Genes regulated by Kctd15 in the developing neural crest

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Abstract

Neural crest (NC) development is controlled precisely by a regulatory network with multiple signaling pathways and the involvement of many genes. The integration and coordination of these factors are still incompletely understood. Overexpression of Wnt3a and the BMP antagonist Chordin in animal cap cells from *Xenopus* blastulae induces a large number of NC specific genes. We previously suggested that Potassium Channel Tetramerization Domain containing 15 (Kctd15) regulates NC formation by affecting Wnt signaling and the activity of transcription factor AP-2. In order to advance understanding of the function of Kctd15 during NC development, we performed DNA microarray assays in explants injected with Wnt3a and Chordin, and identify genes that are affected by overexpression of Kctd15. Among many genes identified we chose Duf domain containing protein 1(*ddcp1*), Platelet-Derived Growth Factor Receptor a (*pdgfra*), Complement factor properdin (*cfp*), Zinc Finger SWIM-Type Containing 5 (*zswim5*), and complement component 3 (*C3*) to examine their expression by whole mount in situ hybridization. Our work points to a possible role for Kctd15 in the regulation of NC formation and other steps in embryonic development.

Key words

Neural crest, Kctd15, DNA microarray, *Xenopus*, gene regulation, transcription factor AP-2

Introduction

The Kctd family of proteins has received increasing attention due to its multiple biological functions and putative roles in human disease. The family is composed of over 20 genes/proteins in different vertebrate species and can be subdivided into seven subgroups on the basis of sequence similarity (Liu *et al.*, 2013, Skoblov *et al.*, 2013). A number of Kctd genes have been associated with human diseases, as summarized by Liu and colleagues (Liu *et al.*, 2013). All members of the family share a Bric-a-brack, Tram-track, Broad complex (BTB) domain usually located in the N-terminal half of the protein. BTB domains, also called POZ domains, are widely involved in protein-protein interactions (Perez-Torrado *et al.*, 2006, Stogios *et al.*, 2005), and thus it is not surprising that acting as adapters is perhaps the best-studied molecular function of Kctd proteins. Kctd5, Kctd6 and Kctd11 bind Cullin 3 and act as adapters mediating the interactions of E3 ubiquitin ligases with their substrates (Balasco *et al.*, 2014, Bayon *et al.*, 2008, Canettieri *et al.*, 2010, Chen *et al.*, 2009, Correale *et al.*, 2011). While other Kctd proteins also have been reported to bind Cullin 3, this has been contradicted in recent work (Smaldone *et al.*, 2015). Kctd8, Kctd12 and Kctd16 associate with GABA receptors, modulating their activity (Rajalu *et al.*, 2015); these genes also show striking left/right asymmetry in their expression in the brain (Gamse *et al.*, 2005). Other family members appear to have very different molecular functions by acting in the regulation of transcription. These include Kctd10, a factor involved in heart development through the inhibition of

Tbx5a activity, apparently by direct interaction between the Tbx and Kctd components (Hu *et al.*, 2014, Tong *et al.*, 2014). In addition Kctd10 also interacts with proliferating cell nuclear antigen and through this interaction affects proliferation (Wang *et al.*, 2009).

We have been interested in the functions of Kctd15 in *Xenopus* and zebrafish development. We found that overexpression of Kctd15 leads to very effective inhibition of the formation of the neural crest (NC), suggesting the possibility that Kctd15 regulates the size of the NC domain in development (Dutta and Dawid, 2010, Groves and LaBonne, 2014). Kctd15 and the closely similar Kctd1 have two distinct molecular functions, inhibition of canonical Wnt signaling (Dutta and Dawid, 2010, Li *et al.*, 2014) and inhibition of the function of transcription factor AP-2 (Ding *et al.*, 2009, Zarelli and Dawid, 2013). Both of these functions may be responsible for the suppression of NC formation by Kctd15 as Wnt signaling and AP-2 activity are essential for NC induction and differentiation (Brewer *et al.*, 2004, de Croze *et al.*, 2011, Ikeya *et al.*, 1997, Knight *et al.*, 2005, Li and Cornell, 2007, Luo *et al.*, 2003, Luo *et al.*, 2005, Saint-Jeannet *et al.*, 1997, Schorle *et al.*, 1996, Simoes-Costa and Bronner, 2015). Based primarily on the strength of the inhibitory effect we believe that Kctd15 suppression of AP-2 activity may be the dominant mechanism in its blocking of NC development. Mutations in KCTD1 in humans are responsible for scalp-ear-nipple (SEN) syndrome (Marneros *et al.*, 2013), but it is not known whether inhibition of AP-2 or Wnt signaling plays a role in the etiology of this disease. KCTD15 is not been reported as the cause of a disease, but numerous studies show an association between this gene and obesity (Gutierrez-Aguilar *et al.*, 2012, Leon-Mimila *et al.*, 2013, Mei *et al.*, 2012, Willer *et al.*, 2009, Williams *et al.*, 2012). It is known that AP-2α regulates the activity of C/EBPα during adipogenesis (Jiang *et al.*, 1998), and AP-2β affects other steps of adipogenesis and insulin resistance (Ikeda *et al.*, 2006, Meng *et al.*, 2010, Tao *et al.*, 2006, Zhang *et al.*, 2014). It is tempting to speculate that the association of KCTD15 with obesity is based on its ability to inhibit AP-2 proteins, but evidence for this hypothesis in vertebrates is not available to date.

