitor cells. One of the critical elements for the formation of neural stem/progenitor cells is the SoxB1 transcription factor family (Kondoh and Kamachi, 2010). In zebrafish, the members of the SoxB1 family (Sox1, 2, 3, and 19) are required for the specification of neural ectodermal fate and the maintenance of neural stem cell fate. In combination with the Pou2/Oct4 transcription factor, Sox2 activates repressors of neuronal differentiation, such as *her3* (Schmidt et al., 2013). Hairy/E(Spl) transcription factors (Hes or Her) have also attracted much attention because of their role in inhibiting neuronal differentiation and maintaining cells in a neural stem/progenitor status. Members of Hes (Her) genes, such as *her3* and *Hes5*, are transcriptional targets of SoxB1 (Engelen et al., 2011; Okuda et al., 2010). The ectopic expression of these Hes/Her proteins results in a downregulation of *neurogenin1* expression, whereas loss-of-function experiments have shown an increase in *neurogenin1* expression. Taken together, the SoxB1/Hes-dependent active inhibition of neurogenesis is the crucial mechanism that expands the pool of neural progenitor cells (Kageyama et al., 2008). However, the instructive role of Hairy/E(Spl) proteins in neural stem/progenitor cells remains unclear. Recent studies have shown that Hairy/E(Spl) may directly bind to genes responsible for the cell cycle. *In vitro* analysis revealed that the expression of HES1 repressed the transcription of the cell cycle inhibitors *Cdkn1a* (encoding p21^{cip1}) (Castella et al., 2000) and *Cdkn1b* (encoding p27^{Kip1}) (Murata et al., 2005). In contrast, Hes1-deficient embryos showed an elevated expression of *Cdkn1b* in the liver and brain (Murata et al., 2005), suggesting that Hairy/E(Spl) proteins directly regulate cell cycle progression. In this study, we provided the first *in vivo* evidence showing that a Hairy/E(Spl) protein, Her2, is required for maintaining the progenitor status by inhibiting neuronal differentiation and is sufficient to induce the proliferation of neural progenitors by inhibiting the expression of *cdkn1a*, *cdkn1ba*, and *cdkn1bb*. These results revealed a novel role of Hairy/E(Spl) proteins in expanding the neural stem/progenitor population by promoting the proliferation of neural stem/progenitor cells and inhibiting neuronal differentiation.

The roles of many Her proteins have been characterized during embryogenesis. For example, Her1, Her4, Her6, Her7, Her11, Her12, Her13.2, and Her15 are essential for somitogenesis (Brend and Holley, 2009; Henry et al., 2002; Pasini et al., 2004; Shankaran et al., 2007; Sieger et al., 2004). We found that Her 2 was also expressed in the presomitic mesoderm, thus suggesting a potential role in somitogenesis, which is worth further investigation. In the developing nervous system, Her4, which is expressed in the proneuronal domains, inhibits the expression of *neurogenin1* and reduces neurogenesis (Takke et al., 1999), whereas our results also showed that Her2 inhibits neuronal differentiation. Given that both Her2 and Her4 are orthologs of mammalian HES5 and that HES5 is also essential for the inhibition of neurogenesis (Ohtsuka et al., 1999), these results suggested an evolutionarily conserved

