

## Cerebellar granular neuron progenitors exit their germinative niche via Barhl1 mediated silencing of T-Cell Factor transcriptional activity

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### SUMMARY

T-Cell Factors (TCFs) are the main transcriptional effectors of Wnt/β-catenin signaling. TCF responsiveness is a hallmark of self-renewal in mouse embryonic, and adult, neural stem cells (NSC). However, *in vivo* contribution(s) of TCF activities in long-lived NSCs are poorly understood. Granule neuron progenitors (GNP) in the upper rhombic lip (URL) are long-lived NSCs which express *Atoh1* and generate cerebellar granule neurons. Using functional and transcriptomic approaches in amphibian, we demonstrate that TCFs are active in the URL, and are strictly necessary for the emergence and maintenance of the GNP germinative zone. We identify BarH-like 1 (Barhl1), a direct target of *Atoh1*, as a gate keeper for GNP exit from the URL, through silencing of TCF transcriptional activity. Our transcriptomic and *in silico* analysis identifies Barhl1/TCF URL target genes, and confirms our functional data. Our study provides *in vivo* evidence that inhibition of TCF repressive activity is necessary for maintenance of the URL, a long-lived neural germinative niche.

### KEYWORDS

Granule Neuron Progenitors, Cerebellum, Upper Rhombic Lip, Neural Stem Cell, Germinative niche, Wnt signaling, TCF/Lef, BarH-like 1.

## INTRODUCTION

The Wnt/β-catenin cell-to-cell signaling pathway coordinates development and is one of the most conserved in the animal kingdom. The large majority of Wnt/β-catenin transcriptional targets are regulated by T-Cell Factor/Lymphoid Enhancer-binding Factor (TCF/LEF) transcription factors (TF)<sup>1,2,reviewed in 3</sup>. Investigation of the developmental fate of Wnt/β-catenin-responsive cells in embryonic and postnatal mouse brains reveals that long-lived NSCs retain neuroepithelial properties, and Wnt/β-catenin responsiveness throughout development<sup>4</sup>. In the adult mouse ventricular-subventricular zone of the lateral ventricles, WNT signaling promotes both NSC self-renewal and neural progenitor cell proliferation, while TCF/LEF activity is detected in deeply quiescent NSC cells<sup>4–7 reviewed in 8</sup>. Moreover, hippocampal quiescent NSC and progenitors in culture exhibit enhanced TCF/LEF1 driven transcription<sup>9</sup>. Taken together, these observations suggest contribution(s) of Wnt driven TCF transcriptional activity in adult NSC biology. However, currently, our understanding of such activities in long-lived NSC remains surprisingly fragmented<sup>8,reviewed in 10–12</sup>.

A crucial component of the central nervous system (CNS) in all jawed vertebrates is the cerebellum, involved in executing motor functions as well as participating in higher cognitive processes such as decision-making, emotional and social behaviour, and expectation of reward<sup>13–15</sup>. The cerebellum has two major stem cell niches: the ventricular zone (VZ) adjacent to the fourth ventricle, which produces all cerebellar GABAergic inhibitory neurons<sup>16–18</sup>; and the upper rhombic lip (URL) which is the origin of glutamatergic excitatory neurons, derived from Atonal homologue 1 (*Atoh1*)-expressing progenitors. The URL gives birth first to the deep cerebellar nuclei (DCN), followed by the unipolar brush cells (UBC) and the granular neuron progenitors (GNP) that in turn produce granule neurons, the predominant neuronal population in the entire CNS<sup>reviewed in 19–21</sup>.

While the VZ appears to be TCF inactive, positive TCF transcriptional activity has been documented in the URL of mice, human and *Xenopus* species<sup>22–25</sup>. Moreover, *in vitro* and *in vivo* studies in mice show that, in contrast to what is observed in NSC and progenitors of the developing CNS, or in the VZ<sup>26,27</sup>, constitutive activation of β-catenin in *Atoh1*+ URL cells does not promote their proliferation<sup>26,28–30</sup>. Taken together, these data indicate that TCF-mediated transcription probably contributes to the URL biology, but its role(s) in this germinative area remains undefined. In addition, they highlight the presence of TCF developmental regulators within this germinative area that have not yet been identified.

The GNP developmental path is marked by expression of specific TF, including ATOH1 which is indispensable for maintaining GNP in an immature state<sup>31</sup>. In amniotes, *Atoh1* expression initiates in the RL, is maintained in the EGL during GNP proliferation, and is lost in differentiated GN that start expressing the basic Helix Loop Helix (bHLH) neurogenic differentiation factor 1 (*Neurod1*). In addition, the Paired box protein 6 (*Pax6*) and BarH-like 1 homeobox protein (*Barhl1*) expressions are markers of GNP commitment<sup>25,32–36</sup>.

Amphibians show a marked development of the cerebellum that displays morphological features resembling those found in higher vertebrates<sup>37,38 reviewed in 39,40</sup>. The few studies performed in the amphibian *Xenopus* pre-metamorphosis reveal the presence of an EGL-like structure that is unique compared with other anamniotes. However, unlike in higher vertebrates, the *Xenopus* EGL lacks any cells that undergo proliferation<sup>41</sup>. These studies also indicate that the developmental processes that lead to the formation of GN, specifically the presence of an *Atoh1*-expressing URL and EGL, and the expression of *Neurod1* are close to those described in higher vertebrates<sup>41,42</sup>. Of Note, the amphibian URL maintains itself until

post-metamorphic stages<sup>38,41</sup>. However, early developmental events leading to GNP induction and EGL formation have not been described in amphibians.

ATOH1 directly induces its own expression as well as the expression of the two homeodomain (HD)-containing TF, BARHL1 and BARHL2, which are mammalian homologues of the *Drosophila* Bar-class HD, BarH1 and BarH2<sup>43–46</sup> reviewed in 3,47. In mice, *Barhl1* cerebellar expression is detected in committed GNP, and persists in the EGL<sup>33,48</sup>. In the developing cerebellum, BARHL1 participates in the generation of the EGL<sup>45</sup>, and is one of the major TF that regulate the radial migration of GNP in a mechanism involving Neurotrophin3 (NT3)<sup>49</sup>. Furthermore, an impairment in GN survival, and an attenuated cerebellar foliation, are observed in *Barhl1*-/- mice<sup>50</sup>. On the other hand, we established that BARHL2 dramatically enhances the transcriptional repressor activity of TCF, and prevents β-catenin driven activation of TCF target genes<sup>51,52</sup>. Immunoprecipitation assays reveal physical interaction between BARHL2 and the two transcriptional repressors of Wnt target genes TCF7L1 and Groucho/Transducin-like Enhancer of Split (Gro/TLE)<sup>52</sup>. However, the role of BARHL2 in cerebellar development has not been investigated, and whether BARHL1 similarly interacts with, and regulates, TCF transcriptional activity is unknown.

Here, we investigated the role of both Tcf transcriptional activity, and Barhl1, in GNP development using *Xenopus* as a model system. We establish that markers of GN early progenitors, EGL, commitment, and differentiation, are conserved in *Xenopus* compared to amniotes. Using gain and loss of function experiments (GOF/LOF), immunoprecipitation, and a *X. tropicalis* Wnt reporter transgenic line, we demonstrate that Tcf-mediated transcriptional activation is strictly necessary for both the emergence and maintenance of the cerebellar URL, and furthermore demonstrate that, in this germinative area, Barhl1 is the main repressor of Tcf transcriptional activity. Barhl1 LOF, through Morpholino (MO)-mediated depletion or Crispr/Cas9 gene knock-out, dramatically increases Tcf transcriptional activity in the URL, leading to a major enlargement of the URL, and significant delays in GNP differentiation. Using a transcriptomic approach, we confirm our functional assays and identify direct and indirect target genes repressed by Barhl1 in the cerebellar URL. Indeed, Barhl1 depletion induces an upregulation of TF involved in maintenance of neural stem/progenitor properties, an enhancement of both Wnt/Tcf and Notch signaling activities, associated with a down regulation of genes involved in inhibiting proliferation, and promoting neural differentiation. Together with *in silico* analysis of Barhl1 target genes regulatory regions, our study confirms that in the amphibian, Barhl1 drives URL stem/progenitor cells out of their germinative niche, towards commitment and differentiation, by repressing TCF transcriptional activity.

## RESULTS

### Spatial and temporal expression of key markers of GNP development are conserved in *Xenopus* compared to higher vertebrate

We performed *in situ* hybridization (ISH) on *X. laevis* tadpoles through pre-metamorphic froglets (stages 35 to 50) and assessed the expression patterns of genes known to be involved in the development of *Atoh1* lineage in rodents, focusing on GN. By using *atoh1* to label the URL and EGL, *pax6* and *barhl1* to mark GNP commitment, and *neurod1* to mark post-mitotic GN, we established a fate map that outlines the developmental progression of *atoh1* lineage cells within rhombomere 1 (R1), located caudal to the midbrain-hindbrain boundary (MHB). The proliferation marker *n-myc* helps define the posterior boundary of R1 (Fig. 1A; Fig. S1B).

We found that *pax6* expression begins at stage 38 (Fig. S1C), while *barhl1* expression begins at stage 39-40 (Fig. 1F), which we used as a landmark of GNP induction. From stage 38 to stage 48, *atoh1* is expressed in the URL and in a layer of 3 to 4 cells bordering the URL, which we consider to be the EGL (Fig. 1B; Fig. S1A). Although the *n-myc* expression pattern is similar to that of *atoh1* in the URL, *n-myc* is also strongly detected in the VZ, in agreement with its expression in proliferating cells (Fig. 1C, Fig. S1B).

We investigated the dynamics of *pax6*, *barhl1*, and *neurod1* expressions within R1 from stage 38 to stage 48 (Fig. 1E-G; Fig. S1C-E). All markers are present in the cerebellar primordium and are first detected in the caudal region of the EGL. During this developmental period, cells expressing *barhl1* and *neurod1* migrate from an external layer partially covering the cerebellar plate towards the inner cerebellar tissue, where they undergo their final differentiation (Fig. 1F, G, Fig. S1D, E). Furthermore, while *Orthodenticle Homeobox 2 (otx2)* expression is typically limited to the posterior lobes of the EGL in amniotes, we detected *otx2* expression in the caudal EGL at stage 39, which was subsequently restricted to the cerebellar plate (Fig. 1D). At stage 48, *Hairy and enhancer of split-4 (hes4)*, a known marker of Notch-active cells and of stemness in *Xenopus*<sup>53,54</sup>, strongly labels both the VZ and the EGL at stage 48 (Fig. 1H). *hes4* is not expressed in mice, while its expression is found in *Xenopus* and in Human. Additionally, we did not observe expression of *barhl2* in the cerebellar anlage at the analysed developmental stages (Fig. S1F).

These observations indicate striking similarities in GNP development between *Xenopus* and amniotes. Specifically, the expression pattern of genes involved in induction and specification of GNP, including *atoh1*, *n-myc*, *pax6*, *barhl1*, *otx2*, *neurod1*, is conserved. As previously reported<sup>41</sup>, we detect the presence of an EGL along the R1 antero-posterior axis marked by *atoh1* and *hes4*. Our observations also indicate a gradient in GNP differentiation, initiated in the caudal EGL at stage 38 and progressing to the rostral part up to stage 50. Starting at stage 50, we report changes in the shape of the URL. Thus, we focused our analysis on cerebellar anlage development between stage 38 and stage 48.

### In the cerebellar primordium, constitutive Tcf inhibition and Barhl1 overexpression produce similar developmental defects in atoh1 expression, URL induction and GNP early commitment/differentiation

We focused our study on the role of Tcf and Barhl1 in URL establishment and maintenance, during the time window where GNP are produced. We used *tcf7l1-Δβcat-GR*, a previously described inducible form of *tcf7l1* which lacks its β-catenin binding domain, and thus acts as a dominant negative and constitutive inhibitor of Tcf transcriptional activity<sup>55</sup> (Fig. S2). Development of the URL and GNP was investigated using either *atoh1*, or *pax6*, *barhl1* and *neurod1* as respectively URL/EGL, and GNP commitment /differentiation markers.

At a high dose, *tcf7l1-Δβcat-GR* overexpression induced a dramatic reduction in the size of the URL, associated with the disappearance of the expression of its key marker *atoh1*. Of note, this effect is restricted to the R1 (Fig. 2Aa-a'', c). At a lower dose, *tcf7l1-Δβcat-GR* overexpression induced a decrease in *atoh1* expression (Fig. 2Ab, c). This decrease is associated with both an increase of expression, and a rostral shift, observed with the three commitment/differentiation markers *pax6*, *barhl1*, and *neurod1* within the R1 (Fig. 2Ba-c). *tcf7l1-Δβcat-GR* effects on *atoh1*, *pax6*, *barhl1* and *neurod1* expression were quantified (Fig. 2Ac-Bd).

In amniotes, BARHL1 is a direct target of ATOH1<sup>45,46</sup>. We next asked whether Barhl1 overexpression impacts early development of the URL and GNP (Fig. S2). We observed that

*barhl1* overexpression phenocopies that of Tcf inhibition. We observed a strong decrease in *atoh1* transcripts levels within the URL (Fig. 2Ca-a", d). Loss of *atoh1* and the URL is associated with an increase of *pax6* and *neurod1* expression, and a concomitant rostral shift in both markers' expression within R1 (Fig. 2Cb-d).

Our data indicate that Tcf transcriptional activity is strictly necessary for the expression of *atoh1* within the URL, and that inhibiting Tcf activity leads to accelerated GNP differentiation. Similarly, overexpression of Barhl1 in the cerebellar primordium results in URL induction defects, associated with premature GNP differentiation.

### In the cerebellar URL, inhibition of Barhl1 maintains GNP in an early progenitor state

To decrease Barhl1 activity within the cerebellar anlage, we designed two morpholinos (MO), *MObarhl1-1* and *MObarhl1-2*, specifically targeting *Xenopus barhl1* mRNA (Fig. S2; S3; Methods). We investigated whether, and by what mechanism(s), Barhl1 Knock-Down (KD) affects the development of the URL, the EGL, and/or GNP development. At stage 42, 45, and 48, depletion of Barhl1 induced an increase in *atoh1* expression. We observed *atoh1* expressing cells spreading across the surface of the cerebellar plate (Fig. 3Aa-a", Ba-a"; Fig. S3B, C, D). This ectopic expansion within the URL and to the EGL is associated with a major decrease in *pax6* expression (Fig. 3Ab, Bb; Fig. S3B, D). Both MO induced the same phenotype, which was quantified (Fig. 3C). At stage 42, the increase in *atoh1* expression in Barhl1-KD embryos is corroborated by a similar increase in *n-myc* expression (Fig. S3C). We further tested the ability of *mbarhl1-GR* to rescue the Barhl1-KD phenotype. *mbarhl1-GR* was co-injected with *MObarhl1-1*, and *neurod1* was used as a marker of GNP differentiation. *MObarhl1-1* and *MObarhl1-2* induced a strong decrease in *neurod1* expression, which was rescued by co-injection of *mbarhl1-GR* (Fig. 3D).

We next asked whether inhibition of Tcf activity compensates for Barhl1-KD using *pax6* as a marker of GNP commitment. As previously observed, Barhl1-KD delayed GNP differentiation process, while *tcf7l1-Δβcat-GR* overexpression accelerated it (Fig. 3Ea-c). *MObarhl1-1* co-injected with two different doses of *tcf7l1-Δβcat-GR* mRNA rescued the phenotype (Fig. 3Ed-f).

These data provide strong evidence that *MObarhl1* acts by specifically inhibiting endogenous *Xenopus* Barhl1 activity. They also indicate that Barhl1 depletion delays differentiation of GNP. Combined, these observations reveal that Barhl1 and Tcf act in opposing ways within the URL and the EGL, and maintain GNP in an early progenitor state.