Beyond NC development and adipogenesis, the two pathways affected by Kctd15, Wnt signaling and AP-2 transcriptional regulation, have wide-ranging roles in development, physiology and disease (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/) (Eckert *et al.*, 2005, Hilger-Eversheim *et al.*, 2000, Hoffman *et al.*, 2007, Orso *et al.*, 2008, Wenke and Bosserhoff, 2010). The role of Kctd15 in different tissues and cell types and the global role of Kctd15 in development have not been studied so far. We investigated Kctd15 function broadly through transcriptome analysis by DNA microarray. As test tissue we chose *Xenopus laevis* animal explants (animal caps) that were injected with Wnt and Chordin (Chd) mRNAs, which induce the expression of NC marker genes (Saint-Jeannet *et al.*, 1997). Recently the transcriptome of animal caps overexpressing Pax3 and Zic1 has been analyzed (Bae *et al.*, 2014, Plouhinec *et al.*, 2014). This procedure is more selective for NC induction (Hong and Saint-Jeannet, 2007, Milet *et al.*, 2013), but we chose Wnt/Chd injection to test broadly for effects of Kctd15 at the neural plate border (NPB). Among genes induced by Wnt/Chd and inhibited by Kctd15 we find many well characterized NC markers as well as several hatching gland markers. We further identify several strongly

affected genes not previously studied in this context whose further analysis may contribute to an understanding of the role of Kctd15 and AP-2 in NC formation and other processes in the embryo.

Materials and methods

Embryo manipulation

In vitro fertilization and *X. laevis* embryo culture were performed as described previously (Wang *et al.*, 2011). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Experiments have been approved by the NICHD Animal Care and Use Committee. Capped mRNA for microinjection was prepared with mMessage mMachine kit (Invitrogen) and purified with RNeasy mini kit (Qiagen).

Total RNA extraction

Total RNA was extracted using the TRIzol reagent (Invitrogen) and precipitated by isopropanol. After DNase I treatment, the total RNA was purified with RNeasy purification kit (Qiagen).

Animal cap assay and RT-PCR

Animal cap assay was performed as described previously (Shi *et al.*, 2015, Zhao *et al.*, 2008). Briefly, animal caps were dissected from stage 9 embryos that had been injected with the indicated mRNAs, and were cultured till the sibling embryos reached stage 18. Total RNA was extracted for microarray or RT-PCR assays. cDNA was synthesized using Superscript III (Invitrogen) following the manufacturer's manual. Primers for RT-PCR are listed in Supplementary Table S1.

Whole mount in situ hybridization

The EST clones were purchased from GE Healthcare. The preparation of digoxigenin-labeled RNA probe and whole mount in situ hybridization were performed as described previously (Shi *et al.*, 2015, Wang *et al.*, 2015). Embryos probed with indicated antisense RNAs were sectioned by a vibratome (Zeiss) to a thickness of 50 μm (Shi *et al.*, 2015).

Affymetrix DNA microarray and data analysis

AC treated as described above were used to prepare RNA; three biological replicates were done for each condition. Biotinylated probe was prepared according to manufacturer's instructions (Affymetrix). Probes were hybridized to *X. laevis* genome arrays 2.0 (Affymetrix). The hybridized arrays were washed by the GeneChip Fluidics station 450, and scanned with the GeneChip Scanner 3000 (Affymetrix). Gene expression profiles were analyzed by the Partek Genomics Suite software (Partek). The microarray data set was submitted to the NCBI GEO database and obtained accession number GSE72391.

Results

Identification of genes regulated by Kctd15 during NC formation

We wished to survey the range of genes that are inhibited by Kctd15 overexpression in the context of NC induction by using Affymetrix DNA microarray. Injection of RNA encoding a Wnt and a BMP inhibitor such as Chordin into *Xenopus* embryos followed by animal cap culture (Fig. 1A) leads to the induction of many NC and additional NPB border genes (Saint-Jeannet *et al.*, 1997), whereas co-injection of Kctd15 inhibits induction (Dutta and Dawid, 2010). Recent analyses by other workers have focused on induction of NC genes more selectively by Pax3 and Zic1 (Bae *et al.*, 2014, Plouhinec *et al.*, 2014). We injected four sets of RNAs, *lacZ* as control (L), *wnt3a+chd* (WC)*, wnt3a+chd+kctd15* (WCK)*,* and *kctd15* (K). We tested several NC markers by PCR and found that, as predicted, *wnt+chd* induced, and *kctd15* inhibited NC marker expression (Fig. 1B). Microarray analysis was then carried out (for raw data see GEO accession number GSE72391). Here we will deal with the two relevant comparisons: WC vs. L, representing genes activated during NPB/NC induction, although additional genes are affected; and WCK vs. WC, representing genes inhibited by Kctd15 in the context of Wnt/Chd overexpression. For further analysis we selected genes with a fold change of two or greater and a p value <0.05. Under the criteria stated above, there are 2869 Affymetrix probe sets that are induced by Wnt/Chd, while 3680 probe sets are suppressed (Supplementary Table S2). Note that Affymetrix probe sets often represent genes repetitively; nevertheless we will use “genes” to stand for “probe set” hereafter. A number of known NPB or NC genes were strongly induced by Wnt/Chd overexpression (Table 1), supporting the microarray results.

Gene ontology (GO) analysis of the NPB/NC transcriptome revealed that GO terms of cellular component organization or biogenesis, cellular component organization, developmental process, anatomical structure morphogenesis, anatomical structure development, single-organism developmental process, single-organism process, single-organism cellular process, system development, and cell morphogenesis are highly represented (Fig. 1C).

We next surveyed the comparison of WCK vs WC, and found that 334 genes are suppressed by addition of Kctd15, while 210 genes were increased under the criteria stated above (Supplementary Table S3). Among the suppressed genes we found all genes listed in Table 1 except *pax3a/b* and *zic1* (see Discussion). Expression of Kctd15 itself was much reduced in WCK animal caps, suggesting that Kctd15 negatively regulates its own expression. Gene ontology analysis showed that GO terms regulation of cell differentiation, positive regulation of cell differentiation, response to inorganic substance, response to estradiol, positive regulation of developmental process, response to estrogen, stem cell differentiation, response to lipid, tissue development, response to oxygen-containing compound are significantly enriched (Fig. 1D). While multiple terms are included, Kctd15 appears to affect most strongly aspects of cell differentiation.