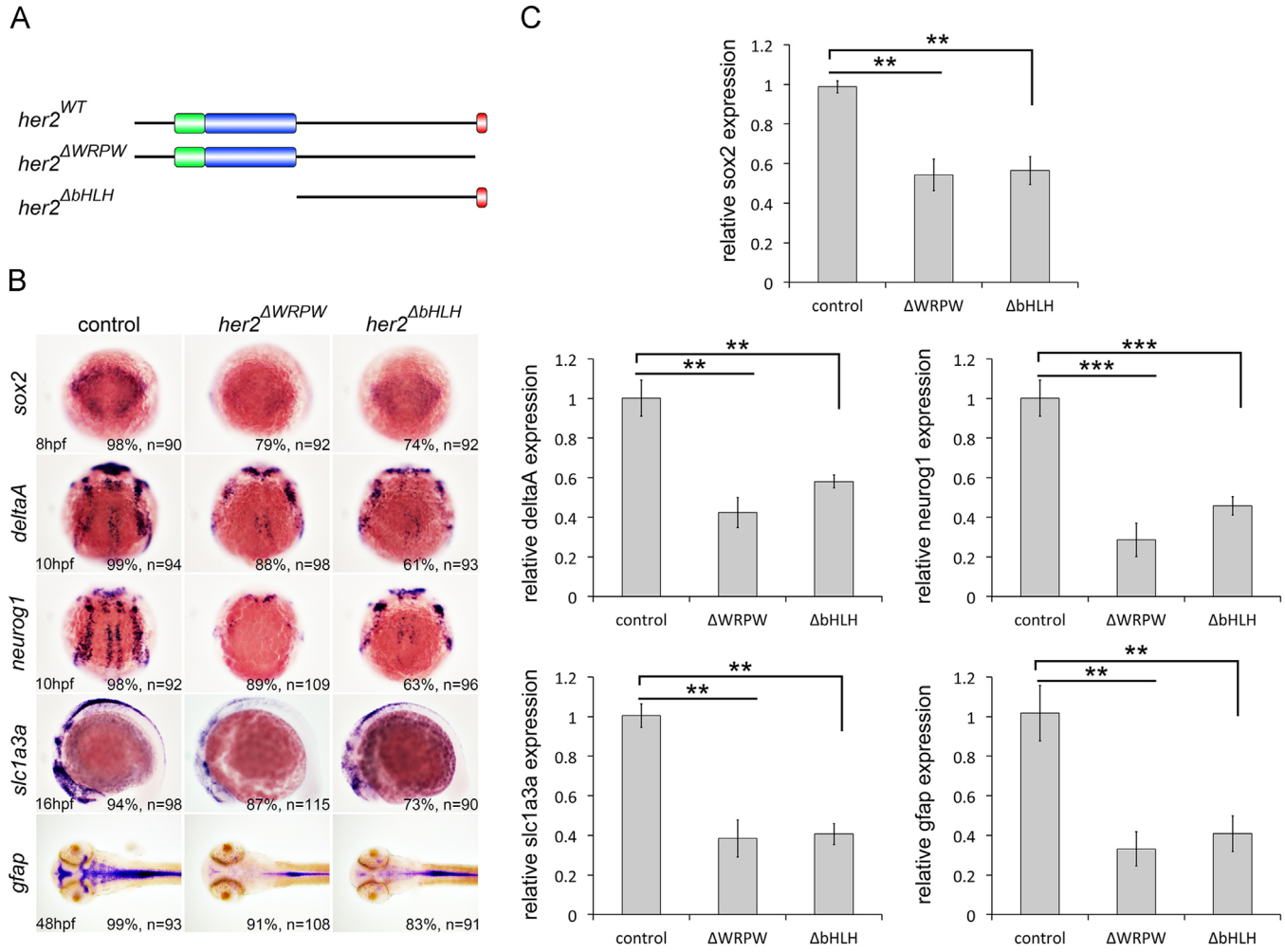

Fig. 8. Resemblance between the phenotypes of *her2*$^{\Delta bHLH}$- or *her2*$^{\Delta WRPW}$-injected embryos and those of *her2* morphants. (A) Schematic illustration of *her2*$^{\Delta bHLH}$ and *her2*$^{\Delta WRPW}$ deletion constructs. (B) *In situ* hybridization showing injection of either *her2*$^{\Delta bHLH}$ or *her2*$^{\Delta WRPW}$ was sufficient to downregulate markers for neural progenitors and neuronal and glial derivatives, as observed in Her2 morphants. The stages of the embryos are shown in the bottom- left corner. These results were confirmed by qPCR (C). *, $p < 0.05$; **, $p < 0.01$.