### Barhl1 limits Tcf transcriptional activity within the cerebellar primordium

We next asked whether Barhl1 directly controls Tcf transcriptional activity within the cerebellar URL. We investigated interactions between Barhl1, Gro4, and Tcf7l1, by performing co-immunoprecipitation (Co-IP) experiments on protein extracts from HEK293T cells transfected with tagged constructs of Tcf7l1, Gro4, and Barhl1 (Fig. 4A; Fig. S2). In agreement with Barhl1 containing two Engrailed Homology-1 (EH1) motifs known to interact with the WD-repeat domain of Gro, Barhl1 co-immuno-precipitated with Gro4 (Fig. 4Aa). Using Tcf7l1 as bait, we further observed that Tcf7l1 could immuno-precipitate Barhl1, in the presence and absence of Gro4. The presence of Barhl1 does not affect the interaction of Tcf7l1 with Gro4 (Fig. 4Ab). Concatemers of the consensus Tcf binding motif have been used to generate Wnt/Tcf reporter lines, such as *Xenopus tropicalis* (*X. tropicalis*) transgenic pbin7LefdGFP line<sup>22,56,57</sup>, which contains one copy of a *wnt* reporter gene. We assessed Tcf activity from stage 42 up to stage

50 using this reporter line and observed a positive Tcf activity in the URL. Contrastingly, we did not detect any Tcf activity in the VZ and the EGL at similar developmental stages. Importantly, up to stage 48 we observed that Tcf activity is stronger at the rostral end of the URL. This asymmetry of expression is lost at stage 50 (Fig. 4Ba-d). In addition, we observed a strong correlation between Tcf activity and *atoh1* expression (Fig. 4Ba'-d'), which appears to be complementary to that of *barhl1* (Fig. 4Bc", c""-d"). Taken together, these data are consistent with our previous observations indicating that Tcf activity is strictly necessary for the induction of *atoh1* expression within the URL.

We next assessed the impact of Barhl1 gain of function (GOF) and loss of function (LOF) on Tcf activity (Fig. 4C). Whereas *mBarhl1* overexpression decreased Tcf activity (Fig. 4Ca), we observed a threefold increase in Tcf activity upon Barhl1 downregulation with *MObarhl1-1* (Fig. 4Cb). In contrast, *MOct* had no effect on Tcf activity (Fig. S4A). The effect was quantified (Fig. 4Cc). We further inhibited Barhl1 by selective knock-out (KO) of *xbarhl1* gene in the pbin7LefdGFP line (F0 generation) using Crispr/Cas9 genome editing technology (Fig. S4B). We observed that Barhl1 KO induced an average twofold increase in Tcf transcriptional activity (Fig. 4Da-c). We assessed phenotypic penetrance in Crispr/Cas9 injected embryos based on *gfp* expression. We observed different levels of phenotypic severities in >70% of injected embryos, ranging from a slight increase in *gfp* expression observed in ~20% of injected embryos to >40% of injected embryos exhibiting strong to complete penetrance as observed by a significant increase in *gfp* expression in the R1 (Fig. 4d).

To determine whether Barhl1 effects were mediated through its interaction with Gro, we used and inducible form of *mBarhl2-EHsGR*, which contains the two EH1 domains of Barhl2 (Fig. S2) and has been previously demonstrated to act as a dominant negative for Tcf repressive activity by competing for Gro binding<sup>52</sup>. Overexpression of *mBarhl2EHsGR* induced a phenotype similar to that of Barhl1-KD in terms of increasing the URL/EGL size at the expense of GNP commitment/differentiation (Fig. 4Ea, b; S4).

Taken together, our data establish that Barhl1 directly interacts with Tcf7l1 and Gro, limiting their transcriptional activity in the cerebellar URL.

### **Through its limiting of Tcf transcriptional activity, Barhl1 allows GNP to leave their early progenitor state and exit the proliferating URL**

The URL germinative zone is characterized by its proliferative state, and its bordering of the roof plate. We first asked whether the EGL is proliferative in *Xenopus*, and second whether the enlargement of the URL/EGL territories observed in Barhl1-KD tadpoles was corroborated with an increased proliferation in the URL and/or within the cerebellar plate. Using immunofluorescence staining for Phosphorylated-Histone H3 (PHH3), a marker of cells undergoing mitosis, counterstained with a cell nuclear marker, we measured proliferation in tadpoles injected with either *MOct* or *MObarhl1-1* at stage 45 and 48. In agreement with previously published data<sup>41</sup>, at both stages, PHH3+ cells were solely detected within the URL (Fig. 5Aa). To investigate the capacity of URL-derived GNP to leave their proliferative state, and to progress along their developmental trajectory, we measured the length of the URL on the injected side compared to the control side in embryos either injected with *MOct* or *MObarhl1-1*. At stage 45 and 48, Barhl1-KD embryos exhibited a 1.2-fold lengthening of the URL on the injected side relative to the control side (Fig. 5Ac-e). Because it was morphologically easier to distinguish the URL from the VZ at stage 48, we performed our following analysis at this later developmental stage (Fig. 5Ab). At stage 48, we measured an average 2-fold increase in the number of PHH3+ cells on the injected side compared to the

control side (Fig. 5Ac, d, f). Moreover, we observed and quantified the presence of proliferating cells in the cerebellar plate, which is normally devoid of PHH3+ cells as observed in tadpoles injected with *MOct* (Fig. 5Ac, d, g). Taken together, these results confirm that in *Xenopus*, the EGL is non proliferative, and show that Barhl1-KD cells are compromised in their ability to leave the URL niche and become postmitotic.

We next investigated whether Tcf inhibition could counteract Barhl1-KD effect on URL extension. As previously observed, whereas Barhl1-KD induced an extension of the URL length, *tcf7l1-Δβcat-GR* overexpression reduced it (Fig. 5Ba-c), and co-injection of *MObarhl1-1* and *tcf7l1-Δβcat-GR* mRNA brought back the URL size to normal (Fig. 5Bd-f).

In conclusion, Barhl1 activity as an inhibitor of Tcf transcription is strictly necessary for URL cells to exit their niche, become postmitotic and enter the EGL.

### Transcriptomic analysis of Barhl1 activity in the developing cerebellum

*barhl1* starts to be significantly expressed in the developing cerebellum at stage 40. To further document Barhl1 activity, we designed an RNA-sequencing experiment allowing the identification of Barhl1 direct and indirect target genes in the early *Xenopus* cerebellum.

We isolated and sequenced RNA from R1 of stage 42 tadpoles previously injected at the 4 cells stage in the 2 dorsal blastomeres with *MObarhl1-1*, *MObarhl1-2* or *MOct* together with *gfp*. Tadpoles were selected for hindbrain injection and R1 were dissected. Samples were compared through differential expression (DE) analysis. Genes with adjusted p-value (pAdj) inferior to 0.001 were selected as significant DE genes (DEG) (Table S1). Principal component analysis of these R1 samples demonstrated that they clustered by Barhl1-KD status (Fig. 6A), indicating that changes in gene transcription were consistent across different clutches. At stage 41-42 we identified 1622 and 830 genes differentially expressed between respectively *MObarhl1-1* and *MObarhl1-2* injected R1, compared with *MOct* injected R1. Amongst these DE genes 575 were common between MOs injected samples (Fig. S6B). A selection of significant upregulated ( $\text{Log2FC} > 0.4$ ) and downregulated ( $\text{Log2FC} < -0.4$ ) genes are represented in volcano plots for both MOs (Fig. 6B). Furthermore, we generated a heatmap representing upregulated and downregulated common DEG for both MOs (Fig. 6C).

As a first approach we performed gene ontology analysis (GO) based on  $\text{pAdj} < 0.001$  DEG using the clusterProfiler algorithm<sup>58</sup> and compared altered biological functions between both Barhl1-KD conditions (Fig. 6D). Our GO analysis reveals that the most significantly upregulated genes act as transcriptional activators when bound to DNA (Fig. 6Da). In agreement with a delay of GNP differentiation the downregulated DEG were found to be involved in axon development and axonogenesis, in addition to neuronal differentiation (Fig. 6Db). Indeed, our differential expression dataset reveals that genes that are the most upregulated in the *MObarhl1-1* and *MObarhl1-2* conditions are involved in adult neural stem cell (NSC) maintenance. For example, *dmrta2* that encode for doublesex and mab-3-related transcription factor a2, also known as *dmrt5*, the orphan nuclear receptor subfamily 2 group E member 1 (*nr2e1*) commonly known as Tailless, that are upregulated with a Log2FC over 1.5. We also observed a down regulation of the Delta/Notch-like epidermal growth factor (EGF)-related receptor (*dner*) ( $\text{Log2FC} \leq -0.5$ ), which has been suggested to be a neuron-specific Notch ligand<sup>59</sup>. Indeed, *dner* has been suggested to inhibit neural proliferation and induce neural and glial differentiation<sup>60</sup>. We also identify Basic Helix-Loop-Helix Family Member E22 (*bhlhe2*), a downstream target of NEUROD1, which is strongly downregulated in Barhl1 depleted R1 ( $\text{Log2FC} \leq -0.5$ )<sup>61</sup>.

Our functional data argue that Barhl1 mostly act through inhibition of TCF transcriptional activity. We first investigated the presence of Barhl1 Cis Regulatory Motifs (CRM) defined as CAATTAC/G and its mirror motif<sup>62</sup>, within the regulatory sequences - 5Kb upstream or downstream of the Transcription Start Site (TSS) - of previously identified DEG common to *MObarhl1-1* and *MObarhl1-2* conditions. We observed that all DEG regulatory regions contain at least 2 Barhl1 CRM, 87.5% contain 5 or more Barhl1 CRM and 40% 10 or more Barhl1 CRM (Table 1A). Thereby our identified DEG appear to be Barhl1 direct target genes. To investigate which Barhl1 target genes are also regulated by TCF we similarly searched for TCF CRM defined as CTTTGAA/CTTTGAT, within the regulatory sequences of previously identified DEG common to *MObarhl1-1* and *MObarhl1-2* conditions (Table 1B; Fig. 6E)<sup>63,64</sup>. We observed that 76% of Barhl1 depleted DEG regulatory regions contain at least one Tcf CRM: 26% contain one CRM, 25% contain two Tcf CRM and 25% contain three and more Tcf CRM (Fig. 6E). Using ISH, we explored changes in two up-regulated DEG. *zic3*, a member of the Zinc Finger of the Cerebellum (Zic) family known to be involved in regulation of neuronal progenitor proliferation versus differentiation, and cerebellar patterning<sup>reviewed in 65–67</sup> ( $\text{Log2FC} \geq 1$ ) and *otx2* that is detected in a subset of GNP ( $\text{Log2FC} \geq 1.2$ ). At stage 41-42 we observed a significant expansion of both *otx2*, and *zic3* expression territories within the cerebellar plate (Fig. 6Fa,b). *zic3* transcripts are present in the URL, and *zic3* regulatory regions contain at least three Tcf CRM (Table 1B). *otx2* regulatory regions contain either no Tcf CRM (*otx2.L*), either 3 Tcf CRM (*otx2.S*). Thereby we argue that *zic3* is a direct target of Tcf, whereas *otx2* genes may be indirect targets of Barhl1 depletion effect on GNP development.

Amongst the DEG, we also observed an upregulation of faithful reporters of Notch pathway activation *hes5* family genes (*hes5.1*, *hes5.2*, *hes5.3*, *hes5.4*), which regulatory regions contain between 1 and 5 Tcf CRM ( $\text{Log2FC} \geq 0.5 \leq 0.91$ ) and HES/HEY-Like Transcription Factor (*hel*t) ( $\text{Log2FC} \geq 2$ ), which regulatory regions lack Tcf CRM. HELT is closely related to Hairy enhancer of split proteins that act as a major downstream effector in the Notch pathway, that is required for the maintenance of NSC, and a proper control of neurogenesis in both embryonic and adult brains<sup>68</sup>.

Finally and importantly amongst the DEG, we identified markers of Wnt pathway activity including one of the bona fide direct target gene of Wnt signalling *sp5* ( $\text{Log2FC} \geq 1.55$ ; 3 Tcf CRM) (Fig.6B,C; Suppl. Table 1), together with *lef1* ( $\text{Log2FC} \geq 1.55$ ; one Tcf CRM) known to be a target of Wnt/β-catenin signalling<sup>69</sup>, two Wnt secreted signals *wnt8b* ( $\text{Log2FC} \geq 1$ ; one Tcf CRM) (Fig. 6Fc), and *wnt2b* ( $\text{Log2FC} \geq 1.3$ ; one Tcf CRM) (Fig. 6Fd), both of which activate the Wnt canonical pathway, and Wnt Ligand Secretion Mediator (*wls*) ( $\text{Log2FC} \geq 0.5$ ; one Tcf CRM), which expression in the URL orchestrates cerebellum development in mice<sup>70,71</sup>. Thereby Barhl1 depletion activates Wnt/Tcf activity throughout the cerebellar anlage, specifically within the URL and the cerebellar plate.

Taken together, our transcriptomic analysis identified direct and indirect Barhl1 target genes. Within the R1 territory, when upregulated these genes are involved in i) the maintenance of neural stem/progenitor properties, ii) the enhancement of Notch activity, iii) promoting Wnt/Tcf activity. When down regulated they are mostly involved in inhibiting proliferation and promoting neural differentiation. Our analysis of Barhl1 target genes regulatory regions confirms our functional analysis demonstrating that Barhl1 mostly acts by inhibiting Tcf transcriptional activity.

## DISCUSSION

This study conducted in amphibians provides evidence that the development of the *Xenopus Atoh1* lineage is similar to that of higher vertebrates. We show that Tcf transcriptional activity is necessary for inducing the cerebellar URL, as well as *atoh1* expression. Furthermore, we demonstrate that Barhl1 plays a critical role in promoting the exit of URL cells from their niche, and initiating their differentiation trajectory towards mature GN. Most importantly, Barhl1 acts primarily by inhibiting Tcf transcriptional activity. Transcriptomic analysis of Barhl1 depletion in the cerebellar anlage confirms our functional study.

### ***Xenopus* represents a novel model for studying cerebellar development**

Our ISH experiments provide a developmental map of GNP development in *Xenopus*, revealing that the processes leading to the emergence of URL derivatives and maturation of GN are similar to those seen in higher vertebrate<sup>37,38</sup> reviewed in 39,40. Amongst the important similarities i) the *Xenopus* URL expresses *atoh1*, is proliferative and is Tcf responsive; ii) *pax6* and *barhl1* are early markers of GNP commitment; iii) GNP migrate out of the URL along the cerebellar surface where they appear as the equivalent of the amniote-like EGL; iv) As in amniotes, postmitotic GN express *neurod1*, and migrate inwardly to form the IGL. Amongst the differences we observed i) a GNP caudal-to-rostral temporal differentiation gradient, with caudal URL differentiating first; ii) Although the EGL express stem/progenitor markers such as *atoh1*, the Notch pathway activity marker *hes4*, and *n-myc*, our proliferation analysis confirmed the absence of proliferating cells within the EGL from earlier stages<sup>41</sup>; iii) Although *Barhl2* is expressed in the amniote EGL<sup>72</sup>, we could not detect this transcription factor in the *Xenopus* cerebellar anlage. Our observations suggest that the tetrapod vertebrate *Xenopus*, the only described anamniote displaying an EGL, could be an alternative useful model for some clinical evaluation of cerebellar developmental defects, especially those related to early cerebellar development<sup>41</sup> reviewed in 39,40.

### **Tcf transcriptional activity is strictly necessary for *atoh1* expression and URL induction**

Our data show that within R1, TCF transcriptional activity is strictly necessary for induction of *atoh1* expression and of the URL territory. Moreover, TCF inhibition is associated with an increase and/or an acceleration of GNP commitment/differentiation. Interestingly, studies performed in mouse neuroblastoma, and neural progenitor cells in culture, identified two TCF/LEF binding sites present in the 3' enhancer region of *Atoh1* that are required for *Atoh1* activation<sup>73</sup>. In these cells, the concomitant inhibition of Notch signaling and activation of WNT/Tcf, appear to be required for *atoh1* expression<sup>73</sup>. In mice low levels of Notch activity are necessary to induce a glutamatergic cell fate in *Sox2*-expressing cerebellar progenitors<sup>74</sup>. Whereas it remains to be demonstrated in amniotes, our data argue that concomitant TCF activation and Notch inhibition are responsible for *atoh1* expression in the cerebellar primordium.