As two recent reports examined gene expression in ACs induced to form NC by pax3 and zic1 injection (Bae *et al.*, 2014, Plouhinec *et al.*, 2014), we tested the overlap between the gene sets in GEO submissions associated with these references and our data, using genes changed by 2-fold or more in

either direction at p<0.05. Among 203 genes increased in the data of Plouhinec et al., 154 are also increased in our data, and among 227 decreased genes, 205 in our set are likewise decreased (Supplementary Table S4). In the comparison with the work of Bae et al., of 713 increased genes 702 are also increased in our data, and among 863 decreased genes 853 are decreased in our data (Supplementary Table S5). Thus the overlap with both sets of data is high.

Developmental expression of selected genes affected by Kctd15

We next used whole mount in situ hybridization to examine the expression pattern of genes encoding Duf domain protein 1 (*ddcp1*), Platelet-derived growth factor receptor a (*pdgfra*), Complement factor properdin (*cfp*), Zinc finger SWIM-Type Containing 5 (*zswim* 5), and Complement component *C3* (Table 2).

Duf domain containing protein 1(*ddcp1*) encodes a protein that has not been characterized, and even the function of the Duf domain is unknown. Signals for *ddcp1* became visible at neurula stages, forming stripes along the anterior and lateral edges of neural fold (Fig 2A,B). Two signal patches at each side with higher intensity can be observed at the future head region (arrows in Fig. 2A). Transverse sections of a stage 18 indicated that staining was localized in a region extending from ectoderm, mesoderm to the endoderm (Fig. 2B). With the progress of neural fold closure, the two signal patches seem to merge and form a short strip at the midline of the head region at tadpole and tailbud stages (Fig. 2C-E,G). Transverse sections identify an additional expression region of *ddcp1* at the stomodeal-hypophyseal anlage (Fig. 2F).

Platelet-derived growth factor receptor (Pdgfr) is a receptor tyrosine kinase that has been identified as a pax3/zic1 target (Bae *et al.*, 2014, Plouhinec *et al.*, 2014). These authors also showed expression of *pdgfra* in the NC region at neurula stages. We found strong expression in the branchial arches in tail bud and tadpole stage embryos (Fig. 3A,B).

Complement factor properdin (Cfp) is a cytoplasmic glycoprotein that is implicated in regulating the alternative complement pathway of the innate immune system (Ali *et al.*, 2014). *Cfp* transcripts can be detected in neurula stage embryos, presenting as two diffuse stripes extending laterally from the dorsal midline of stage16 embryos. Expression of *cfp* was observed in the neural tube, otic vesicle, and dorsal region of the branchial arches at tadpole stages (Fig. 3D,E).

Zinc Finger SWIM-Type Containing 5 (*zswim5*) encodes a protein that remains to be characterized. Whole mount in situ hybridization indicates that *zswim5* was not expressed before gastrula stages (Fig. 4A). Staining was first detected in the dorsal blastopore lip at the onset of gastrulation (Fig. 4B). The positive region expanded and covered the prospective neural plate at mid gastrula stage (Fig. 4C). At the early neurula stages, *zswim5* was expressed at the anterior border of the neural plate, partially overlapping with the NC region (Fig. 4D-F), and this expression was further confirmed by transverse and sagittal sections (Fig. 4G,H). Weak signals were also detected along the lateral border of the neural plate, extending posteriorly. At mid and late neurula stages, *zswim5* was detected in the

anterior border of the neural plate and the NC (Fig. 4I,J). At tail bud and tadpole stages, *zswim5* expression can be detected in the brain and anterior portion of spinal cord. In addition, it was also detected in the eye, especially in the retina region (Fig. 4K-R). It is notable that some regions of forming cranial nerves were also stained (pointed by arrow in Fig. 4L) (Wang *et al.*, 2011).

Complement component 3 (C3) is one of the key components in activation of the complement system in higher vertebrates. Its activation is required for both classical and alternative complement activation pathways. *C3* transcripts became visible from the early neurula stages onward (Fig. 5A,B). At stage 14, the *C3* transcripts were detected at the edge of the forming neural plate. Staining for *C3* was specifically observed in the NC at stage 16, and this expression was confirmed by sectioning (Fig. 5C,D). At the tail bud and tadpole stages, a strong *C3* signal was observed in the trunk region, whereas the head was much less stained. Transverse sections of embryos after whole mount in situ hybridization revealed that the *C3* transcripts are located in a region corresponding to the somatic and splanchnic layer of lateral plate mesoderm, and ventral mesoderm including the blood island (Fig. 5E-H). At stage 35, *C3* stains the heart primordium in addition to intense endodermal expression (Fig. 5I). *C3* expression in *Xenopus* has previously been reported by McLin and colleagues (McLin *et al.*, 2008). While there are some differences in detail our results confirm strong NC expression at stage 16-17, and strong endodermal expression at later stages.

To validate the microarray data, we examined the expression of *zswim5* and *C3* upon overexpression of *kctd15* by in situ hybridization. We injected *kctd15* mRNA into either both blastomeres of two-cell stage embryos or two dorsal blastomeres of four-cell stage embryos, and checked the expression of *zswim5* and *C3*. Overexpression of *kctd15* dramatically reduced *zswim5* and *C3* staining, consistent with our microarray data (Fig. 6; Table 2).

Discussion

Further exploration of the regulatory network that controls NC formation is essential for understanding this developmental process in the normal embryo and the pathological conditions due to disturbed NC development. Kctd15 was previously implicated in the regulation of NC formation, as overexpression of Kctd15 causes strong inhibition of NC development (Dutta and Dawid, 2010). In order to further understand the molecular events regulated by Kctd15, we performed DNA microarray analysis to identify genes that are affected by Kctd15 during NC formation.

