行政院國家科學委員會專題研究計畫 期末報告

探討 diras 家族蛋白及其相關基因在斑馬魚胚胎發育所扮演的解色

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 101-2311-B-040-001-
執 行 期 間 ：101 年 08 月 01 日至 102 年 07 月 31 日
執 行 單 位 ：中山醫學大學生化暨生物科技研究所

計 畫 主 持 人：許立松

計畫參與人員：碩士班研究生-兼任助理人員：吳崇毓
碩士班研究生-兼任助理人員：洪銘謙
碩士班研究生-兼任助理人員：簡寬穎
博士班研究生-兼任助理人員：葉期瑋

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 10 月 16 日

中 文 摘 要 ： 與典型的Ras GTPase相較之下，DI-RAS基因在生物功能上扮演一個不同的角色。最近，人類的DI-RAS基因已被鑑定並顯示無法活化下游MAPK和ELK-1的轉錄。由線蟲模式中的證據已指出，線蟲的DI-Ras同源基因，DRN-1，其專一表現於在神經元細胞，同時亦證明DRN-1在運動神經元中負責調節突觸活性與活動，然而，DI-RAS基因如何參與神經元的發展仍然有許多未知之處。在本篇實驗中，我們利用斑馬魚動物模式，鑑定出斑馬魚中di-rasla和di-raslb基因，並顯示出這兩個基因在演化過程中具有高度的保守性。藉由原位雜交和RT-PCR分析顯示di-rasla和di-raslb 屬於zygotically；相較於其他組織，di-rasla和di-raslb大量表達於腦組織中。利用顯微注射反譯股寡核苷酸制基因的表達，發現di-rasla與di-raslb的缺失明顯的導致軸突缺陷和三叉神經節的數目下降，這些缺陷可利用注射全長而非C端刪除的變異種的小鼠Di-rasl mRNA達到回復的效果。此外，在Tg（huc：gfp）的轉基因魚動物模式中注射反譯股寡核苷酸後，其胚胎的神經元急劇損失。因此，根據我們的研究，di-rasla 和 di-raslb 在斑馬魚的神經新生過程中扮演重要角色。

中文關鍵詞： 神經, 斑馬魚, di-ras基因

英 文 摘 要 ： Di-ras was belonged to a distinct subfamily of Ras GTPases that play a diverse role in biological function compare to canonical Ras family. Recently, human DI-RAS has been identified and fails to trigger downstream MAPK activation and transactivation of Elk-1. Although the evidence from C. elegans model suggest that drn-1, Caenorhabditis elegans Di-Ras homologue, was more restricted in neuron cells and has been shown to modulate synaptic activity in motor neurons, the biological role of Di-Ras in neuron development remains unknown. Here, we identified zebrafish di-rasla and di-raslb gene and provide evidences that both gene were highly conserved. Using whole mount in situ hybridization (WISH) and RT-PCR analysis revealed di-rasla/lb were zygotically deposited and specifically expressed in brain tissue. Interestingly, knockdown of di-rasla and di-raslb gene expression by microinjection of morpholino antisense oligonucleotides led to obvious axon defects and decreased the population of trigeminal

ganglion, these effects could be rescue by co-injection of full-length mouse di-ras1 mRNA but not C-terminal truncated counterpart. Furthermore, the di-rasla and di-raslb MO-injected embryos appeared dramatic loss in the huC population of neuron in Tg(huC:GFP) model. Based on ours studies, di-rasla and di-raslb may play a critical role in neurogenesis during zebrafish development.

英文關鍵詞： Neuron, zebrafish, di-ras

附件一

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

探討 diras 家族蛋白及其相關基因在斑馬魚胚胎發育所扮演的角色

計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC 101－2311－B－040－ 001－
執行期間：101 年 8 月 1 日至 102 年 7 月 31 日

執行機構及系所：中山醫學大學生畫計生物科技研究所

計畫主持人：許立松
共同主持人：
計畫參與人員：葉期瑋

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□移地研究心得報告
□出席國際學術會議心得報告
□國際合作研究計畫國外研究報告

處理方式：除列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

中　華　民　國　102 年　10 月 3 日

附件二

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估 ■達成目標 □ 未達成目標（請說明，以 100 字為限） □ 實驗失敗 □ 因故實驗中斷 □ 其他原因 說明：
2. 研究成果在學術期刊發表或申請專利等情形： 論文：□已發表 □未發表之文稿 ■撰寫中 □無 專利：□已獲得 □申請中 □無 技轉：□已技轉 □洽談中 □無 其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500 字為限）