We did not investigate which Tcf/Lef isoforms are transcriptionally active in the amphibian URL. However, our transcriptomic data reveal that *lef1* is one of the most up-regulated DEG in the absence of *barhl1*, suggesting that it is present in the URL and could mediate Wnt signaling in this germinative niche. Three of the four Tcf isoforms (Tcf7l2, Tcf7 and Lef1), mostly act as transcriptional activators, whereas the fourth (Tcf7l1) mostly acts as a transcriptional

repressor<sup>reviewed in 2</sup>. Transcriptomic analyses of human cerebellar development reveal that the transcriptional activator TCF7 is active in the human URL<sup>32</sup>, whereas Tcf7l2 is detected in the mouse URL<sup>33</sup>. In both species, Tcf7l1 is associated with differentiated GNs<sup>25,32</sup>.

It is well documented that inhibition of TCF7l1-mediated repression is at the core of mouse embryonic stem cell (ESC) self-renewal and pluripotency. In contrast, enhancement of TCF7l1 repressive activity blocks mESC self-renewal, and allows mESC to differentiate, even in the presence of Wnt signaling<sup>75–82</sup> reviewed in 3,12,83. In adult mice, canonical WNTs are produced by both NSCs and astrocytes, and WNT/β-catenin signalling stimulates both NSC self-renewal and neural progenitor cell proliferation<sup>4–7,9</sup> reviewed in 8. At least up to stage 50, we observed that the entire amphibian URL is Tcf active whereas the VZ is not. Early observation using electronic microscopy reported that during the premetamorphic phase, the cerebellum remains in an immature state and that a well-defined EGL up to 8 cells layers is likely to be established by the end of the prometamorphic phase<sup>38</sup>. Taken together these observations indicate that the cerebellar URL displays features of an adult NSC niche. Our data provide new *in vivo* evidence that Tcf activity is strictly necessary for NSC niche maintenance and function.

### In the URL, *barhl1* promotes GNP exit from their germinative niche, towards commitment and differentiation

R1 territory is correctly established in embryos either lacking or overexpressing *barhl1*, arguing that Barhl1 is not involved in the establishment of the cerebellar anlage. Yet both our functional and transcriptomic data show that Barhl1 activity is strictly necessary for development of URL-derived GNPs. Whereas Barhl1 overexpression decreases the size of the URL, and promotes GNP commitment/differentiation, MO-mediated depletion of Barhl1 induces an enlargement of the URL associated with a marked delay in the GNP differentiation process.

Our transcriptomic analysis is consistent with our phenotypic observations. Barhl1-depleted DEG identification reveals that most significant upregulated genes regulate URL cell behavior either by acting on the fine equilibrium between a proliferative state and commitment and / or in maintenance of their stem/progenitor features. Indeed, *dmrt2* (*dmrt5*) expression is specific to neural stem/progenitor cells and has been shown to maintain NSC self-renewing ability<sup>84</sup>. In neural progenitor cells derived from mESC, Dmrt2 maintains proliferation by binding to a target of Notch signaling, *Hes1*, and upregulates its expression, which will further inhibit neuronal differentiation through repressing the transcription of proneural genes<sup>84</sup>. In the rodent developing and adult brain, the primary function of the orphan nuclear receptor *Nr2e1* (also known as *Tlx*) is to maintain NSC pools in an undifferentiated, self-renewing state preventing their premature differentiation<sup>85–88</sup> reviewed in 89,90. In mice, *otx2* is expressed in GNPs during their massive expansion in the EGL<sup>91</sup>, and its expression is associated with the high proliferation rate of GNP<sup>92</sup>. However, the exact role of *otx2* in GNP development has not yet been elucidated. Finally, another upregulated URL target gene is *zic3*. Although *zic3* activity in the URL has not been described in mice, *zic3* is involved in maintaining pluripotency in both ESC<sup>93,94</sup>, and neural progenitor cells<sup>95</sup>.

On the other hand, most downregulated DEG are involved in terminal neuronal differentiation, including dendrite development, and axonogenesis. One example is *Bhlhe2*, which in mice is expressed in the inner EGL, and is a downstream target of *Neurod1* in migrating and in differentiated GNs<sup>61</sup>. *In vitro* KD experiments in primary GN culture indicate that *bhlhe22* is a regulator of post-mitotic GN radial migration towards the IGL<sup>96</sup>.

## In the cerebellar primordium Barhl1 acts through repression of Tcf transcriptional activity

Similar to what we previously described for Barhl2<sup>52</sup>, in mammalian cells Barhl1 physically interacts with both Gro/Tle and Tcf7l1. In R1, Barhl1 overexpression phenocopies inhibition of Tcf transcriptional activity, and decreases Tcf activity. Conversely, Barhl1 depletion dramatically increases Tcf transcriptional activity in the URL. Both the increase in URL length, and the delay in GNP commitment/differentiation induced by Barhl1 depletion, are compensated by co-expression of a constitutive inhibitory form of Tcf7l1. Finally, Barhl1-KD embryos display a massive increase of Wnt activity throughout the cerebellar anlage.

Over 75% of Barhl1 depleted DEG regulatory regions contain at least one Tcf CRM; these include markers of the URL and EGL including *zic3*, *hes5* family genes, and *wls*. In line with our data in the rodent telencephalon, *Dmrt2* is transcriptionally activated by a stabilized form of beta-catenin and inhibited by a dominant-negative form of TCF<sup>86</sup>. Tcf7l1 directly represses transcription of *Lef1*, which is stimulated by Wnt/β-catenin activity<sup>69</sup>. These data argue that Barhl1 drives GNP out of the URL via Tcf-mediated repression and that Barhl1 LOF and GOF phenotypes are, at least partly, the result of alteration of its inhibitory effect on Tcf transcriptional activity.

## Barhl1 activates Notch activity in the cerebellar primordium

*Hes4*, a marker of Notch activity and stemness in *Xenopus*<sup>53</sup>, is detected at stage 48 in the VZ, the URL, and the EGL. Our RNA-seq analysis reveals that depletion of Barhl1 leads to a significant upregulation of Notch pathway activity. Among upregulated components of Notch signaling, we identified the bHLH TF *helt* (also known as *Heslike* and *Megane*). In mice *Helt* is expressed in undifferentiated neural progenitors where it acts as transcriptional repressor of proneural genes<sup>97,98</sup>. Similarly, Barhl1 depletion in R1 leads to upregulation of *hes5* genes, which are known to inhibit neuronal differentiation by directly repressing proneural genes. In rodents, levels of Notch activity regulate the early progenitor choice between inhibitory (Notch +) and excitatory GN (Notch -) fate in the VZ<sup>74</sup>. In agreement with its described function in maintaining cells in a primitive state, Notch has been suggested to prevent early GNP differentiation<sup>99</sup> reviewed in 100,101, yet its exact function in developing GNP is still debated. Overexpression of a dominant negative form of Barhl2, which binds to Gro/Tle and counters its inhibitory activities, increases the URL/EGL size at the expense of GNP commitment/differentiation. Gro/Tle acts as a corepressor of both TCF and Enhancer of split E(spl), a major transcriptional repressor of Notch target gene activation, including proneural genes<sup>102</sup> reviewed in 3. Our findings suggest that there may be as yet unknown crosstalk between Wnt/Tcf and Notch signaling pathways in the maintenance of the cerebellar URL/EGL<sup>reviewed in 103</sup>.

## Barhl genes in amphibian versus mammalian cerebellar development

In mice, *Barhl1* and *Barhl2* transcripts are detected in the outer URL and the posterior EGL from E11.5 onwards<sup>32,45,48,50,72,104,105</sup>. scRNA-sequencing analysis of mouse cerebellar cells reveals that *Barhl1* is associated with early GNP differentiation, whereas *Barhl2* expression is uniquely associated with early fate commitment in the *Atoh1* lineage<sup>33</sup>. Barhl1 and Barhl2 are highly conserved through evolution<sup>reviewed in 3</sup>, and their functional conservation is evidenced

through studies in various species, including mouse, *C. elegans* and the acorn worm *Saccoglossus kowalevskii*<sup>106,107</sup>. Our data demonstrate that in the amphibian URL, Barhl1 mostly acts through inhibiting TCF activity. It remains to be investigated which of the *Barhl* TF inhibits TCF activity in mammals.

### **Biological significance of our findings**

Our study reveals previously undescribed roles for TCF and Barhl1 in the early development of URL-derived GNP s. We show that Barhl1 is the main repressor of Wnt/TCF activity in this germinative area. Our analysis reveals a set of Barhl1 target genes and opens the way for further characterization of relevant targets in order to create a global picture of GNP development and for further investigations of their relevance in adult NSC niche biology.

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### **AUTHOR CONTRIBUTIONS**

Conceptualization, J.BR., and B.C.D.; Methodology, J.BR., M.D., A.E., and B.D., Software, M.D.; Investigation, J.BR., M.D., A.E., A.A., and B.C.D.; Data curation, J.BR., and M.D., Writing – Original Draft, J.BR., and B.C.D.; Writing — Review & Editing, J.BR., M.D., A.E., and B.C.D.; Visualisation, J.BR., and B.C.D.; Funding Acquisition, B.C.D.; Resources, J.BR., M.D., A.E., and B.C.D.; Supervision, B.C.D.;

### **DECLARATION OF INTERESTS**

The authors declare no competing interests.

### **STAR METHODS**

### **EXPERIMENTAL MODEL**

#### ***Xenopus* embryos care and husbandry**

*X. laevis* embryos were obtained by conventional methods of hormone-induced egg laying and *in vitro* fertilization and were staged according to<sup>108</sup>. *X. tropicalis* transgenic Wnt reporter pb1n7LefdGFP has been generated as previously described<sup>22,56,57</sup>. Briefly, the synthetic Wnt-responsive promoter consists of 7 copies of TCF/LEF1 binding sites and a TATA box driving destabilized green fluorescent protein (eGFP) and a polyA sequence. *gfp* expression reveals Wnt/TCF activity. *X. tropicalis* embryos were obtained by *in vitro* fertilization. Experimental procedures were specifically approved by the ethics committee of the Institut de Biologie Paris Seine (IBPS) (Authorization 2020-22727 given by CEEA #005) and have been carried out in strict accordance with the European community directive guidelines (2010/63/UE). B.D. carries the authorization for vertebrates' experimental use N°75-1548.

## METHOD DETAILS

### Plasmids design and preparation

*mbarhl1-HA-GR* contains the full-length *mbarhl1* sequence (two Engrailed-Homology (EH1) motifs, Nuclear Localization Signal (NLS); Homeodomain HD); and the C-terminal part), followed by an HA tag at the C-terminal part. This construct is inducible as it contains a glucocorticoid receptor which can be activated by dexamethasone (10uM). Dexamethasone-inducible *mbarhl2-EHs-GR* contains the first 182 amino acids (a.a) of mouse Barhl2 full-length cDNA, which correspond to the N-terminal EHs Gro-binding domains, and has been shown to act as a dominant negative<sup>52</sup>. The full-length *mbarhl1-HA-GR* and truncated *mbarhl2-EHs-GR* constructs were generated in pCS2+ by Vector Builder. Non-inducible *mbarhl1-Myc* and *xbarhl1-Flag* were generated by GeneScript. Peptide sequences of the tags used are the following: HA (YPYDVPDYA); FLAG (DYKDDDDK) and MYC (EQKLISEEDL). The constitutive repressor pCS2-Tcf7l1-Δβcat-GR was a gift from H. Clevers<sup>55</sup>, and consists of the full-length Tcf7l1 lacking the β-catenin-binding domain (BCBD), which reinforces its repressive activity. Constructs used for immunoprecipitation assay are pCS2+ *mbarhl1-3xFlag-HA* which was generated by Vector Builder. It contains the full-length *mbarhl1* sequence followed by three Flag tags and one HA tag at the C-terminal part. pCS2+ *Myc-Tcf7l1*, pCS2+ *Flag-Gro4* and pCS2+*GroHA* have been previously described<sup>52</sup>. All necessary sequences were obtained from NCBI database. Constructs were validated by western blot on extracts from injected embryos or cell lysates.

### mRNA synthesis, morpholino oligonucleotides (MOs) and *Xenopus* injection

Capped messenger RNAs (mRNAs) were synthesized using the mMessage mMachine kit (Invitrogen) and resuspended in RNase-free H<sub>2</sub>O. Antisense morpholino oligonucleotides (MOs) were generated by Gene Tools. ATG start-site *MObarhl1-1* and *MObarhl1-2* were designed to block initiation of xBarhl1 protein translation. The MO were designed in a region overlapping the translation initiation site, so that they do not recognize mouse *Barhl1* or *xbarhl2* mRNA (Fig. S3). To establish the specificity of the MO effect, we tested the ability of *MObarhl1-1* and *MObarhl1-2* to specifically inhibit translation of *xbarhl1* mRNA. Flag-tagged *xbarhl1* (*xbarhl1-flag*) or myc-tagged *mBarhl1* (*mBarhl1-myc*) were co-injected with *MObarhl1-1*, or *MObarhl1-2*, or a control MO (*MOct*) (Fig. S2). Western blot analysis on extracts from injected embryos confirmed a *MObarhl1*-mediated dramatic decrease in *Xenopus* Barhl1 protein levels, while *MOct* had no effect. We also observed that *MObarhl1-1* did not decrease *mBarhl1-myc* protein levels (Fig. S2; S3). *MObarhl1-1* was used for both *X. laevis* and *X. tropicalis* as the

mRNA sequence of *barhl1* is highly conserved between both species, more specifically in the region on which MO*barhl1* is hybridized. Standard control MO from gene tools was used in this study. MO sequences and doses are summarized in table 2.

*Xenopus* embryos were injected unilaterally in one dorsal blastomere at the four and eight-cell stage together with *gfp* as a tracer for phenotype analysis by *in situ* hybridizations (ISH), except for CRISPR/Cas9 genome editing and RNAseq analysis (see corresponding sections in material and methods). MOs were heated for 10 min at 65°C before usage. Injected embryos were transferred into 3% Ficoll in 0.3X Marc's Modified Ringer's (MMR) buffer (stock solution: 1M NaCl, 20 mM KCl, 20 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 50 mM HEPES pH 7.4). 10nl of mRNA or MO solution was injected together with a tracer in *X. laevis* while 5nl were injected in *X. tropicalis*. In *X. laevis*, MOs or mRNAs were co-injected with *gfp* mRNA (100 pg). MOs or mRNAs were co-injected with *mcherry* (100 pg) in *X. tropicalis*. Concentration of injected mRNA and MOs per embryo have been optimized in preliminary experiments. The minimal mRNA or MO quantity that induced the specific phenotype without showing toxicity effects was used. For embryos injected with inducible constructs, half of the injected embryos were treated with 10µM dexamethasone at stage 35, while the other half were left untreated and served as control. All necessary *Xenopus* sequences were obtained from <https://www.xenbase.org/entry/>.

### ***in situ* hybridization**

Embryos were staged according to <sup>108</sup>, and collected at the desired stage, then fixed in PFA4% for 1-2 hours at room temperature and dehydrated in 100% MeOH. ISH were performed using digoxigenin (DIG)-labeled probes. Antisense RNA probes were generated for the following transcripts: *atoh1*, *barhl1*, *hes4*, *neurod1*, *pax6*, *n-myc*, *otx2*, *zic3*, *wnt2b*, *wnt8b* and *gfp* according to the manufacturer's instructions (RNA Labeling Mix, Roche). pCS2-Gfp is a gift from David Turner (University of Michigan, Ann Arbor, MI, USA). pBSK+xBarhl1 is a gift from Roberto Vignali (Unità di Biologia Cellulare e dello Sviluppo, Pisa Italy). pCS2-Atoh1 is a gift from G. Schlosser (University of Galway, Ireland). pBSK+Wnt2b was a gift from S. Sokol (Icahn School of Medicine at Mount Sinai, NY, USA). pBSK+Wnt8b was a gift from J Christian (University of Utah, USA). ISH was processed following the protocol described by (El Yakoubi et al., 2012; Sena et al., 2019). DISH was processed as described by <sup>109</sup>. For *X. laevis* embryos, following rehydration, the eyes and ectoderm overlying the anterior neural tube were removed, which allows to skip the further Proteinase K (PK) treatment. Dissections weren't performed on *X. tropicalis* embryos which were treated with PK. In both cases, bleaching was carried out, and samples were incubated with the probes overnight. Alkaline phosphatase-conjugated anti-DIG or anti-FLUO antibodies (Roche) were incubated 3 hours at room temperature. Enzymatic activity was revealed using NBT/BCIP (blue staining) and INT/BCIP (red staining) substrates (Roche). Following ISH, post-fixation was carried out in PFA 4% and the neural tubes of control and injected *X. laevis* embryos were dissected in PBS-0.1% Tween and stored in 90% glycerol. *X. tropicalis* embryos were stored in PFA 4%. Dissected neural tubes or embryos were photographed on a Leica M165 FC microscope equipped with Leica DFC320 camera using the same settings to allow direct comparison. Dorsal and lateral views of the dissected neural tubes were photographed.