In the system we used, *Xenopus* animal caps induced by inhibition of BMP and activation of Wnt signaling, NC differentiation is elicited together with other events. Yet this system allows a broad evaluation of genes that are affected during the induction process. We found activation of expression of multiple genes known to be characteristic of NC development, some of which are illustrated in Table 1. In line with previous findings, we also detected components involved in modulating Wnt (Shisa), retinoic acid

(rarres1, cyp26c1, cyp26a1), and BMP (bambi) signaling, and other genes identified in earlier work. In addition, we have identified a number of novel genes that are induced under these conditions and future analysis will be needed to elucidate their possible functions in NC specification and differentiation.

During the NC specification, coordination of *pax3* and *zic1* gene activity is essential and sufficient to initiate this complex process (Bae *et al.*, 2014, Garnett *et al.*, 2012, Hong and Saint-Jeannet, 2007, Milet *et al.*, 2013, Monsoro-Burq *et al.*, 2005, Sato *et al.*, 2005). Two recent papers reported the target gene profile of Pax3 and Zic1, both of which utilized inducible constructs in order to identify genes specifically involved in NC specification, EMT induction, and migration. Comparison of our results with those of (Bae *et al.*, 2014) and (Plouhinec *et al.*, 2014) shows a considerable degree of overlap (see Results).

The focus of our study has been to identify genes whose activation during manipulation of Wnt and BMP signaling is sensitive to Kctd15. In the subset of genes shown in Table 1, all except *pax3a/b* and *zic1* are strongly inhibited. As Kctd15 strongly inhibits AP-2 function in addition to lesser inhibition of Wnt signaling (Dutta and Dawid, 2010, Zarelli and Dawid, 2013), these results suggest that *pax3* and *zic1* expression in the neural border might be independent of AP-2. Using morpholino-mediated knock-down and enhancer studies, de Croze et al. (de Croze *et al.*, 2011) found that expression of *pax3,* but not of *zic1* depends on AP-2 function in this system. A possible explanation for this difference may be that different members of the AP-2 family may be involved with different Kctd15 sensitivity, or that the particular *pax3* enhancer studied confers Kctd15 resistance on AP-2 molecules bound to it. In any case, it is of interest that Kctd15 can inhibit expression of multiple genes lower in the NC induction hierarchy, but does not affect two genes at its top.

Members of *sox* gene family, notably *sox8*, *sox9* and *sox10,* are strongly activated by NC induction and are very susceptible to overexpression of *kctd15*. All three genes belong to the *soxE* group, which are known to be important to NC formation (Haldin and LaBonne, 2010). The effect of Kctd15 on *soxE* gene expression is likely through inhibition of AP-2 function, known to be upstream of the *sox* genes in NC formation (de Croze *et al.*, 2011). In addition, Kctd15 may have a role in chondrogenesis and osteogenesis as Sox9 and Sox10 are essential in these two developmental processes (Kozhemyakina *et al.*, 2015, Long and Ornitz, 2013). Some matrix metallopeptidases including adam33, mmp3 and mmp28a, and the genes involved in ER stress and the secretory pathway such as creb3l2 and dnajb9 are also inhibited by ectopic Kctd15 (Supplementary Table S3), suggesting again a broad spectrum of developmental processes that can be regulated by Kctd15.

We have examined the expression of some genes induced by Wnt/Chd and repressed by Kctd15, *ddcp1*, *pdgfra*, *cfp, zsmim5* and *C3*, by in situ hybridization, and further confirmed that *zswim5* and *C3* are inhibited by overexpression of Kctd15. We chose these genes because their possible role in NC formation was not previously noted, or noted but not studied extensively. We find expression of these genes variously in the neural border, premigratory NC, subsequent NC derivatives, and also in unrelated

tissues. These observations suggest that Kctd15 may affect the development of multiple cells and tissues in embryogenesis. The large number of genes affected by Kctd15 in our experiments further suggest that the molecular function of Kctd15 might be in the regulation of transcription rather than in other pathways as indicated for different members of the Kctd family (Balasco *et al.*, 2014, Bayon *et al.*, 2008, Canettieri *et al.*, 2010, Chen *et al.*, 2009, Correale *et al.*, 2011). Our results provide a framework for future studies into the biological role of Kctd15.

Figure legends

Fig. 1. Transcriptome analysis of animal explants induced by co-injection of *wnt3a* and *chd* mRNA. (A) Schematic diagram of experimental procedures. (B) Kctd15 suppressed the expression of NC marker genes in animal cap assays. Both blastomeres of two-cell stage embryos were injected with the indicated mRNAs. RT-PCR was performed to examine expression of *snai2*, *sox9*, and *foxd3*. Co-overexpression of *wnt3a* and *chd* strongly induced the expression of these genes, and the induction was suppressed by addition of *kctd15*. *Odc* was the loading control. WE, whole embryos. (C,D) The top ten enriched GO terms in differentially expressed genes in the comparison of (B) induced to control (WC/L), and (C) induced/inhibited to induced (WCK/WC) animal caps.

Fig. 2. Spatial expression pattern of *ddcp1* encoding a Duf domain protein. (A-C) *ddcp1* was expressed along the edge of neural plate. Notably, two stronger staining patches (arrows) were detected in whole mount embryos (A) and transverse sections (B). With the closure of neural tube, the two signaling patches merged and were located at the frontal region of the head (D-G). Ant, anterior view; lat, lateral view. Ac, archerteron; ev, eye vesicle; bv, brain vesicle; hb, hindbrain.

Fig. 3. Spatial expression pattern of *pdgfra* and *cfp*. (A-B) Strong signals of *pdgfra* were detected in branchial arches at tail bud and tadpole stages. (C-E) Cfp signals were detected first at mid neurula stage, and appear as two stripes at the anterior neural plate indicated by red arrows (C). The signals were detected in brain, lens, and ear vesicle at tail bud and tadpole stages (D, E).