role between HES5, Her2, and Hes4 in the inhibition of neuronal differentiation. In contrast, *her5* and *her11* are specifically expressed in the midbrain-hindbrain boundary where they act as prepattern genes to prevent neurogenesis in the midbrain-hindbrain boundary (Geling et al., 2003; Geling et al., 2004). Our results that *her2* was also expressed in the midbrain-hindbrain boundary suggest a role for *her2* in the formation of the midbrain-hindbrain boundary. However, the conformational structure of the midbrain-hindbrain boundary was unaffected in Her2-knockdown embryos, suggesting a possible compensatory function of other Her proteins in the midbrain-hindbrain boundary in Her2 morphants, which remains to be confirmed. Her2 was also expressed in many specific brain structures during segmentation stages. Related to this finding, *her6*, which is expressed in the thalamic complex, determines thalamic neurogenesis and the decision between glutamatergic and GABAergic fates (Scholpp et al., 2009). Therefore, whether Her2 is implicated in the formation of brain structures remains to be discovered.

Hairy/E(Spl) proteins form homodimers or heterodimers with other Hairy/E(spl) factors, following which they directly bind to their target proneural genes to repress transcription and inhibit neuronal differentiation. They also form heterodimers with bHLH activators, such as ASCL1 and E47, to inhibit their transcriptional activity (Kageyama et al., 2008). Although we found that the knockdown of Her2 resulted in premature neurogenesis, which could be rescued by concomitant overexpression of *her2* cRNA, overexpressing Her2 alone failed to efficiently inhibit the formation of neuronal precursors. This suggests that Her2 needs to form a heterodimer with other Hairy/E(spl) factors to inhibit neuronal differentiation. The expression of *her2* in the proneuronal domains is similar to that of *her4*, *her8a*, *her12*, and *her15.1* (Bae et al., 2005; Chung et al., 2011), suggesting that Her4, Her8a, Her12, and Her15.1 are potential heterodimeric partners for Her2 to facilitate the inhibition of neurogenesis; however, this remains to be confirmed.

We also revealed Her2 as the first Her protein that is sufficient to induce gliogenesis. In mammals, an aberrant expression of Hes1 or Hes5 promotes the generation of Müller glia in the retina, whereas the inactivation of Hes1 or Hes5 causes defects in Müller glial development (Furukawa et al., 2000; Hojo et al., 2000). However, in contrast with the retina, the aberrant expression of Hes1 or Hes5 in the embryonic brain inhibits both neurogenesis and gliogenesis (Ohtsuka et al., 2001). Therefore, the function of Her2 is not evolutionarily conserved within the mammalian homologs. Previous studies have shown reduced neurogenesis and increased gliogenesis in a double-mutant mouse model of proneural genes *Ascl1* and *Neurod4* or *Ascl1* and *Neurog2*, suggesting that proneural genes regulate neuronal *versus* glial fate determination (Nieto et al., 2001; Tomita et al., 2000). Thus, a possible explanation is that Her2 repressed proneural gene expression and consequently induced the expression of glial markers and reduced proneural markers. However,

we found that Her2 alone was not sufficient to inhibit the expression of proneural genes but could induce glial differentiation, suggesting that Her2 plays an instructive role in gliogenesis, independent of its role in inhibiting neurogenesis. In addition, we found that a Notch deficiency had little effect on the expression of *her2* after neurogenesis, which raised the question of whether *her2* is regulated by Notch signaling or whether it also responds to other signaling pathways during gliogenesis. These questions need to be further analyzed.

Conclusion

The *Hairy/E(Spl)* repressor genes play an essential role in neural development by maintaining progenitor cells and regulating binary cell fate decisions; however, their role in neural progenitor cells remains unclear. We showed that Her2 not only maintains progenitor characteristics by inhibiting neuronal differentiation but also promotes the proliferation of progenitors by inhibiting the cell cycle inhibitors. This suggests that Her2 governs these two mechanisms to provide the proper development of the progenitor pool. Her2 also instructively induces glial differentiation. Therefore, Her2 has multiple activities depending on the cell type and developmental stages and is critical for nervous system development.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.10.018.

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