### **Immunofluorescence**

Immunofluorescence was carried out as previously described<sup>51</sup>. The entire brains of wild-type (WT) and MO-injected *X. laevis* embryos were carefully dissected and transferred into a tube containing PBS-0.1% Tween, where they were progressively permeabilized. Samples were incubated with primary antibody (anti-Phospho-Histone H3; Upstate Biotechnology Cat#06–570; d1:500) at 4°C overnight. Cellular nuclei were stained with bisBenzimide (BB) (Sigma) which was added to the solution containing diluted secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG; Invitrogen; d1:500) and incubated at 4°C overnight. Neural tubes were captured on a Zeiss Axio Observer.Z1 microscope equipped with apotome. Acquisitions were taken using the Z stack tool from the most superficial layer to deeper layers.

### **Immunoprecipitation in transfected HEK293T cells**

HEK293T cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) (Gibco). Cells were transfected with expression vectors for *pCS2-mbarhl1-3xFlag-HA*; *pCS2-mbarhl1-Myc*; *pCS2-Tcf7l1-Myc*; *pCS2-Gro-Flag* and *pCS2-Gro-HA* encoding tagged proteins using the Phosphate Calcium method. Plasmids coding for pCS2+ or pSK+ were used as a supplement to ensure that cells in different dishes were transfected with the same quantity of expression vectors and plasmids (a total of 2 µg). Thirty-six hours post-transfection, cells were harvested and lysed in ice-cold lysis buffer (20 mM Tris pH7.6, 150 mM NaCl, 1% Triton, 1 mM EDTA) supplemented with completeTM protease inhibitor (Roche). Cell lysates were centrifuged 15min at 14,000 rpm. Protein complexes were precipitated from the cell lysates with anti-c-Myc antibody (clone 9E10). Protein complexes were then precipitated with protein A-Sepharose beads (Sigma) pre-washed with lysis buffer. Immunoprecipitated proteins were eluted from protein A beads by heating beads in Laemmli sample loading buffer (BioRad).

### **Western blot**

Western blot (WB) analysis was performed on protein extracts from injected/WT *Xenopus* embryos, and on extracts from transfected HEK293T cells. *Xenopus* embryos were injected with *mbarhl1HAGR*, *xbarhl1Flag*, *mBarhl1Myc*, *mBarhl2EHsGR* mRNA at the two-cells stage, targeting both blastomeres. Proteins were extracted at stage 10 with lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5%NP40, 5mM EDTA supplemented with a cocktail of protein inhibitors). WB was carried out using the conventional methods. Proteins were separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked using 5% milk and incubated with the corresponding primary and secondary antibodies diluted in 5% milk (summarized in tables 3 and 4). Proteins were detected with Western Lightning Plus-ECL (Perkin Elmer Life Sciences). Membrane stripping was carried out between two staining steps using stripping buffer (Thermo Scientific) for the removal of primary and secondary antibodies from the membranes. ChemiDoc MP Imaging System (BioRad) was used for imaging the blots.

### **CRISPR/Cas9**

Three CRISPR target sites (*barhl1-1*: GAGTCGGACGAGGCCATGGAAGG), *barhl1-2*: ACCAGCTCTGTGCGACAGAAATGG, *barhl1-3*: AGAGTTGGACTCCGGGCTGGAGG) cutting respectively at 2, 37 and 230 bp from the beginning of the coding sequence were selected for their high predicted specificity and efficiency using CRISPOR online tool (<http://crispor.tefor.net/>). Alt-R crRNA and tracrRNA were purchased from Integrated DNA

Technologies (IDT, Coralville, IA, USA) and dissolved in duplex buffer (IDT) at 100 $\mu$ M each. cr:tracrRNA duplexes were obtained by mixing equal amount of crRNA and tracrRNA, heating at 95°C for five minutes and letting cool down to room temperature. gRNA:Cas9 RNP complex was obtained by incubating 1 $\mu$ L 30 $\mu$ M Cas9 protein (kindly provided by TACGENE, Paris, France) with 2 $\mu$ L cr:tracrRNA duplex in a final volume of 10  $\mu$ L of 20mM Hepes-NaOH ph 7.5, 150mM KCl for 10 min at 28°C. *X. tropicalis* one-cell stage embryos were injected with 2nL of gRNA:Cas9 RNP complex solution and were cultured to the desired stage. For coinjection, the three complexes were mixed at equal quantity.

Single embryo genomic DNA was obtained by digesting for 1h at 55°C in 100  $\mu$ L lysis buffer (100 mM Tris-HCl pH 7.5, 1 mM EDTA, 250 mM NaCl, 0.2% SDS, 0.1  $\mu$ g/ $\mu$ L Proteinase K), precipitating with 1 volume of isopropanol and resuspended in 100 $\mu$ L PCR-grade water. The region surrounding the sgrNA binding sites was amplified by PCR using *X. tropicalis* *Xt\_barhl1\_F* (CAGCTCCTCCGACTTTGTG) as forward primer and *Xt\_barhl1\_R* (GTTGCCCGTTGCTGGAATAA) as reverse primer. CRISPR efficiency was assessed by T7E1 test <sup>110</sup> on mono-injected embryos and by detecting deleted fragments on coinjected embryos.

### RNA-sequencing and data analysis

*X. laevis* embryos were injected with three different conditions: *MObarhl1-1*; *MObarhl1-2* and *MOct* in the two dorsal blastomeres at four cells stage. At stage 42, neural tubes were extracted in RNase-free conditions, and the rhombomere 1 which includes the URL was carefully dissected. For each condition, three biological replicates were collected. Each replicate contains three rhombomeres, which was the optimal number to get the minimal RNA concentration required for this experiment (Total RNA concentration was ~30ng per sample). Briefly, total RNA was extracted using the TRIzol reagent (ambion) according to the manufacturer's instructions. The overall RNA quality was assessed using Agilent High Sensitivity RNA ScreenTape System. Samples with an RNA Integrity Number (RIN) > 9 were used for subsequent analysis.

Sequencing was performed using Illumina NovaSeq (paired-end sequencing) by Next Generation Sequencing Platform (NGS) (Institut Curie). RNAseq data processing was performed using Galaxy server of ARTBio platform (IBPS).

Data sets were aligned against the *X. laevis* v10.1 genome assembly downloaded with its corresponding annotation file from Xenbase <sup>111</sup>. Alignment was made using two read mapping programs, STAR v2.7.8a <sup>112</sup> and HISAT2 v2.2.1 <sup>113</sup>. Quality control checks were assessed using FastQC v0.73 <sup>114</sup> and summarized in a single report generated by MultiQC v1.9 <sup>115</sup>. As both alignment programs provided comparable results, we proceeded with STAR alignment tool. The number of aligned reads was counted by featurecounts tool v2.0.1 <sup>116</sup>. Finally, we used the DESeq2 v2.11.40.6 package <sup>117</sup> to determine differentially expressed genes (DEG) from count tables. In the present study, genes with adjusted p value pAdj<0.001 were selected as significant DEG. Venn diagrams were produced with JVenn v2021.05.12 <sup>118</sup>. Volcano Plots v0.0.5 were generated to show significant upregulated and downregulated genes, only a selection of DEG names were represented.

Further analysis and data visualization were performed using R v4.2.1package. A heatmap was generated to visualize gene expression across the samples. To overcome the lack of

Xenopus gene ontology (GO) annotation, we replaced *X. laevis* gene symbols with the Human orthologs. Functional enrichment analysis was performed using the *compareCluster* function of ClusterProfiler v4.8.1<sup>56</sup> to identify GO-term enrichment amongst DEG with pAdj<0.001 as threshold. It provides the biological processes, cellular components, and molecular functions of DEG and compares each of the three subgroups between both knockdown conditions.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Image processing and analysis

For ISH performed on embryos injected unilaterally, comparison of the expression levels between injected and control sides was assessed using a specific macro from ImageJ v2.1.0/1.53c<sup>119,120</sup>. The macro functions based on the RGB color mode. RGB images are split into three channels (red, green, and blue) and pixel values corresponding only to the blue channel are recorded, excluding the red and green channels, since the signal recorded on the blue channel represents the expression levels. For each image, the region of interest (ROI) was specified, and its dimensions were fixed, such that the same ROI is placed on the control and injected side of the embryo which prevents any subjectivity in ROI determination. Measured are the area corresponding to the blue signal; the mean or average value of signal within the selected ROI; and the integrated density which is the equivalent of the product of area and mean, as it sums the values of pixels in the selection. In this study, ratio of integrated density measured in the injected *versus* control side was assessed. The macro is available from the authors upon request and will be available as a plug-in in ImageJ.

The same macro was used for the analysis of CRISPR/Cas9-injected embryos, except that ROI was placed in all the rhombomere 1 as the entire embryo was targeted. The mean of int. density values of control embryos was compared to each individual int. density value of control and injected embryo. Phenotype penetrance was evaluated by counting and classifying embryos based on the intensity of *gfp* expression increase.

For immunofluorescence, Z-stack images were reconstructed and processed using ImageJ v2.1.0/1.53c. PHH3-positive cells were counted, and the length of the RL was measured on the control and injected side. Ratio of PHH3-positive cells and RL length in the injected *versus* control side was measured.

For the same experiment, all images were acquired using the same magnification and camera settings. In this way, all images were processed in a standardized manner, such that results are objectively analyzed. Final images were processed with Adobe Photoshop (v24.00).

### Statistical analysis

Three independent experiments were performed for each condition analyzed. Dissected neural tubes and embryos were analyzed individually, and the results were pooled for data representation. Statistical analyses were implemented with R. Normality in the variable distributions was assessed by the Shapiro-Wilk test. Furthermore, the Levene test was performed to probe homogeneity of variances across groups. Variables that failed the Shapiro-Wilk or the Levene test were analyzed with non-parametric statistics using the one-way Kruskal-Wallis analysis of variance on ranks followed by Nemenyi test post hoc and Mann-

Whitney rank sum tests for pairwise multiple comparisons. Variables that passed the normality test were analyzed by means of one-way ANOVA followed by Tukey post hoc test for multiple comparisons or by Student's *t* test for comparing two groups. A *p*-value of <0.05 was used as a cutoff for statistical significance. Results are presented as the means ± SEM. The statistical tests are described in each figure legend.

	MO sequence	Dose	Reference
<i>MObarh1-1</i>	CCCAAATCCGTTAGACCCTTCATG	15ng	This study
<i>MObarh1-2</i>	AAAGCCTGTTCGACTCTCACAAATG	20ng	This study
<i>MOct</i>	CCTCTTACCTCAGTTACAATTATA	20ng	GeneTools

**Table 2: Morpholino (MO) oligonucleotide sequences used in this study**

Primary Ab	Source	Host	Dilution	Use
Barh12	Covalab	Rabbit	1:500	Western Blot
HA epitope	Roche High Affinity clone 3F10	Rat	1:1000	Western Blot
c-Myc epitope	Santa Cruz Biotechnology clone 9E10	Mouse	1:5000	Western Blot
Flag epitope	Sigma-Aldrich F7425	Rabbit	1:1000	Western Blot (Extracts from HEK293T)
Flag epitope	Sigma-Aldrich F3165	Mouse	1:1000	Western Blot (Extracts from <i>Xenopus</i> embryos)
Actin epitope	Sigma-Aldrich A2066	Rabbit	1:2000	Western Blot

**Table 3: Primary antibodies (Ab) used in this study**

Secondary Ab	Source	Host	Dilution	Use
HRP anti-mouse IgG	Jackson ImmunoResearch 115-035-003	Goat	1:10 000	Western Blot
HRP anti-rabbit IgG	Jackson ImmunoResearch 111-035-003	Goat	1:10 000	Western Blot
HRP anti-rat IgG light chain specific	Jackson ImmunoResearch 112-035-175	Goat	1:10 000	Western Blot

**Table 4: Secondary antibodies (Ab) used in this study**

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## MAIN FIGURES

### Figure 1: Temporal and spatial expression pattern of genes involved in granule neuron progenitors' (GNP) development

(A) Neural tube dissection and analysis. Shown on the left is a representation of stage (st.) 45 *X. laevis* embryo. Following ISH, neural tubes are dissected as shown on the middle (entire neural tube) and right (a focus on the rhombomere 1 (R1)) panels. The proliferation marker *nmyc* is expressed in the upper rhombic lip (URL) (blue arrow), the ventricular zone VZ (white arrow). Red dotted lines delineate rhombomere 1 (R1) located caudal to the midbrain-hindbrain boundary (MHB). *nmyc* marks proliferating progenitors at the boundary between R1 and R2 and is used as a marker of cerebellar primordium's caudal limit. (B) ISH analysis of GNP markers in *X. laevis* embryos at the indicated Nieuwkoop and Faber stages. Shown are dorsal and lateral views of the R1. From st. 41 to st. 48 stem/progenitor markers *atoh1* (Ba-b'), *nmyc* (Ca-b'), and *hes4* (Ha, a') display a strong expression in the URL and in the EGL. *hes4* and *nmyc* are also detected in the VZ. (Da, a') *otx2* expression is first detected in caudal EGL and becomes restricted to the cerebellar plate (CP) (green arrow) at st. 48 (Db, b'). At st. 41 committed GNP markers *pax6* (Ea, a') and *barhl1* (Fa, a'), together with the differentiation marker *neurod1* (Ga, a') are detected in the caudal EGL and the cerebellar plate. As development proceeds, transcripts for these markers are detected in the CP and their expression significantly increases in this area (E-G, b, b'). Fully differentiated GNs settling in the internal granule layer (IGL) are stained with *neurod1* as observed in lateral views of st. 48 *X. laevis* embryos. The CP is devoid of *atoh1*, *hes4*, and *nmyc* expressions. CP: cerebellar plate; VZ: ventricular zone; URL: upper rhombic lip; EGL: external granule layer; R: rhombomere; MHB: midbrain-hindbrain boundary. Scale bar 150μm.