Fig. 4. Spatial expression pattern of *zswim5*. *Zswim5* was first detected at the dorsal blastopore lip in early gastrula (A, B), and then was preferentially expressed in the future neural plate at later gastrula stages (C). At early neurula stages, *zswim5* signals were restricted at the anterior neural plate, partially covering the NC region (D-F). (G,H) Transverse section (G) and sagittal section (H) of stage15 embryos. Red arrows point to stained regions. At mid neurula stages, *zswim5* signals were restricted in NC and anterior border of the neural plate (I,J). (K-R) *Zswim5* expression at tail bud and tadpole stages. *Zswim5* was mainly expressed in the anterior neural tube including forebrain, midbrain, hindbrain, and anterior portion of the spinal cord. In addition, signals were also detected in the retina of the developing eye and the forming cranial nerves (pointed by red arrows). N' and P' indicate the planes of the sections shown in

N and P, respectively. Ac, archenteron; bt, blastocoel; ev, eye vesicle; eym, endodermal yolk mass; fg, foregut; hb, hindbrain; mb, midbrain; nt, notochord; rt, retina; and sc, spinal cord.

Fig. 5. Spatial expression pattern of complement component C3 (*C3*)**.** *C3* signals were not detected by late gastrula stages (A). Its expression appeared at the anterior border of the neural plate, and then became restricted at the NC (B, C). NC expression was confirmed by sectioning (D). *C3* expression in the NC declined quickly, and an expression domain gradually appeared at the ventral region of the trunk (E,F). Sections from stage 32 embryos indicated that *C3* was expressed in the somatic and splanchnic layer of lateral plate mesoderm just internal to the epidermis. G, sagittal section; H, transversal section. Endodermal expression is prominent at later stages (I). Arrow points to an expression domain in the heart primordium. Ac, archenteron; bl, blood island; edy, endodermal yolk mass; ep, epidermis; lpm, lateral plate mesoderm; nc, neural crest; nt, neural tube; nd, notochord; sm, somite; vm, ventral mesoderm.

Fig. 6. Overexpression of Kctd15 inhibits expression of *zswim5* and *C3*. Both blastomeres of two-cell stage (A1,B1) or two dorsal blastomeres of four-cell stage embryos (A2,B2) were injected with *kctd15* mRNA, and the injected embryos were collected at about stage 16. The expression *zswim5* and *C3* was strongly inhibited compared to those in control embryos (A,B). (A1) 72%, 13 of 18 embryos; (A2), 95%, 19 of 20 embryos; (B1), 58%, 11 of 17 embryos, and (B2) 73.7%, 14 of 19 embryos.

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Supplementary Information

Table Legends

Table S1. PCR primers used in this study.

Tables S2 – S5. In these tables, only genes (Affymetrics probe sets) are listed that are changed in the relevant comparison by two-fold or more at a p value of <0.05. In Tables S2 and S3, positively changed genes are highlighted in red and negatively changed genes are highlighted in yellow. Complete sets of original data have been deposited at GEO under accession number GSE72391.

Table S2. Genes increased or decreased by two-fold or more in the comparison of AC transcriptomes after injection with Wnt3a+Chd as compared to LacZ (control).

Table S3. Genes increased or decreased by two-fold or more in the comparison of AC transcriptomes after injection with Wnt3a+Chd+Kctd15 as compared to Wnt3a+Chd.

Table S4. Comparison of changes in gene expression elicited in AC by Wnt3a+Chd (our data, Table S2) and changes in response to Pax3+Zic1 (data of Plouhinec et al., 2014).

Table S5. Comparison of changes in gene expression elicited in AC by Wnt3a+Chd (our data, Table S2) and changes in response to Pax3+Zic1 (data of Bae et al., 2014).

Table 1

Induction and repression by Kctd15 of known genes characteristic for neural border and NC.

Gene	Unigene	WC vs L, Fold	WC vs L, p	WCK vs WC, Fold	WCK vs WC, p
foxd3a[+#]	Xl.525	144	1.22E-05	-5.23	0.013
pax3b[#]	Xl.45266	129	9.44E-05	N. S.	
foxd3b[+#]	Xl.523	119.3	3.93E-05	-5.65	0.018
zic1[#]	Xl.1796	105.3	4.44E-05	N. S.	
pax3a[#]	Xl.49495	84.86	3.71E-04	N. S.	
sox9a[+#]	Xl.1690	51.5	8.00E-05	-14.13	0.0011
lmx1b.1[+#]	Xl.12464	38.52	4.39E-06	-2.57	0.0013
sox10[#]	Xl.1588	36.2	0.00359	-12.56	0.02
sox8[+]	Xl.29789	34.28	2.34E-05	-5.4	0.0011
snail2a[+#]	XL.3818	26.6	0.0009033	-20.54	0.0015
twist1b[+#]	Xl.56708	10.8	6.86E-05	-3.21	0.0061
tfap2e[#]	Xl.50785	4.427	0.000404	-2.2	0.016

WC, Wnt+Chd, referring to RNA from animal caps injected with these mRNAs; L, LacZ; WCK, Wnt+Chd+Kctd15. N.S., change not significant.

[+] Genes identified in Table 2 or Table 3 of (Bae *et al.*, 2014).

[#] Genes identified in Table 2 or Table S1 of (Plouhinec *et al.*, 2014).

Table 2

Microarray values for induction and inhibition of genes tested by in situ hybridization.

Gene	Unigene	WC vs L, Fold	WC vs L, p	WCK vs WC, Fold	WCK vs WC, p
ddcp1	Xl2.13675	27.0745	3.54E-06	-4.09502	0.00135358
pdgfra	Xl2.20029	16.6366	2.33E-05	-3.5796	0.00230006
cfp	Xl2.41202	8.49842	4.00E-06	-2.36589	0.00214363
zswim5	Xl2.40416	3.04663	0.0003077	-2.40434	0.00142697
c3	Xl2.44891	14.404	0.0166839	-6.64701	0.0951221

See Table 1 for abbreviations.