我們研究首先發現了班馬於中 di-ras1 基因的表現模式，並且利用反譯股寡核苷酸制基因的表達進而發現 di-ras1 基因與神經細胞的發育有密切關聯，此研究結果顯示當斑馬魚的diras1 被抑制後會影響到 trigeminal ganglion 神經的發育，此成果可以當為日後研究此trigeminal ganglion 發育的重要指標。

摘要

與典型的 Ras GTPase 相較之下，*DI-RAS* 基因在生物功能上扮演一個不同的角色。最近，人類的 *DI-RAS* 基因已被鑑定並顯示無法活化下游 MAPK 和 ELK-1 的轉錄。由線蟲模式中的證據已指出，線蟲的 *DI-Ras* 同源基因，*DRN-1*，其專一表現於在神經元細胞，同時亦證明 DRN-1 在運動神經元中負責調節突觸活性與活動，然而，*DI-RAS* 基因如何參與神經元的發展仍然有許多未知之處。在本篇實驗中，我們利用斑馬魚動物模式，鑑定出斑馬魚中 *di-ras1a* 和 *di-ras1b* 基因，並顯示出這兩個基因在演化過程中具有高度的保守性。藉由原位雜交和 RT-PCR 分析顯示 *di-ras1a* 和 *di-ras1b* 屬於 zygotically；相較於其他組織，*di-ras1a* 和 *di-ras1b* 大量表達於腦組織中。利用顯微注射反譯股寡核苷酸制基因的表達，發現 *di-ras1a* 與 *di-ras1b* 的缺失明顯的導致軸突缺陷和三叉神經節的數目下降，這些缺陷可利用注射全長而非 C 端刪除的變異種的小鼠 *Di-ras1* mRNA 達到回復的效果。此外，在 *Tg（huc：gfp）* 的轉基因魚動物模式中注射反譯股寡核苷酸後，其胚胎的神經元急劇損失。因此，根據我們的研究，*di-ras1a* 和 *di-ras1b* 在斑馬魚的神經新生過程中扮演重要角色。

Abstract

Di-ras was belonged to a distinct subfamily of Ras GTPases that play a diverse role in biological function compare to canonical Ras family. Recently, human *DI-RAS* has been identified and fails to trigger downstream MAPK activation and transactivation of Elk-1. Although the evidence from *C. elegans* model suggest that drn-1, Caenorhabditis elegans Di-Ras homologue, was more restricted in neuron cells and has been shown to modulate synaptic activity in motor neurons, the biological role of Di-Ras in neuron development remains unknown. Here, we identified zebrafish *di-ras1a* and *di-ras1b* gene and provide evidences that both gene were highly conserved. Using whole mount *in situ* hybridization (WISH) and RT-PCR analysis revealed *di-ras1a/1b* were zygotically deposited and specifically expressed in brain tissue. Interestingly, knockdown of *di-ras1a* and *di-ras1b* gene expression by microinjection of morpholino antisense oligonucleotides led to obvious axon defects and decreased the population of trigeminal ganglion, these effects could be rescue by co-injection of full-length mouse *di-ras1* mRNA but not C-terminal truncated counterpart. Furthermore, the *di-ras1a* and *di-ras1b* MO-injected embryos appeared dramatic loss in the *huC* population of neuron in *Tg(huC:GFP)* model. Based on ours studies, *di-ras1a* and *di-ras1b* may play a critical role in neurogenesis during zebrafish development.

Key word: Neuron, zebrafish, di-ras, trigeminal ganglion

Introduction

The Ras GTPase superfamily acts as an important role in wide variety biological processes including proliferation, differentiation, signal transduction, protein synthesis and cell metabolism[1-3]. According to their sequence homology and biochemical properties, the Ras superfamily is further divided into five subfamilies: Ras, Rho, Rab, Ran, and Arf[3]. The Rho-family GTPases are belonged to Ras superfamily. Recent intensive evidence also imply these family are key regulators of the actin cytoskeleton, cell adhesion[4-5] and cell polarization[6], especially for neuron cell. *Ras* genes play critical roles in mediating cell proliferation, differentiation and survival during development[7-8]. Furthermore, several studies have also determined the role of Ras family proteins in neuronal plasticity that involved the modification of synapses and the intrinsic excitability of neurons in the brain[9-11]. These studies demonstrate that signaling cascade of Ras family proteins could even modify neuronal function and structure.