### Figure 2: Tcf activity is required for the induction of the URL and its inhibition by Barhl1 is necessary for the proper progression of GNPs development

(A) Overexpression of *tcf7l1* inhibits/abolishes *atoh1* expression in a dose dependent manner. ISH analysis of *atoh1* expression in the rhombomere 1 (R1) showing dorsal views (a, b) and lateral views of control sides (a', b') and injected sides (a'', b'') of stage 45 *X. laevis* embryos unilaterally injected with 200pg (a, a', a'') and 100pg (b, b', b'') of inducible *tcf7l1-Δβcat-GR*. The non-injected side is an internal control. (B) Forced expression of *tcf7l1* increased GNP differentiation. ISH analysis of the commitment/differentiation markers *barhl1*, *pax6* and *neurod1* (a-c) in stage 45 *X. laevis* embryos. (C) *barhl1* overexpression phenocopies defects of *tcf7l1* overexpression. Dorsal views showing *atoh1*, *barhl1* and *neurod1* (a, b, and c respectively) expressions in the R1 primordium of stage 45 *X. laevis* embryos injected with *mBarhl1GR* (200pg). Lateral views of *atoh1* expression in control side (a') and injected side (a'') are shown. Integrated densities (*IntDen*) of markers' expressions were measured. Ratio of markers expression in injected side over control side is represented (Ac; Bd; Cd) and indicated as mean ± s.e.m. Dotted lines separate injected and control sides. Scale bar 150μm. Square brackets delineate R1. Dex: dexamethasone; *inj*: injected side. Statistical analysis C: One-way ANOVA one way anova ( $F_{(2,31)}=437.5$ ;  $p < 0.001$ ) followed by post hoc Tukey test. Bd, Cd: student's t-test. Data are presented as means ± SEM.\*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

### Figure 3: In the cerebellar URL, antagonistic activities of Barhl1 and Tcf are required for the proper development of GNPs

(A-D) Morpholino (MO)-mediated inhibition of Barhl1 induces an ectopic expansion of *atoh1* in the upper rhombic lip (URL) and cerebellar plate and delays GNP differentiation. *in situ* hybridization (ISH) of stage (st.) 45 *X. laevis* embryos unilaterally injected with (A) *MObarhl1-1* (15ng) and (B) *MObarhl1-2* (20ng). The non-injected side is an internal control. Shown are dorsal views of *atoh1* (Aa; Ba), *pax6* (Ab; Bb), and *neurod1* (Da; Db) expressions in the cerebellar anlage. Lateral views of *atoh1* expression in control sides (Aa', Ba') and injected sides (Aa'', Ba'') are shown. (C) Quantification of (A) and (B). (Dd-c) *MObarhl1* phenotype is rescued by *mBarhl1* overexpression. ISH analysis showing rescue of *neurod1* expression in embryos co-injected with *MObarhl1-1* and *mBarhl1* mRNA. (Dd) Quantification of (D). (E) Inhibition of Tcf activity compensates for Barhl1 depletion. ISH analysis of *pax6* expression in the cerebellar anlage of stage 48 *X. laevis* embryos unilaterally injected with (Ea) *MObarhl1-1* (15ng), (Eb) *tcf7l1-Δβcat-GR* at 100pg and (Ec) *tcf7l1-Δβcat-GR* at 200pg. *pax6* expression was rescued when *MObarhl1-1* (15ng) was co-injected with *tcf7l1-Δβcat-GR* at 100pg (Ed) and at 200pg (Ee). (f) Quantification of (E). Ratio of markers expression in injected side over control side is indicated as mean ± s.e.m. Dotted lines separate injected and control sides. Scale bar 150μm. *inj*: injected side. Statistical analysis was carried out using student's t-test. C:*atoh1*:One-way ANOVA( $F_{(2,21)}=19.9$ ;  $p < 0.001$ ) followed by post hoc Tukey test. *pax6*: One-way ANOVA ( $F_{(2,14)}=8.63$ ;  $p = 0.004$ ) followed by post hoc Tukey test. Dd:Kruskal-Wallis test (Chi square=35.6  $p < 0.001$ , df=3) followed by Nemenyi test post hoc. Ef:One-way ANOVA( $F_{(4,31)}=32.9$ ;  $p < 0.001$ ) followed by post hoc Tukey test. Data are presented as means ± SEM\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

#### **Figure 4: Barhl1 physically interacts with Tcf7l1 and Gro and limits Tcf transcriptional activity**

(A) HEK293T cells were transfected with plasmids encoding indicated tagged proteins. Cell lysates were immunoprecipitated (IP) by anti-cMyc antibody. Input and IP samples were subjected to western blot analysis using indicated antibodies. Equal amounts of protein lysates were loaded on SDS-gel. (Aa) Barhl1 interacts with Groucho 4 (Gro4) and Tcf7l1. The interaction between Barhl1 and Tcf7l1 is detected in the presence and in the absence of Gro. (B) Tcf activity is detected in the URL in an area overlapping with that of *atoh1*, and complementary to that of *barhl1*. ISH in *X. tropicalis* pb1n7LefdGFP line at indicated stages (st.) showing *gfp* (Tcf activity) (Ba, b, c, d), *atoh1* (Ba', b', c', d') and *barhl1* (Bc'', d'') expression patterns. (Bc'') DISH showing expression of *barhl1* (blue) and *gfp* (red). Dorsal views of one side of the embryos are shown. (C, D) Barhl1 limits Tcf transcriptional activity *in vivo*. ISH analysis of *gfp* expression in *X. tropicalis* pb1n7LefdGFP embryos injected either unilaterally with (Ca) *mBarhl1GR* (200pg) and (Cb) *MObarhl1-1* or before division with (Db) *Crispr-barhl1*. Embryos injected with *Crispr-barhl1* were compared to their wild-type (WT) siblings. (E) Interaction between Barhl1 and Gro is required for Barhl1 function. *mBarhl2EHsGR* only contains the two EH1 motifs of Barhl2 and acts as a dominant negative by capturing Gro. (Ea) ISH showing *atoh1* expression in injected versus control side. Integrated densities (*IntDen*) of markers' expressions are measured. Ratio of markers expression in injected side over control side is represented (Cc; Dc; Eb) and indicated as mean ± s.e.m. Percentage of phenotype penetrance is quantified in embryos injected with *Crispr-barhl1* versus WT embryos based on indicated criteria. Dotted lines separate injected and control sides. Scale bar 150μm. *inj*: injected side. Statistical analysis Cc: One-way ANOVA( $F_{(2,35)}=111.3$ ;  $p < 0.001$ ) followed by post hoc Tukey test was carried out using Eb: student's t-test. Data are presented as means ± SEM \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

### Figure 5: In the URL, Barhl1 activity as an inhibitor of Tcf transcription is required for GNP s to exit their germinative zone and become post-mitotic

(A) Barhl1-KD induces an increase in the URL length associated with increased proliferation within this compartment. (a-d) Imaging of cerebellar anlage of stage 48 *X. laevis* tadpoles unilaterally injected with *MOct* and *MObarhl1-1* (15 ng). Collected neural tubes were stained for the mitotic marker PhosphoHistone-H3 (PHH3) (green) merged with bisbenzimidole (BB) (red). In *Xenopus*, the EGL is devoid of proliferating cells. (e-g) Quantifications of (A). The ratio of (e) measured URL length and (f) PHH3+ cells in injected side over control side are represented. (Ac, d) PHH3 positive cells are ectopically detected in the cerebellar plate (Ac, d white arrow heads) of injected embryos. (g) Percentage of PHH3+ cells located inside the URL compared to that located outside the URL were quantified on the injected side and on the control side. (E) The abnormal increase in URL length is rescued upon co-inhibiting Tcf and Barhl1 activities. ISH analysis of *n-myc* expression in the cerebellar anlage of stage 48 *X. laevis* embryos unilaterally injected with (Ba) *MObarhl1-1* (15ng), (Bb) *tcf7l1-Δβcat-GR* at 100pg and (Bc) *tcf7l1-Δβcat-GR* at 200pg. *n-myc* marks the boundaries between different rhombomeres which allows the exact measurement of URL length. URL length was rescued when *MObarhl1-1* (15ng) was co-injected with *tcf7l1-Δβcat-GR* at 100pg (Bd) or at 200pg (Be). Ratio of URL length in injected side over control side is represented (Bf) and indicated as mean ± s.e.m. Dotted lines separate injected and control sides. Scale bar 150μm. *inj*: injected side; cp: choroid plexus; URL: Upper Rhombic Lip; VZ: Ventricular Zone; R1-R2: Rhombomere 1 and 2; MHB: Midbrain-Hindbrain Boundary. Statistical analysis Ae, Af: was carried out using student's t-test. Bf: One-way ANOVA( $F_{(4,49)}=65.1$ ;  $p < 0.001$ ) followed by post hoc Tukey test. Data are presented as means ± SEM \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

### Figure 6: RNA-sequencing data processing and analysis

(A) Principal Component Analysis (PCA) plots were obtained based on RNAseq data aligned with STAR and reads counted using feature-counts. Three samples have been generated for each condition. Sample groups are represented by different colors as indicated. Each dot refers to a sample. Samples showing similar gene expression profiles are clustered together. (B) **Volcano plots** showing a selection of significant DEGs with  $p\text{Adj} < 0.001$  in (a) *MObarhl1-1* vs *MOct* and (b) *MObarhl1-2* vs *MOct*. Upregulated genes with  $\text{Log2FC} > 0.4$ , and downregulated genes with  $\text{Log2FC} < -0.4$  are shown. Red and blue dots indicate significant DEGs that are upregulated and downregulated, respectively. Grey dots denote RNAs with non-significant difference. PCA and volcano plots were generated using Galaxy. (C) **Differentially expressed genes (DEGs) visualization** Heatmap displaying expression profiles of most significantly upregulated and downregulated DEGs for each condition (*MObarhl1-1* vs *MOct* and *MObarhl1-2* vs *MOct*). Each row represents a gene, and each column represents a sample. Results are shown as a gradient from blue (downregulated) to dark orange (upregulated). Heatmap is generated using R package. (D) **Gene ontology enrichment comparison**. Shown on Y axis are the altered molecular functions (a) and biological processes (b) for selected (A) upregulated ( $\text{Log2FC} \geq 0.4$ ,  $\text{PAdj} < 0.001$ ), and (b) downregulated ( $\text{Log2FC} \leq -0.4$ ,  $\text{PAdj} < 0.001$ ) DEGs respectively. Enrichment analysis comparing functional profiles among *MObarhl1-1* and *MObarhl1-2* was performed on the DEGs in common between both conditions. Results are visualized as a dot plot based on indicated gene counts and adjusted p-values for enrichment. Dot size corresponds to the count of differentially expressed genes associated with the molecular function or the biological pathway, and dot color refers to the adjusted P-value for enrichment. (E) **TCF Cis Regulatory Motifs (CRM) in regulatory regions of MOBarhl1 DEGs**: pie chart of % of *MObarhl1* DEGs containing either no TCF CRM

(orange), one TCF CRM (grey), two TCF CRM (yellow) and three or more TCF CRM (blue) located 5Kb upstream or downstream of their TSS. (F) **ISH analysis of 4 DEGs:** Dorsal views R1 territory of st. 42 *X. laevis* embryos unilaterally injected with *MObarhl1-1* using *wnt8b*, *wnt2b*, *zic3*, *otx2* as ISH probes as indicated. *inj*: injected side.

**Table 1: Barhl1 and TCF Cis Regulatory Motif (CRM) on regulatory regions of Barhl1 depleted DEGs.** We explore the putative transcription factor-target relationships of Barhl1 (A) and Tcf (B) on Barhl1 depleted DEGs ( $P_{Adj} < 0.001$ ,  $\text{Log2FC} \geq 0.45$  or  $\text{Log2FC} \leq -0.45$ ). We applied R packages Biostrings (v2.64) and GenomicFeatures (v1.48) and determine potential (A) Barhl1 binding sites (5'-C-A-A-T-T-A-C/G-3') (and the mirror sequence (5'-G/C-T-A-A-T-T-G-3'))<sup>60</sup>, or (B) TCF binding sites (5'-C-T-T-T-G-A/T-A-3') (and the mirror sequence (5'-T-A/T-C-A-A-A-G-3'))<sup>61,62</sup> 5Kb upstream and downstream of the Transcription Start Site (TSS) of DEGs using *X. laevis* v10.1 genome assembly downloaded with its corresponding annotation file from Xenbase. For each gene identified through its EntrezID and its symbol, is indicated the sequence of the detected putative CRM and its position within the gene locus.