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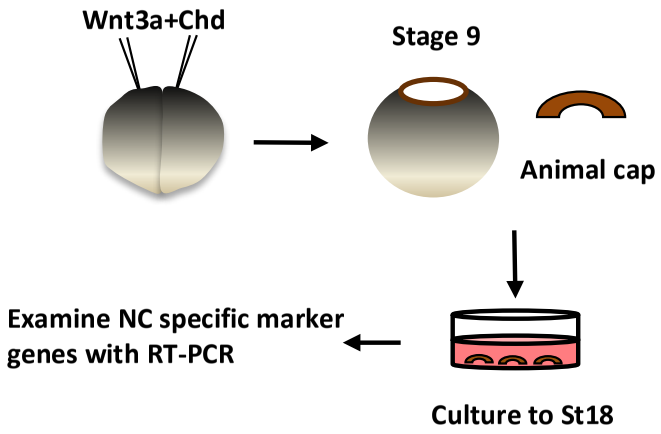

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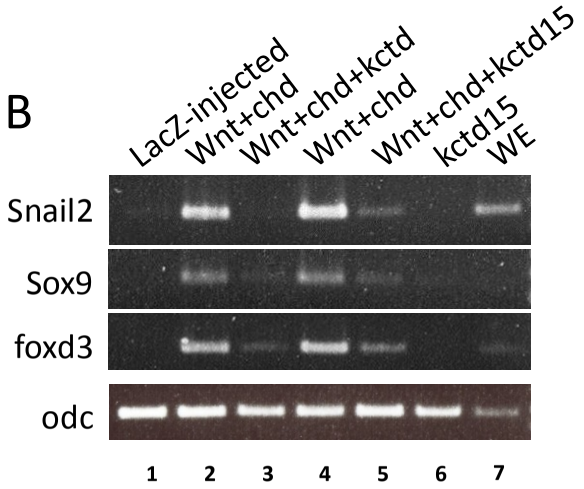

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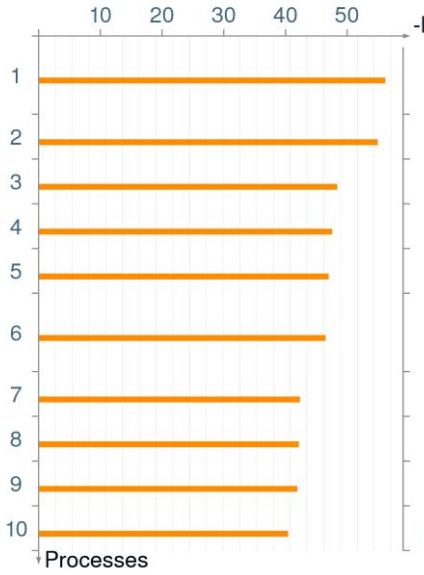
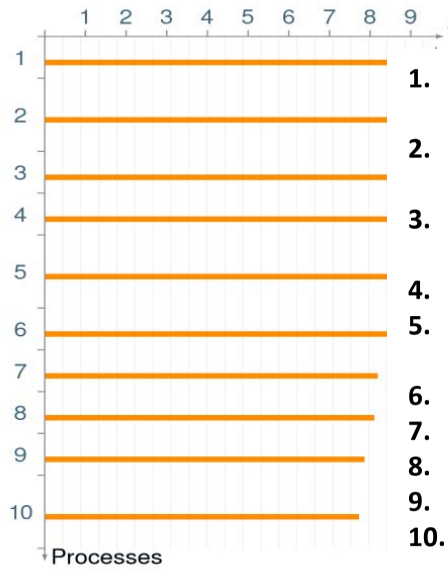


1. Cellular component organization or biogenesis
2. Cellular component organization
3. Developmental process
4. Anatomical structure morphogenesis
5. Anatomical structure development
6. Single-organism developmental
7. Single-organism process
8. Single-organism cellular process
9. System development
10. Cell morphogenesis

D



1. Regulation of cell differentiation
2. Positive regulation of cell differentiation
3. Response to inorganic substance
4. Response to estradiol
5. Positive regulation of developmental process
6. Response to estrogen
7. Stem cell differentiation
8. Response to lipid
9. Tissue development
10. Response to oxygen-containing compound