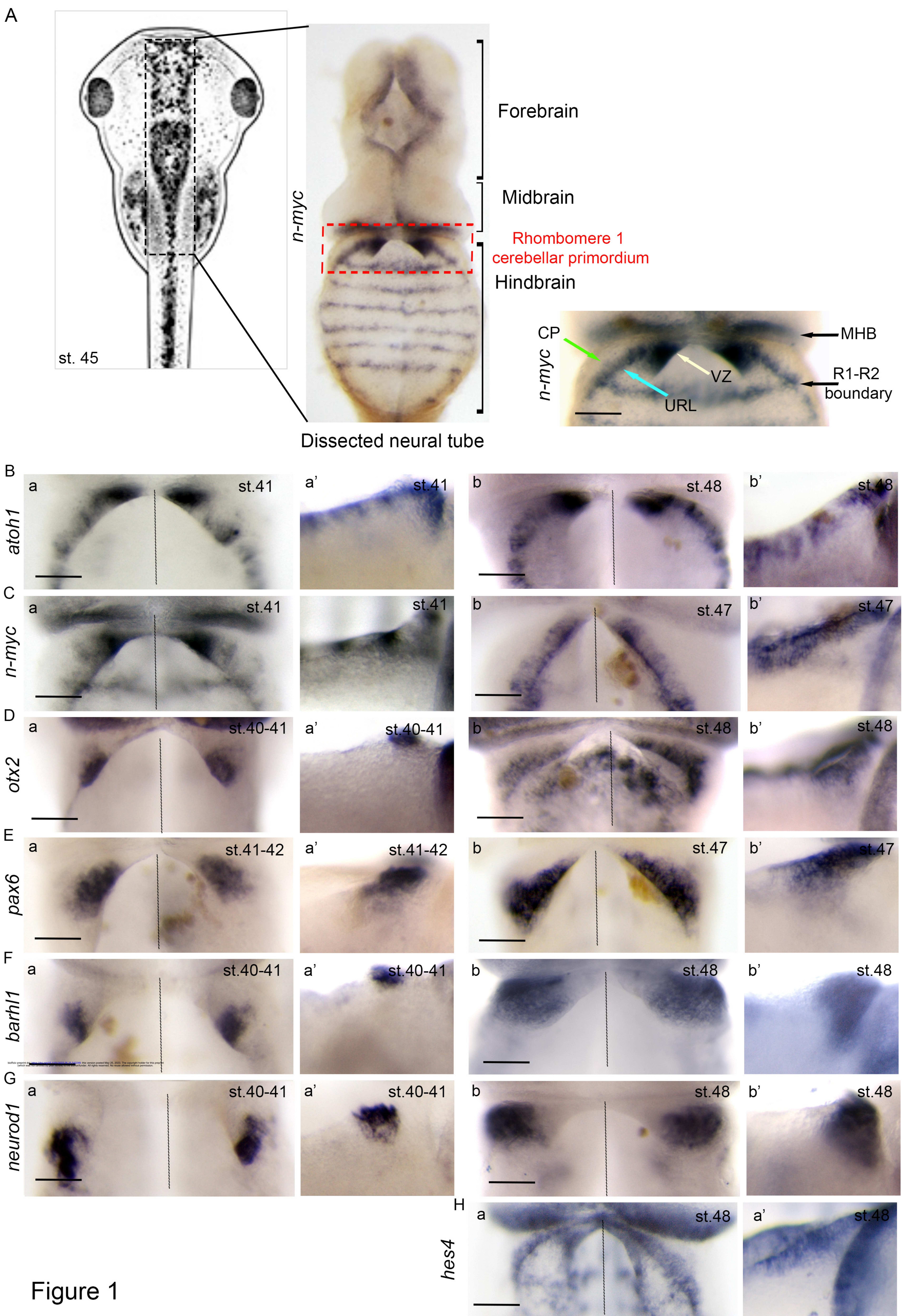



Figure 1




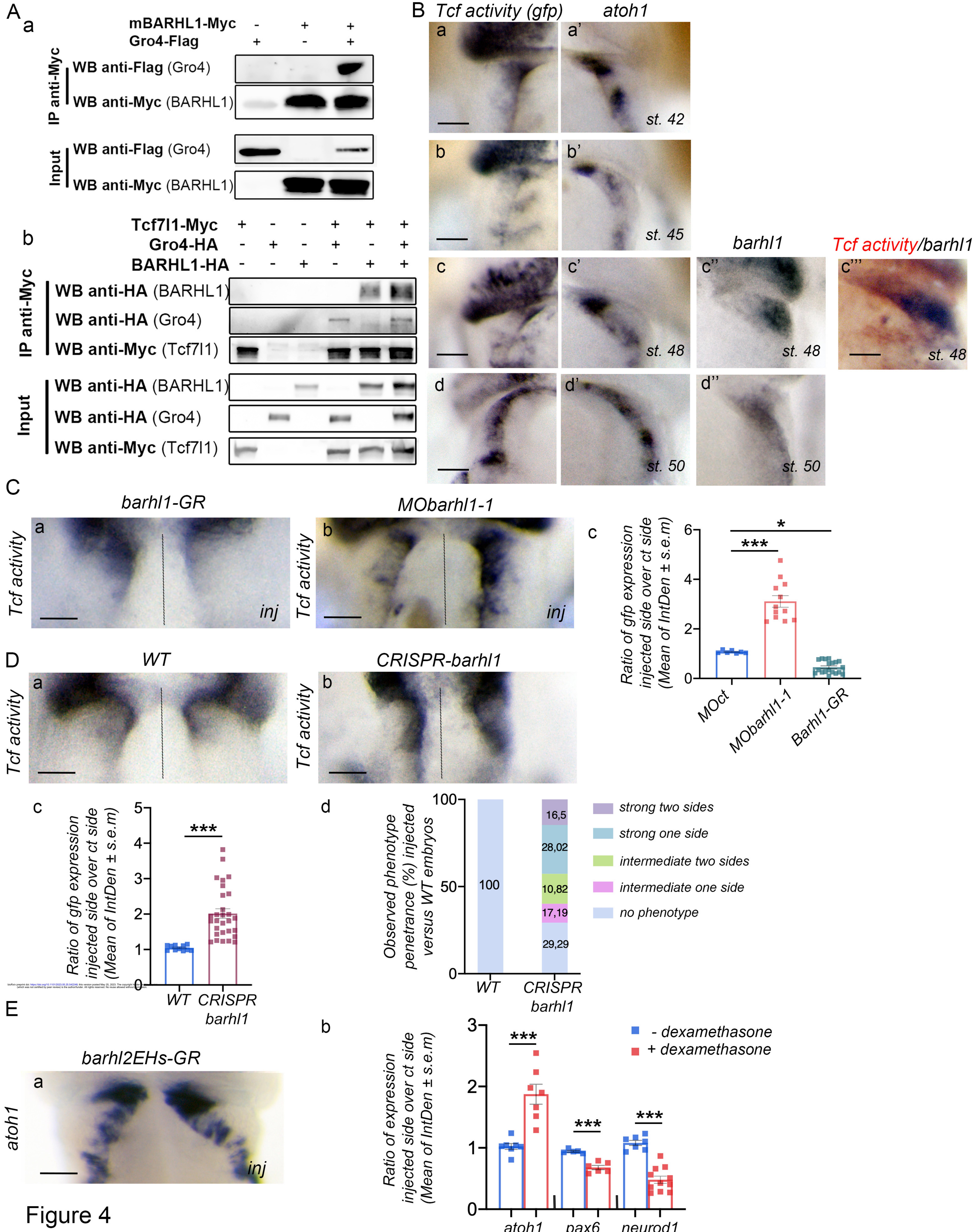


Figure 3




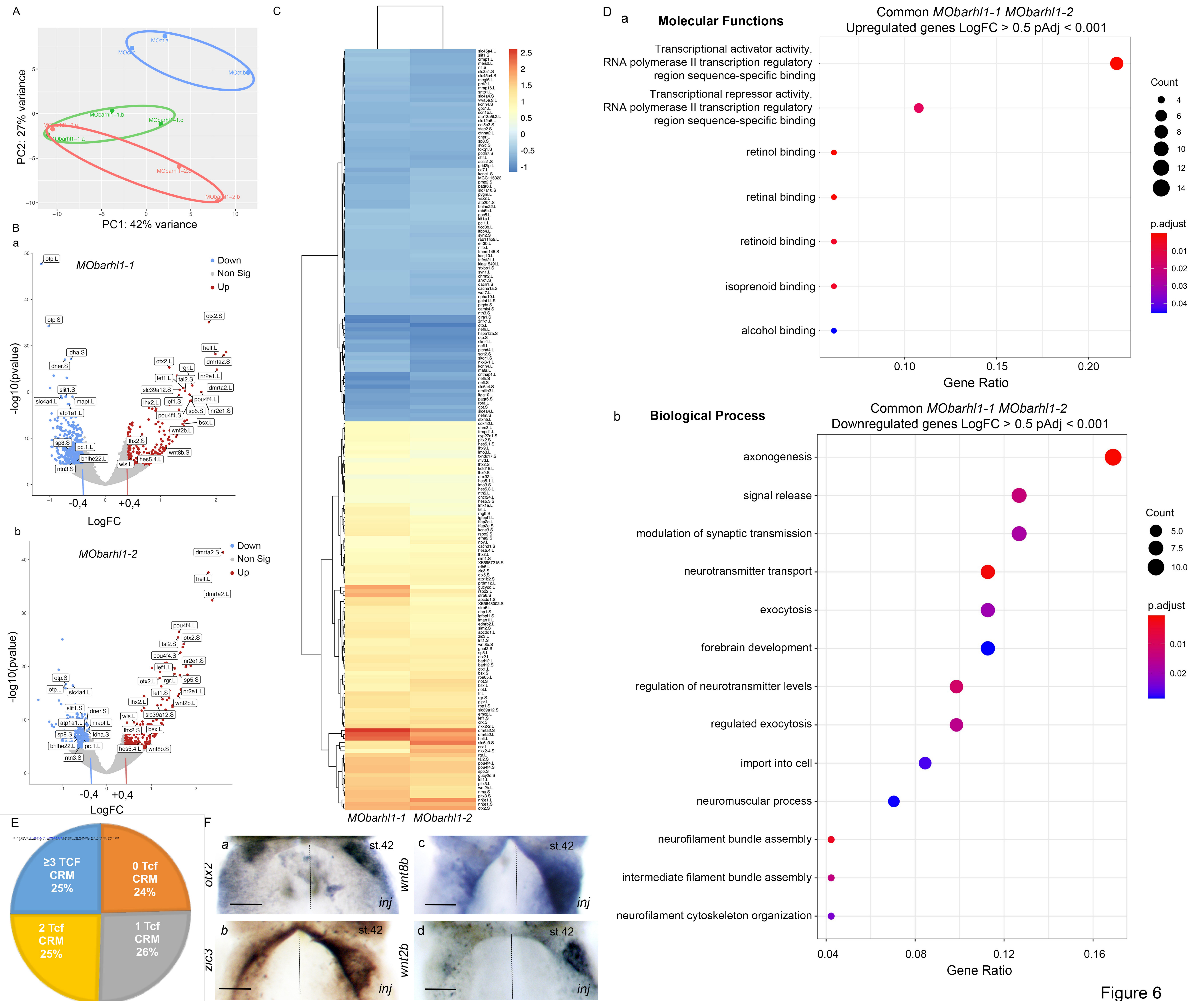


Figure 5



ENTREZID	SYMBOL	Start	End	length	strand	Sequence1	Position1	Sequence	Position2	Sequence	Position3	Sequence	Position4	Sequence	Position4	Sequence	Position5
108717782	acss1.S	30631415	30683070	51656	-	CAATTAG	13714:13721	CAATTAC	27330:2733	CAATTAG	29291:2929	CAATTAG	57936:5794				
503669	ahcy.L	43014140	43030456	16317	+	GTAATTG	1336:1342	GTAATTG	11023:1102	GTAATTG	30161:3016	GTAATTG	30213:3021	CTAATTG	30764:3077	CTAATTG	41612:41618
108710331	ank1.L	139543765	139560010	16246	-	CAATTAC	197:203	CAATTAG	3190:3196	CAATTAC	13842:1384	CAATTAC	14119:1412	CAATTAC	24962:2496	CAATTAC	35066:35072
100036926	ank1.S	22497428	22627635	130208	+	GTAATTG	3984:3990	GTAATTG	15472:1547	GTAATTG	27277:2728	CTAATTG	37819:3782	GTAATTG	43474:4348	GTAATTG	54999:55005
447402	apcdl1.L	111193133	111231649	38517	-	CAATTAC	18010:1801	CAATTAG	32939:3294	CAATTAC	51920:5192						
108695251	apcdl1.S	90867467	90898266	30800	-	GTAATTG	14220:1422	CTAATTG	17674:1768	GTAATTG	18069:1807	CTAATTG	19051:1905	CTAATTG	19187:1919	GTAATTG	30012:30018
108697887	apl1p1.S	109607779	109638526	30748	+	CTAATTG	3271:3277	GTAATTG	8467:8473	CTAATTG	18382:1838	CTAATTG	34384:3439	CTAATTG	51174:5118	CTAATTG	54165:54171
398223	app.L	17761661	17906707	145047	+	CTAATTG	3013:3019	CTAATTG	4506:4512	GTAATTG	6378:6384	GTAATTG	9332:9338	CTAATTG	13294:1330	CTAATTG	25928:25934
379251	app.S	16800553	16933427	132875	-	CAATTAC	7832:7838	CAATTAG	9571:9577	CAATTAG	11331:1133	CAATTAC	26220:2622	CAATTAG	43737:4374	CAATTAC	45770:45776
108716978	atpl13a5.L,2.L	112349351	112395883	46533	-	CAATTAG	9449:950	CAATTAC	6229:6235	CAATTAC	16154:1616	CAATTAG	24346:2435	CAATTAC	38876:3888	CAATTAC	38894:3890
399285	atpl1L	716832	733112	16281	-	CAATTAC	7548:7554	CAATTAC	8646:8652	CAATTG	17326:1733	CAATTAG	19491:1949	CAATTAC	21911:2191	CAATTAC	34864:34870
108708028	atpl1b1.L	83806655	83819946	13292	-	CAATTAC	17936:1794	CAATTAC	23136:2314	CAATTAC	34031:3403	CAATTAG	39829:3983	CAATTAG	40891:4089		
444626	atpl1b2.S	131833350	131838868	5519	-	CAATTAG	8941:8947	CAATTAC	45503:4550								
446855	atpl2b4.L	122980777	131679797	179901	+	GTAATTG	9633:9639	CTAATTG	9715:9721	GTAATTG	9804:9811	GTAATTG	12466:1247	CTAATTG	13181:1318	CTAATTG	17594:1760
108715812	atpl2b4.S	103816801	104014544	197744	+	GTAATTG	10205:1021	CTAATTG	11586:1159	CTAATTG	16128:1613	CTAATTG	18197:1820	CTAATTG	19838:1984	GTAATTG	20261:2026
734627	atpl6v1a.L	2599141	2644208	45068	+	GTAATTG	4750:4756	CTAATTG	49001:4900								
100301951	barh1L	101225765	101320203	4439	+	CTAATTG	9252:9258	GTAATTG	13202:1320	CTAATTG	20176:2018	CTAATTG	21709:2171	GTAATTG	24610:2461	CTAATTG	49049:49055
398182	barh1S	84616978	84621442	4465	+	CTAATTG	12168:12171	GTAATTG	22691:2269	GTAATTG	23320:2332	GTAATTG	25807:2581	GTAATTG	26362:2636	CTAATTG	53983:53985
108719273	bhlhe22.L	126287814	126290806	2993	+	GTAATTG	6234:6240	GTAATTG	21660:2166	GTAATTG	21995:2200	GTAATTG	29890:2989	CTAATTG	33680:3368	GTAATTG	36345:36351
108697000	bsx.L	79604595	79608039	3445	-	CAATTAC	5266:5272	CAATTAC	6168:6174	CAATTAC	9491:9497	CAATTAC	13646:1365	CAATTAC	13670:1367	CAATTAC	14781:14787
108697964	bsx.S	68824825	68828862	4038	+	GTAATTG	10464:10471	GTAATTG	13658:13661	GTAATTG	15828:15831	GTAATTG	24023:24024	CTAATTG	28330:2833	CTAATTG	41723:41725
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373828	caca1a.S	125362502	125539424	176923	+	GTAATTG	4495:4501	GTAATTG	26464:26474	CTAATTG	35644:35651	CTAATTG	39710:39714	GTAATTG	47422:4742	GTAATTG	49247:4925
108707217	camk4.L	161438128	161537363	99236	-	CAATTAC	2638:2644	CAATTAG	10826:1083	CAATTAC	21652:21654	CAATTAC	23565:2357	CAATTG	25423:2542	CAATTAC	35891:35897
108704358	chrm2.L	5237056	5318866	81811	+	CTAATTG	15581:15588	CTAATTG	20646:2065	CTAATTG	22593:22599	CTAATTG	32047:3205	CTAATTG	33877:3388	CTAATTG	34388:34394
108696042	cistrn3.L	6324066	6349999	25934	+	GTAATTG	1824:1830	GTAATTG	25965:2597	CTAATTG	35825:3583						
444580	cistrn3.S	4392785	4420685	27901	+	GTAATTG	21613:21614	CTAATTG	26938:2694	GTAATTG	47765:4777						
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379593	coxl2.L	19030637	19035071	4435	-	CAATTAC	961:967	CAATTAC	16832:16834	GTAATTG	17728:17734	CAATTAC	39973:39974				
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734422	crmp1.L	26676228	26724958	48731	+	CTAATTG	26859:26861	GTAATTG	43623:4362								
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444710	ctnna2.L	15276253	16316751	1040499	+	GTAATTG	20981:2098	CTAATTG	32986:3299	GTAATTG	41281:4128	CTAATTG	44166:4417	CTAATTG	51657:5166	CTAATTG	51776:51782
735141	cyp27c1.S	51031782	51046442	14661	+	CTAATTG	1492:1499	GTAATTG	1577:1578	GTAATTG	1618:1618	GTAATTG	29826:2983	GTAATTG	42196:4220	CTAATTG	45188:45194
779068	dach1.L	118521901	118753480	231580	+	CTAATTG	3064:3070	CTAATTG	5075:5081	GTAATTG	9593:9599	GTAATTG	10210:10211	GTAATTG	13885:1389	CTAATTG	15167:15175
444688	dhcr24.L	93054708	93073865	19158	-	CAATTAC	10207:1021	CTAATTG	13039:1304	CAATTAG	13580:13584	CAATTAG	15634:1564	CAATTAC	20942:2094	CAATTAG	26469:26475
444269	dhrs3.L	99627471	99645567	18097	+	GTAATTG	10880:10881	CTAATTG	24359:24361	CTAATTG	41426:4143	GTAATTG	41893:4189	GTAATTG	43943:4394	GTAATTG	52337:52343
495093	dxh2.L	19175507	19767878	52372	+	GTAATTG	9136:9142	GTAATTG	17145:1715	GTAATTG	26989:26999	GTAATTG	47526:4753	GTAATTG	48634:4864	GTAATTG	51487:51495
108719909	dx5.S	26933539	26939186	5648	-	CAATTAC	40047:4005	CAATTAC	43788:4379	CAATTAG	56370:5637						
108714311	dmrta2.L	91058234	91062116	3883	-	CAATTAG	2377:2383	CAATTAC	3372:3378	CAATTAC	7255:7261	CAATTAC	22450:2245	CAATTAC	50577:5058		
734181	dmrta2.S	75902854	75906665	3812	-	CAATTAG	2881:2887	CAATTAC	3962:3968	CAATTAG	47391:4739	CAATTAC	46604:4661	CAATTG	47391:4739	CAATTAC	49757:49765
447353	dner.L	138821407	138940168	118762	-	CAATTAG	5728:5734	CAATTAC	10674:1077	CTAATTG	5483:5489	GTAATTG	6118:6124	GTAATTG	9469:9475	CTAATTG	9510:9516
733218	dner.S	116522613	116640582	117970	-	CAATTAC	8775:8781	CAATTAC	23734:2374	CAATTAC	24685:2469	CAATTAC	34061:3406	CAATTAC	41496:4150	CAATTAG	47732:47738
108698468	ednb2.L	57360733	57373837	13105	+	GTAATTG	12292:1229	GTAATTG	16288:1629	GTAATTG	23329:23333	GTAATTG	23329:23333	GTAATTG	28068:2807	CTAATTG	32474:32480
108706841	efna2.S	90276200	90339974	123279	-	CAATTAC	5315:5321	CAATTAG	7988:7990	CAATTAG	9785:9792	CAATTAC	10236:1024	CAATTAC	28541:2854		
378621	efnb3.L	155585401	155629439	44039	-	CAATTAG	199:205	CAATTAC	19570:1957	CAATTAC	55239:5524	CAATTAG	56253:5625	CAATTAC	58376:5838		
108717253	efrb3.L	164941521	165040302	101512	+	GTAATTG	5615:5621	GTAATTG	15139:1514	GTAATTG	1949:1995	GTAATTG	28890:2889	GTAATTG	36806:3681	GTAATTG	39787:39795
108704605	emilin3.S	38678349	38691669	13321	+	GTAATTG	401:407	GTAATTG	6670:6676	GTAATTG	14780:1481	GTAATTG	17408:17412	GTAATTG	20671:2067	GTAATTG	43707:43715
444481	emx2.L	4320493	4329940	9448	+	GTAATTG	10286:1029	CTAATTG	13660:1366	CTAATTG	17852:1785	GTAATTG	31618:3162	GTAATTG	32450:3245	GTAATTG	34808:34814
108708233	epha10.L	87446557	87510675	64119	+	GTAATTG	3523:3529	GTAATTG	5583:5589	GTAATTG	6359:6365	GTAATTG	10903:10904	GTAATTG	43116:4312	GTAATTG	55403:55405
108699897	fgd1.S	36747935	36824003	76069</													