GO analysis of Wnt+Chd vs Lac Z

GO analysis of Wnt+chd+kctd15 vs wnt+chd

Figure 1

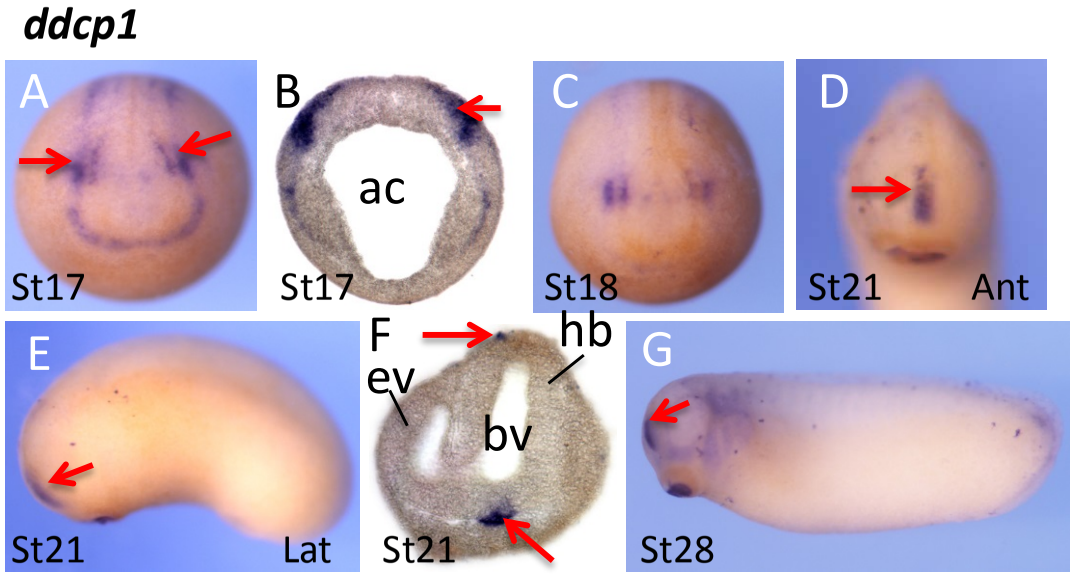

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Figure 2

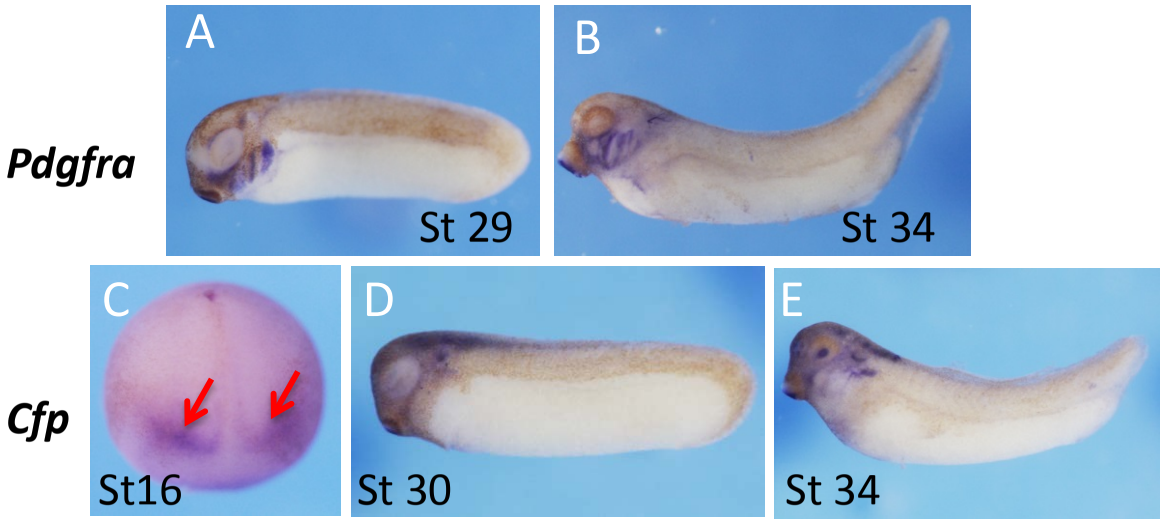
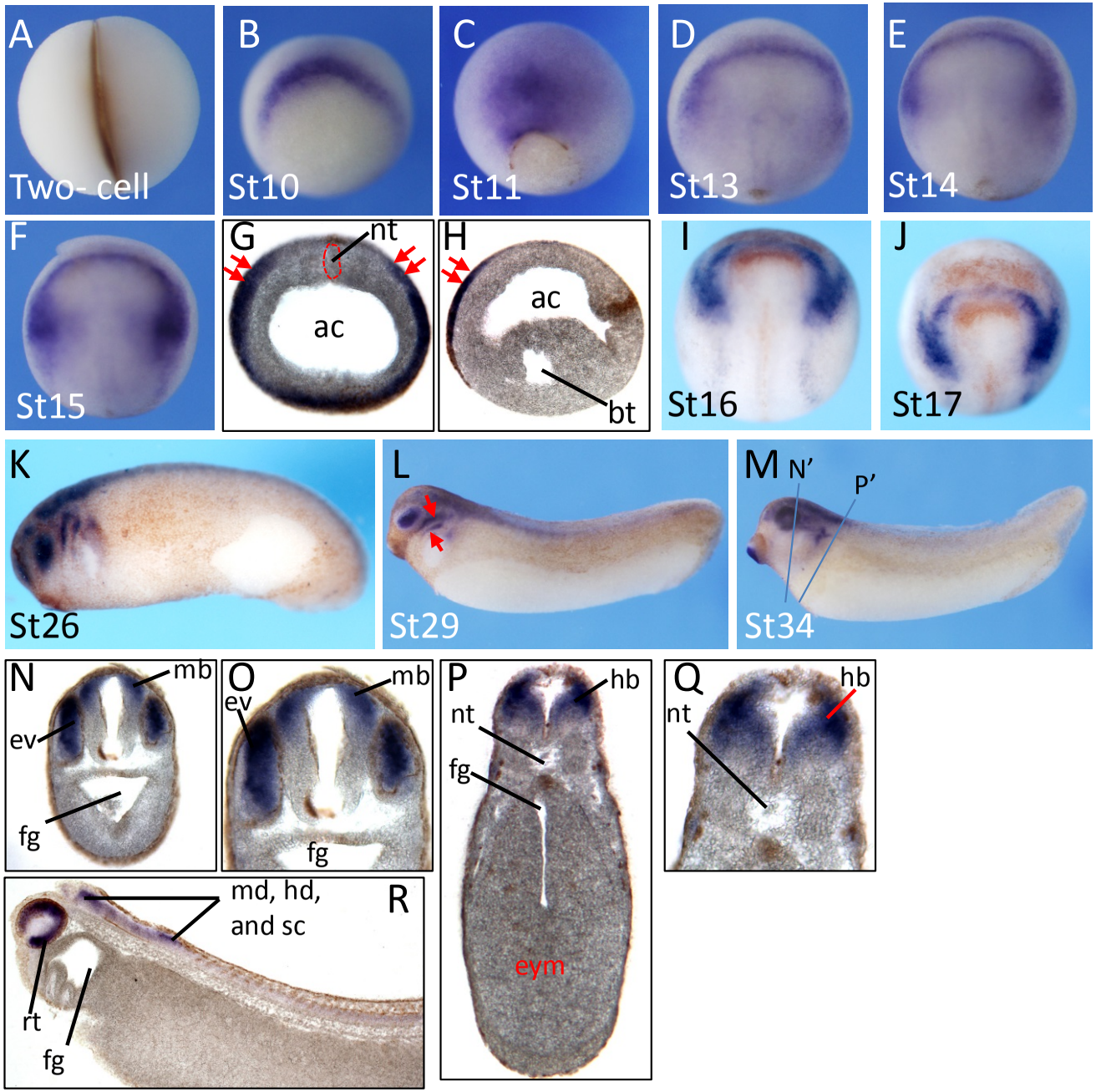