373694	ntn1.L	44922889	44973436	50548	CAATTAG	1670:1676	CAATTAG	2877:2883	CAATTAC	7470:7476	CAATTAG	21909:2191	CAATTAC	39030:3903	CAATTAG	42154:42160	
108703174	ntn3.S	99754837	99826023	71187	+	CTAATTG	4879:4885	CTAATTG	7776:7782	CTAATTG	14952:1495	CTAATTG	26159:2616	CTAATTG	36667:3667	CTAATTG	38345:38351
100127305	ntn5.L	110797030	110869283	72254	-	CAATTAG	27766:27771	CAATTAG	40887:4089	CAATTAC	45871:4587	CAATTAC	46629:4663				
108717966	otp.L	201728385	201738028	9644	-	CAATTAG	2570:2576	CAATTAG	21151:2115	CAATTAC	22413:2241	CAATTAC	26326:2633	CAATTAG	32718:3272	CAATTAG	44950:44956
108707273	otp.S	173569661	173578934	9274	-	CAATTAG	731:737	CAATTAG	4469:4475	CAATTAG	10972:1097	CAATTAC	21706:2171	CAATTAG	32533:3253	CAATTAC	41824:41830
394326	otx1.L	31685746	31692153	6408	-	CAATTAC	14505:1451	CAATTAC	23833:2383	CAATTAG	36199:3620	CAATTAC	42664:42674	CAATTAG	43782:4378	CAATTAC	52832:52838
432013	otx2.L	95776826	95783694	6869	+	CTAATTG	994:1000	GTAATTG	3224:3230	CTAATTG	6228:6234	CTAATTG	13517:1352	GTAATTG	15978:1598	CTAATTG	17295:17301
399342	otx2.S	6466178	6474324	8147	-	CAATTAG	20308:2031	CAATTAG	26756:2676	CAATTAG	32493:3249	CAATTAG	34524:3453	CAATTAC	42169:4217	CAATTAC	45009:45019
734441	pqr6.L	13131650	13132936	18287	-	CAATTAG	6457:6463	CAATTAG	9290:9296	CAATTAC	36365:3637	CAATTAG	37465:3747	CAATTAG	49334:4934	CAATTAC	57136:57142
447230	pqr6.S	101050289	101078138	27850	-	CAATTAG	4674:4680	CAATTAC	18507:1851	CAATTAC	45965:4597	CAATTAG	59599:5960				
398811	pc1.L	30954393	31113591	159199	+	GTAATTG	7242:7248	CTAATTG	30671:3067	CTAATTG	44671:4467	CTAATTG	53548:5353	GTAATTG	57754:5776		
108696974	pcdn10.L	66880836	66949508	68673	-	CAATTAG	4755:4761	CAATTAG	8576:8582	CAATTAC	12680:1268	CAATTAC	22095:2210	CAATTAC	22957:2296	CAATTAC	39432:39438
108704222	pcdn7.S	23255963	23831275	575313	+	CTAATTG	302:308	GTAATTG	793:799	CTAATTG	19631:1963	CTAATTG	23145:2315	CTAATTG	29035:2904	CTAATTG	37810:37816
447238	phyhip1.S	7878133	7949239	71107	+	CTAATTG	8287:8293	GTAATTG	31141:3114	GTAATTG	40296:4030	CTAATTG	58577:5858	CTAATTG	59924:5993		
444658	ptpnc1.S	54192135	54249643	57509	+	CTAATTG	7460:7466	GTAATTG	1917:1917	CTAATTG	23704:2371	GTAATTG	37249:3725	GTAATTG	43394:43400		
373586	ptx2.S	56647765	56666204	18440	+	CTAATTG	1602:1608	CTAATTG	14059:14061	CTAATTG	32755:3276	GTAATTG	38455:3846	CTAATTG	54638:5464	GTAATTG	56451:5645
398183	ptx3.L	41587535	41635262	47728	-	CAATTAC	2525:2531	CAATTAC	4634:4640	CAATTAC	9693:9699	CAATTAC	9740:9746	CAATTAG	19670:1967	CAATTAG	21145:2115
373824	ptx3.S	32817243	32831254	14012	-	CAATTAC	12767:1277	CAATTAC	20656:2066	CAATTAC	25778:2578	CAATTAC	47706:4771	CAATTAC	52118:5212	CAATTAC	52199:5220
445848	ptcb4.S	26892020	27052040	160021	+	GTAATTG	2917:2923	CTAATTG	22083:2208	CTAATTG	33761:3376	GTAATTG	34344:3435	CTAATTG	36711:3671	GTAATTG	38721:3872
397805	pxna1.L	133875694	134130925	255232	-	CAATTAG	11699:1170	CAATTAC	15806:1581	CAATTAG	20165:2017	CAATTAG	20371:2037	CAATTAC	21683:2168	CAATTAG	21859:2186
447098	ppm2.S	109853538	109862789	9252	+	GTAATTG	4616:4622	GTAATTG	15830:1583	CTAATTG	28000:2800	CTAATTG	33146:3315	CTAATTG	35582:3558	CTAATTG	38882:3888
108698467	pou4f1L	57171968	57174719	2752	+	GTAATTG	85:91	CTAATTG	4269:4275	CTAATTG	15666:1567	CTAATTG	19622:1962	GTAATTG	2389:2383	CTAATTG	29732:29738
373807	pou4f1.S	74055954	74057472	1519	+	GTAATTG	1661:1667	CTAATTG	4296:4302	GTAATTG	17742:1774	CTAATTG	21856:2186	GTAATTG	22251:2225	CTAATTG	29643:29645
379544	prdm12.L	887245	8885268	8024	-	CAATTAG	17799:1780	CAATTAG	28982:2898	CAATTAG	2906:2951	CAATTAG	2997:2993	CAATTAG	33231:3323	CAATTAG	38930:3893
108703933	prt2.L	135195041	135219049	24009	-	CAATTAC	1093:1099	CAATTAG	3306:3312	CAATTAG	3509:3515	CAATTAC	8172:8178	CAATTAC	11329:1133	CAATTAG	23802:23808
108717211	ptchd4.L	157887809	157953598	65790	+	GTAATTG	20596:2060	CTAATTG	28993:2899	GTAATTG	32566:3257	CTAATTG	34348:3435				
444723	ptgs.D	27277189	27280672	3484	-	CTAATTG	15519:1552	CTAATTG	21767:2177	GTAATTG	45687:4569						
379862	pygm.L	37406515	37450030	43516	+	GTAATTG	10833:1083	GTAATTG	11824:1183	GTAATTG	15466:1547	GTAATTG	29168:2917	GTAATTG	35701:3570		
431959	rab11fp5.L	9885324	9916083	30760	-	CAATTAC	718:724	CAATTAC	14337:1434	CAATTAC	35182:3518	CAATTAC	42022:4202	CAATTAC	52972:5297	CAATTAC	59927:5993
108717144	rab6b.L	139929362	139954012	24651	-	CAATTAG	2465:2471	CAATTAC	6249:6255	CAATTAC	15427:1543	CAATTAC	17018:1702	CAATTG	19296:1930	CAATTAG	22125:2213
108718191	rbp1.S	115965914	115980872	14959	-	CAATTAC	3020:3026	CAATTAC	5998:6004	CAATTAC	13808:1381	CAATTAC	17333:1733	CAATTAC	18905:1891	CAATTAG	19699:19705
444623	rdhs.L	143860108	143871386	11279	+	CTAATTG	794:800	CTAATTG	1038:1044	CTAATTG	6311:6317	GTAATTG	7543:7549	CTAATTG	24006:2401	GTAATTG	39034:3904
444753	rgr.L	52560896	52597415	36520	-	CTAATTG	10052:1005	GTAATTG	18186:1819	GTAATTG	28912:2891	GTAATTG	47211:4721	GTAATTG	4745:4775	CTAATTG	49625:49631
494721	rgr.S	43030908	43045862	14955	+	GTAATTG	1740:1746	GTAATTG	8508:8514	GTAATTG	10075:1008	CTAATTG	14724:1473	GTAATTG	16885:1689	GTAATTG	27516:2752
108712845	rbp1.S	44829028	44843412	14385	-	CAATTAC	3726:3732	CAATTAC	6696:6702	CAATTAC	7585:7591	CAATTAG	14103:1410	CAATTAC	17460:1746	CAATTAG	24458:24464
399383	rngt.S	73324344	7381598	157255	+	CTAATTG	16471:1647	GTAATTG	24194:2420	CTAATTG	24595:2460	GTAATTG	30629:3063	CTAATTG	50765:5077	CTAATTG	51548:51554
108711313	rola.R	99083952	99132453	48502	-	CAATTAG	32188:3219	CAATTAC	36087:3609	CAATTAG	37665:3767						
447613	rpe65.L	777973831	77985824	11994	+	GTAATTG	360:366	GTAATTG	4177:4183	GTAATTG	8314:8320	GTAATTG	14655:1466	CTAATTG	31649:3165	CTAATTG	36293:36295
397938	rpl27a.S	69071921	69081562	9642	-	CAATTAC	8655:8661	CAATTAG	22444:2245	CAATTAC	26895:2690	CAATTAG	32236:3224	CAATTAC	32399:3240	CAATTAG	33468:33474
100101273	rpl28.S	93487155	93494448	7294	-	CAATTAC	1911:1917	CAATTAC	4878:4884	CAATTAC	13895:1390	CAATTAC	17600:1760	CAATTAC	24111:2411	CAATTAG	26762:26768
496383	rsp2.L	144263290	144347989	84700	-	CAATTAC	7038:7044	CAATTAG	15665:1567	CAATTAG	18278:1828	GTAATTG	26125:2613	CTAATTG	29396:2940	CTAATTAC	55852:55858
108719754	rsp2.O	120015407	120113213	97807	-	CAATTAC	3376:3382	CAATTAC	3696:3702	CAATTAC	5074:5080	CAATTAC	12764:1277	CAATTAG	16938:1694	CAATTAC	23302:23308
108696901	scn1b1.S	133638758	133937341	28584	+	GTAATTG	2662:272	GTAATTG	2121:2127	GTAATTG	3347:3353	GTAATTG	41053:4105	GTAATTG	42914:4292	GTAATTG	44742:44748
108695492	scrt2.S	137254708	137271763	17056	+	GTAATTG	5812:5818	CTAATTG	9966:9972	CTAATTG	15577:1558	GTAATTG	20397:2040	CTAATTG	58313:5831	CTAATTG	58941:58947
100505449	sfxn5.L	9773225	9863167	89943	-	CAATTAC	56:62	CAATTAC	7011:7017	CAATTAG	12422:12424	CAATTAC	28870:2887	CAATTAC	34877:3488	CAATTAC	36138:36144
108711341	shf.S	101318640	101434109	115470	+	CTAATTG	8436:8442	GTAATTG	16457:16461	CTAATTG	17600:1760	CAATTAC	26197:2620	GTAATTG	33990:33994	CTAATTG	45713:45715
108717957	sim1.S	6869840	68762369	63930	+	GTAATTG	3974:3980	CTAATTG	4428:4434	GTAATTG	1137:1174	GTAATTG	27364:2737	CTAATTG	28747:2875	CTAATTG	34999:35005
373634	sim2.S	21371380	21424282	52903	-	GTAATTG	9952:9958	GTAATTG	11269:1127	GTAATTG	22190:2219	CTAATTG	29149:2915	CTAATTG	40917:4092	CTAATTG	51334:51340
108711507	skor1.L	124253950	124280009	26060	-	CTAATTG	1294:1300	CTAATTG	1946:1952	CTAATTG	4479:4485	CTAATTG	8707:8713	CTAATTG	9941:9947	CTAATTG	11787:1179:
108712800	skor1.S	36508318	36528035	19718	-	CAATTAC	12734:1274	CTAATTG	21759:2176	CTAATTG	27596:2760	CTAATTG	32319:3232	CAATTAG	36728:36734		
100380948	slc12a5.L	356154539	35693170	51632	-	CAATTAC	15190:1519	CAATTAC	17858:1786	CAATTAC	25709:2571	CAATTAC	27511:2751	CAATTAC	30950:3095	CAATTAG	33895:33901
494763	slc2a1.L	139293680	139354264	60585	+	GTAATTG	18225:1823	GTAATTG	20512:2051	GTAATTG	23410:23414	GTAATTG	40932:40934				
108697841	slc2a1.S	112562130	112612031	51072	-	GTAATTG	1541:1547	CTAATTG	17545:1755	GTAATTG	51645:5167						
108719869	slc39a12.S	21235717	21263894	28178	-	CAATTAC	1978:1984	CAATTAG	5323:5329	CAATTAC	5863:5869	CAATTAC	6818:6824	CAATTAG	17184:1719	CAATTAG	17369:17375
446661	slc40a1.S																