Figure 3

Zswim 5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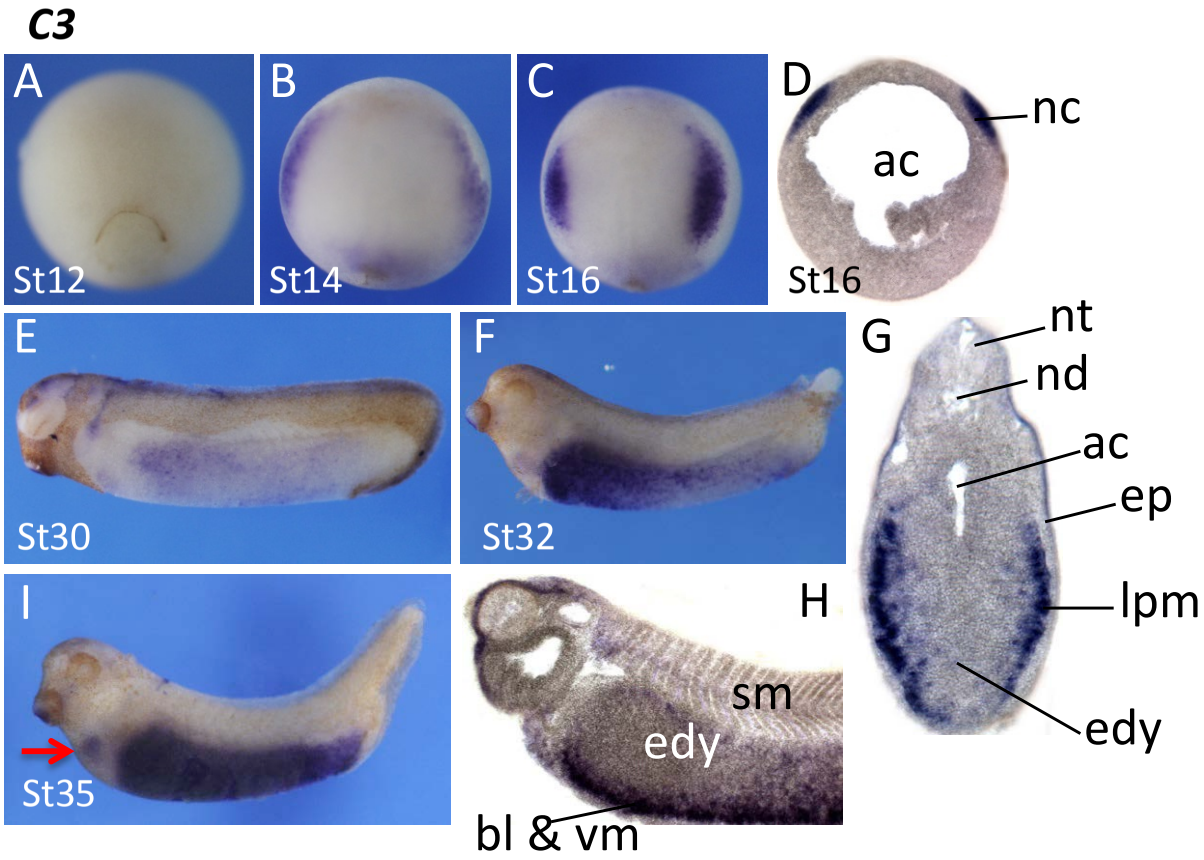
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Figure 5

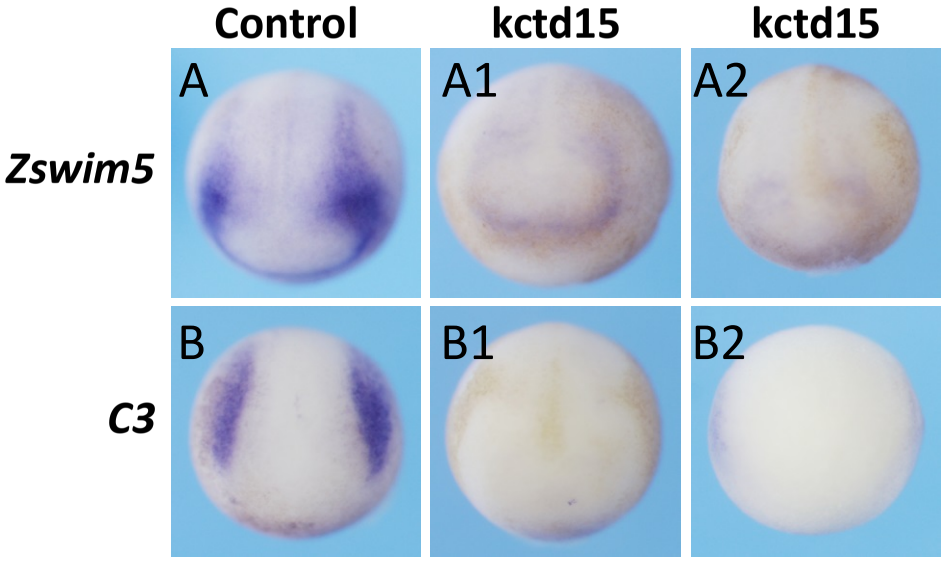

Figure 6