108719921	sp8.S	31765303	31770097	4795		CAATTAG	696:702	CTAATTAC	9811:9817	CAATTAC	12022:1202	CAATTAG	18920:1892	CAATTAG	19291:1929	CAATTAG	28647:2865:
108702173	stac2.S	2200107	2219302	19196	+	CTAATTG	5466:5472	CTAATTG	10849:1085	GTAATTG	42961:4296	GTAATTG	45897:4590	CTAATTG	47768:47774	CTAATTG	48591:4859:
108698026	stk32b.L	26913633	27021267	107635		CAATTAC	1599:1605	CAATTAC	5215:5221	CAATTAG	5513:5519	CAATTAC	12455:1246	CAATTAC	23036:2304	CAATTAC	24074:2408:
108711302	stra6.L	98290744	98325559	34816	+	GTAATTG	12825:1283	GTAATTG	15031:1503	CTAATTG	18811:1881	GTAATTG	21333:2133	GTAATTG	25078:2508	CTAATTG	29542:2954:
108712985	stra6.S	60370714	60391574	20861	-	CAATTAG	13202:1320	CAATTAC	1710:1714	CAATTAC	19505:1951	CAATTAG	19847:1985	CAATTAG	20168:2017	CAATTAC	25118:2512:
399128	stxbp1.S	37814169	37852485	38317		CAATTAG	14274:14284	CAATTAG	25713:25714	CAATTAC	26612:2661	CAATTAC	28730:28734	CAATTAC	34617:3462	CAATTAG	40285:40291
108695332	sulf1.S	105510768	105600014	89247	+	CTAATTG	797:803	CTAATTG	3349:3355	CTAATTG	4287:4293	CTAATTG	12436:12444	GTAATTG	12972:1297	CTAATTG	16331:1633:
108707265	sv2c.S	172712415	172811520	99106	+	CTAATTG	783:789	GTAATTG	4801:4807	GTAATTG	7839:7845	GTAATTG	15730:1573	GTAATTG	18221:1822	GTAATTG	20281:2028:
399153	syn1.L	1103444	1130181	26738		CAATTAC	10754:10761	CAATTAG	14908:1491	CAATTAG	14915:1492	CAATTAC	33841:3384	CAATTAC	58924:5893	CAATTAC	59579:5958:
447574	syn1.S	26392849	26419082	26234	+	GTAATTG	7007:7013	GTAATTG	7030:7036	CTAATTG	8751:8757						
734421	syn2.S	131990000	132073108	83109	+	CTAATTG	23797:2380	GTAATTG	32391:3239	GTAATTG	32509:3251	CTAATTG	47041:4704	CTAATTG	54670:5467	GTAATTG	54772:5477:
108706352	tal2.S	109849414	109852485	3072	+	GTAATTG	4764:4770	CTAATTG	20579:20584	GTAATTG	21546:2155	GTAATTG	23901:2390	GTAATTG	25074:2508	CTAATTG	26912:2691:
398723	tlf.L	139885473	139908189	22717	+	GTAATTG	17856:17861	GTAATTG	21204:2121	CTAATTG	21547:2155	CTAATTG	22830:2283	CTAATTG	25475:2548	CTAATTG	30526:3053:
735028	tfap2e.L	84926644	84951512	28509		CAATTAG	529:535	CAATTAC	1761:1767	CAATTAC	7080:7086	CAATTAC	8640:8646	CAATTAC	10027:1003	CAATTAC	21391:2139:
108709553	tfap2e.S	73303372	73331065	27694		CAATTAC	17412:17411	CAATTAC	22018:22024	CAATTAC	51256:51261	CAATTAC	53516:5352				
446285	tldcb3b.L	135292067	135303961	11895		CAATTAC	36410:36411	CAATTAC	45077:4508	CAATTAG	52422:52424	CTAATTG	53186:5319	CAATTAG	54310:5431	CAATTAG	58702:58708
108697767	tmem145.S	94735298	94796405	61108	+	GTAATTG	607:613	GTAATTG	11874:1188	GTAATTG	12811:1281	CTAATTG	14989:1499	CTAATTG	18499:1850	CTAATTG	22452:22458
447630	tmem255a.L	367049443	36733163	28221	-	CAATTAG	3738:7384	CAATTAG	26443:2644	CAATTAC	55627:5563	CAATTAG	57833:5783				
108717212	tnfrsf21.L	158227220	158287816	60597	-	CAATTAG	837:843	CAATTAG	5211:5217	CAATTAG	13648:13654	CAATTAC	17749:1775	CAATTAG	24741:2474	CAATTAG	41691:4169:
734865	txndc17.S	40413056	40423919	10864		CAATTAG	7567:7573	CAATTAC	20379:20384	CAATTAC	23510:2351	CAATTAC	40910:40914	CAATTAG	56116:5612		
108698670	vsx2.L	73205871	73223554	19484	+	GTAATTG	2583:2589	CTAATTG	3005:3011	CTAATTG	6618:6624	CTAATTG	13998:1400	CTAATTG	16514:1652	GTAATTG	21344:2135:
443849	vwa5a.2.L	229002038	229020083	18046	-	CAATTAG	3229:3235	CAATTAC	6044:6050	CAATTAG	14298:14304	CTAATTAC	18609:18611	CAATTAC	44794:4480	CAATTAC	49314:4932:
108716834	wasf1.L	80885973	80948210	62238	+	GTAATTG	9703:9709	GTAATTG	16632:1663	CTAATTG	20137:2014	CTAATTG	28312:28314	CTAATTG	29678:2968	GTAATTG	33289:3329:
444059	wdr7.L	219997784	220241100	243317		CAATTAC	5450:5456	CAATTAG	18182:1818	CAATTAC	20486:2049	CAATTAC	27205:2721	CAATTAC	34894:3490	CAATTAG	37884:3789:
735023	wls.L	78023374	78051373	28000	+	CTAATTG	17263:1726	CTAATTG	18224:1823	CTAATTG	18528:18534	GTAATTG	23109:2311	GTAATTG	37509:3751	GTAATTG	45363:4536:
378566	wnt2b.L	77464633	77502777	42645		CAATTAG	5193:5199	CAATTAC	8167:8173	CAATTAG	11822:11824	CAATTAG	19722:19724	CAATTAC	34656:3466	CAATTAG	38525:3853:
399098	wnt8b.S	31487371	31513813	26443	+	CTAATTG	1426:1432	GTAATTG	6454:6460	CTAATTG	11835:1184	GTAATTG	13579:13583	GTAATTG	25399:2540	CTAATTG	30561:3056:
108707089	XB5848002.	137458090	137460566	2477		CAATTAG	10951:1095	CAATTAC	12157:1216	CAATTAG	14839:1484	CAATTAC	16639:1664	CAATTAC	17403:1740	CAATTAG	24999:2500:
108706749	XB5957215.S	65462924	65509161	46238		CAATTAC	1805:1811	CAATTAC	30493:3049	CAATTAC	32972:3297	CAATTAG	35497:3550	CAATTAG	39081:3908	CAATTAG	40359:4036:
108698501	zic3.L	61479845	61485940	6096	+	GTAATTG	413:419	CTAATTG	17057:1706	CTAATTG	22184:2219	CTAATTG	31242:3124	CTAATTG	40292:4029		
394375	zic3.S	78158858	78162564	3707	+	CTAATTG	1367:1373	GTAATTG	3146:3152	GTAATTG	4899:4905	CTAATTG	4948:4954	CTAATTG	21749:2175	CTAATTG	23111:2311:
108701051	znfx1.L	36784608	36808405	23798	-	CAATTAC	261:267	CAATTAG	4734:4740	CAATTAG	5795:5801	CAATTAG	10925:1093	CAATTAG	13592:13594	CAATTAC	22097:2210:

ENTREZID	SYMBOL	Start	End	length	strand	Sequence1	Position1	Sequence	Position2	Sequence	Position3	Sequence	Position4	Sequence	Position4
108717782	acss1.S	30631415	30683070	51656	-	TTCAGAAG	63:69	TTCAGAAG	1186:1192	TTCAGAAG	1606:1612				
503669	ahcy.L	43014140	43030456	16317	+	CTTGAA	770:776	CTTGAT	6231:6237	CTTGAA	8258:8264				
108710331	ank1.L	139543765	139560010	16246	-	ATCAAAG	3997:4003	ATCAAAG	8676:8682						
100036926	ank1.S	22497428	22627635	130208	+	CTTGAA	5813:5819	CTTGAT	9569:9575						
447402	apcdd1.L	111193133	111231649	38517	-										
108695251	apcdd1.S	90867467	90898266	30800	+	CTTGAA	2902:2908	CTTGAA	5695:5701	CTTGAA	6521:6527	CTTGAT	8657:8663	CTTGAA	8687:8693
108697887	apl1p1.S	109607779	109638526	30748	+	CTTGAT	2785:2791	CTTGAT	9243:9249	CTTGAT	9901:9907				
398223	app.L	17761661	17906707	145047	+	CTTGAT	2775:2781	CTTGAT	7018:7024	CTTGAA	8653:8659				
379251	app.S	16800553	16933427	132875	-	ATCAAAG	9607:9613								
108716978	atp13a5I.2.L	112349351	112395883	46533	-	ATCAAAG	2621:2627								
399285	atp1a1.L	716832	733112	16281	-	ATCAAAG	3072:3078								
108708208	atp1b1.L	83806655	83819946	13292	-										
444626	atp1b2.S	131833350	131838868	5519	-										
446855	atp2b4.L	122988077	123167977	179901	+										
108715812	atp2b4.S	103816801	104014544	197744	+										
734627	atp6v1a.L	2599141	2644208	45068	+										
100301951	barhl2.L	101225765	101230203	4439	+	CTTGAA	4756:4762								
398182	barhl2.S	84616978	84621442	4465	+	CTTGAA	8359:8365								
108719273	bhlhe22.L	126287814	126290806	2993	+	CTTGAT	4429:4435	CTTGAT	4712:4718						
108697000	bsx.L	79604595	79608039	3445	-	ATCAAAG	6332:6338								
108697964	bsx.S	68824825	68828862	4038	+	CTTGAT	237:243	CTTGAA	6767:6773	CTTGAA	8515:8521				
108714103	ca7.L	60958000	60982049	24050	+										
108715529	cachd1.S	65179606	65266838	87233	-	TTCAAAG	501:507	TTCAAAG	1965:1971	TTCAAAG	9925:9931				
373828	cacna1a.S	125362502	125539424	176923	+										
108707217	camk4.S	161438128	161537363	99236	-	ATCAAAG	286:292								
108704358	chrm2.L	5237056	5318866	81811	+										
108696042	clstn3.L	6324066	6349999	25934	+	CTTGAT	3912:3918	CTTGAA	9792:9798						
444580	clstn3.S	4392785	4420685	27901	+										
108701021	cntnap1.L	33971993	34025896	53904	+	CTTGAT	6269:6275	CTTGAA	7214:7220						
108713331	col5a3.S	122174819	122285652	110834	+	CTTGAA	2426:2432								
379593	cox4i2.L	19030637	19035071	4435	-	ATCAAAG	317:323	TTCAAAG	1052:1058	TTCAAAG	3876:3882				
108718496	cpne4.L	54675311	54874125	198815	-										
734422	crmp1.L	26676228	26724958	48731	+	CTTGAT	8310:8316								
108706537	crmp1.S	16514921	16550190	35270	+										
398118	crx.L	70907385	70911313	3929	-										
373653	crx.S	85300054	85305321	5268	-	TTCAAAG	413:419								
444710	cttna2.L	15276253	16316751	1040499	+										
735141	cyp27c1.S	51031782	51046442	14661	+	CTTGAT	314:320								
779068	dach1.S	118521901	118753480	231580	+	CTTGAA	4004:4010	CTTGAT	5254:5260						
444688	dhcr24.L	93054708	93073865	19158	-	ATCAAAG	1834:1840								
444269	dhrs3.L	99627471	99645567	18097	+	CTTGAT	3795:3801	CTTGAA	7339:7345	CTTGAA	7505:7511	CTTGAA	7595:7601		
495093	dhx32.L	19715507	19767878	52372	+	CTTGAT	6972:6978	CTTGAT	7608:7614	CTTGAT	7681:7687				
108719909	dlx5.S	26933539	26939186	5648	-	TTCAAAG	2908:2914	TTCAAAG	7319:7325	TTCAAAG	9802:9808				
108714311	dmrta2.L	91058234	91062116	3883	-	ATCAAAG	4436:4442	TTCAAAG	7362:7368						
734181	dmrta2.S	75902854	75906665	3812	-	ATCAAAG	4443:4449	TTCAAAG	7295:7301						







100380948	slc12a5.L	35641539	35693170	51632	-	ATCAAAG	306:312	TTCAAAG	2663:2669							
494763	slc2a1.L	139293680	139354264	60585	+											
108697841	slc2a1.S	112562130	112613201	51072	+											
108719869	slc39a12.S	21235717	21263894	28178	-	ATCAAAG	5719:5725	TTCAAAG	5728:5734	TTCAAAG	9454:9460					
446661	slc40a1.S	82213415	82229157	15743	-	TTCAAAG	3968:3974	TTCAAAG	9147:9153							
108719439	slc45a4.L	158368034	158431635	63602	+	CTTGAT	740:746									
108695461	slc45a4.S	132742338	132812415	70078	+											
108711128	slc4a4.L	86532823	86621277	88455	-	ATCAAAG	6989:6995									
108706771	slc4a4.S	70481573	70533883	52311	-	TTCAAAG	8418:8424	TTCAAAG	8450:8456							
108720038	slc6a3.S	63839322	63876812	37491	+	CTTGAA	926:932	CTTGAT	1481:1487							
108709176	slc6a4.S	5573782	5647981	74200	+	CTTGAT	1519:1525	CTTGAT	1580:1586	CTTGAA	4072:4078	CTTGAA	4104:4110			
108704092	slc7a10.S	50688467	50733293	44827	+	CTTGAA	3294:3300	CTTGAT	9938:9944							
380270	slit1.S	21612366	21845457	233092	-	TTCAAAG	3089:3095	TTCAAAG	4084:4090							
779307	snrpg.S	31620636	31630323	9688	+	CTTGAA	2204:2210	CTTGAT	7786:7792							
108719398	sntb1.L	149457904	149565163	107260	-	ATCAAAG	5715:5721									
735180	sp5.L	75610256	75613281	3026	+	CTTGAA	56:62	CTTGAT	4661:4667	CTTGAT	4857:4863	CTTGAT	5895:5901	CTTGAT	8042:8048	
378650	sp5.S	66202552	66207578	5027	+	CTTGAT	3643:3649	CTTGAT	5451:5457	CTTGAT	5647:5653	CTTGAT	9358:9364			
108719921	sp8.S	31765303	31770097	4795	-	TTCAAAG	4487:4493									
108702173	stac2.S	2200107	2219302	19196	+	CTTGAT	7506:7512									
108698026	stk32b.L	26913633	27021267	107635	-	TTCAAAG	430:436	ATCAAAG	8938:8944							
108711302	stra6.L	98290744	98325559	34816	+	CTTGAA	2400:2406	CTTGAT	4634:4640	CTTGAT	8180:8186	CTTGAT	8412:8418			
108712985	stra6.S	60370714	60391574	20861	-	TTCAAAG	2542:2548	TTCAAAG	4492:4498	TTCAAAG	4550:4556	ATCAAAG	4680:4686	TTCAAAG	5259:5265	
399128	stxbp1.S	37814169	37852485	38317	-	ATCAAAG	251:257	TTCAAAG	3011:3017	TTCAAAG	7732:7738					
108695332	sulf1.S	105510768	105600014	89247	+	CTTGAA	1007:1013	CTTGAT	1602:1608	CTTGAT	6947:6953					
108707265	sv2c.S	172712415	172811520	99106	+	CTTGAA	3401:3407	CTTGAA	7965:7971	CTTGAA	9504:9510					
399153	syn1.L	1103444	1130181	26738	-	ATCAAAG	9241:9247									
447574	syn1.S	26392849	26419082	26234	+											
734421	syn2.S	131990000	132073108	83109	+	CTTGAA	2634:2640	CTTGAA	7431:7437							
108706352	tal2.S	109849414	109852485	3072	+	CTTGAA	2416:2422	CTTGAA	8395:8401							
398723	tf.L	139885473	139908189	22717	+	CTTGAA	953:959	CTTGAA	1447:1453							
735028	tfap2e.L	84926644	84955152	28509	-	TTCAAAG	1024:1030	TTCAAAG	3141:3147	TTCAAAG	4266:4272					
108709553	tfap2e.S	73303372	73331065	27694	-	TTCAAAG	3810:3816	ATCAAAG	6177:6183							
446285	tlcd3b.L	135292067	135303961	11895	-	TTCAAAG	202:208	ATCAAAG	9748:9754							
108697767	tmem145.S	94735298	94796405	61108	+	CTTGAA	1088:1094	CTTGAT	3735:3741							
447630	tmem255a.L	36704943	36733163	28221	-											
108717212	tnfrsf21.L	158227220	158287816	60597	-	ATCAAAG	3437:3443									
734865	txndc17.S	40413056	40423919	10864	-	TTCAAAG	8323:8329	TTCAAAG	9874:9880							
108698670	vsx2.L	73205871	73225354	19484	+	CTTGAA	2841:2847	CTTGAT	4101:4107							
443849	vwa5a.2.L	229002038	229020083	18046	-											
108716834	wasf1.L	80885973	80948210	62238	+	CTTGAA	974:980	CTTGAT	1461:1467	CTTGAT	2936:2942					
444059	wdr7.L	219997784	220241100	243317	-											
735023	wls.L	78023374	78051373	28000	+	CTTGAA	2400:2406	CTTGAA	3362:3368	CTTGAT	4675:4681	CTTGAA	8975:8981			
378566	wnt2b.L	77464633	77507277	42645	-	TTCAAAG	2381:2387	ATCAAAG	9625:9631							
399098	wnt8b.S	31487371	31513813	26443	+	CTTGAT	552:558	CTTGAT	7700:7706	CTTGAA	9219:9225					
108707089	XB5848002.S	137458090	137460566	2477	-	TTCAAAG	5373:5379	ATCAAAG	9122:9128							
108706749	XB5957215.S	65462924	65509161	46238	-	TTCAAAG	98:104	TTCAAAG	2371:2377							

108698501	zic3.L	61479845	61485940	6096	+	CTTGAT	4171:4177	CTTGAT	6641:6647	CTTGAT	7522:7528	CTTGAT	9877:9883		
394375	zic3.S	78158858	78162564	3707	+	CTTGAT	4103:4109	CTTGAT	6581:6587	CTTGAT	9835:9841				
108701051	znfx1.L	36784608	36808405	23798	-	ATCAAAG	2674:2680	ATCAAAG	3815:3821						