More recently, Ellis et al. and Kontoni et al. have identified novel Ras members, Di-Ras proteins, which belonging to a distinct subfamily of Ras GTPases[12-13]. There are three different Di-Ras proteins are designated Di-Ras1/Rig, Di-Ras2 and Di-Ras3/ARHI in human. RNA transcripts of Di-Ras1/Di-Ras2 are specifically expressed in adult human brain tissues. Likewise, Di-Ras1/Di-Ras2 display the low level of intrinsic nucleotide hydrolysis activity and predominantly exists in GTP-bound state. Due to the competition between Di-Ras1 and H-Ras, forced expression of Di-Ras1 does not interact with Raf kinase, fails to trigger downstream MAPK activation and transactivation of Elk-1[12-13]. Indeed, Di-Ras1 has been described as potential tumor suppressor that attenuates the growth of human glioblastomas. Nevertheless, Di-Ras1 exhibit the faster rate on intrinsic guanine-nucleotide exchange than other Ras-family GTPases[14]. Besides, the evidence from *C. elegans* model suggest that drn-1, Caenorhabditis elegans Di-Ras homologue, was more restricted in neuron cells and required for neuromuscular junctions. Despite Caenorhabditis elegans Di-Ras homologue, drn-1, has been shown to modulate synaptic activity in motor neurons[15], the biological role of Di-Ras1 in neuron development remains scarce.

In recent decades, zebrafish becomes an popular vertebrate model organism in scientific research[16], especially in the fields of neurobiology and neurodegenerative diseases[17]. Because of some advantages, zebrafish has now been considered an excellent research tool in laboratory. In contrast with other models like flat worm, sea slug and fruit fly, zebrafish offers a compromise between system complexity and practical simplicity. Most importantly, the nucleotide sequence of zebrafish genes is often found highly similar to the mammalian counterparts, and the functional proteins has also been found to be even more evolutionarily conserved[18-19]. In this study, we described identification and functional analysis of zebrafish *di-ras1a/1b* gene.

Method and Material

Maintenance of zebrafish-The zebrafish AB strain and transgenic *Tg* (*huC:gfp*) fish were kind gifts from the Taiwan Zebrafish Core Facility (Academia Sinica, Taiwan) and Dr. Chang-Jen Huang (Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan), respectively. All of the fish were raised and maintained under standard conditions at 28.5 $^{\circ}$C under a 14 h light/10 h dark cycle[20]. The developmental stages of the embryos were determined according to previously described methods[21]. All of the animal handling protocols were approved by the Chung Shan Medical University Animal Care Committee. Images of the fluorescent signals were captured using a Zeiss LSM510 confocal microscope.

RNA extraction and RT-PCR analysis-Total RNA was isolated from different tissues and stages in adult zebrafish using Trizol (Invitrogen). First-strand cDNA was synthesized from 3 μg of total RNA by MMLV reverse transcriptase (Promega) according to the manufacturer's instructions. The full coding region of di-ras1a/1b were amplified by RT-PCR using zebrafish cDNA as a template and the following primer sets: *di-ras1a* forward: 5'-GCA GCA TGC CAG AGC AGA G- 3' and *di-ras1a* reverse: 5'-GGC AGC ATA CAA ATC ATG AC-3'. *di-ras1b* forward: 5'-GAT GCC TGA GCA GAG CAA CG- 3' and *di-ras1b* reverse: 5'-GCA AGT CTA CAT GAC ACT GC-3'. Wild type mouse *di-ras1* forward: 5′-ATG CCA GAA CAG AGC AAT GAC-3′ and reverse: 5′-GGG CTC ACA TGA GCG CGC AC-3′ ; C-terminal truncate mouse *di-ras1* reverse: 5′-TCACTTGCCCTTGATGCGGTCAG -3′. The PCR was performed under the following conditions: 94 °C, 30 s; 52 °C, 60 s; 72 °C, 60 s for 30 cycles.

Microinjection of antisense morpholino (MO) oligonucleotides and synthetic mRNA-MO oligonucleotides were designed and synthesized by Gene Tools, LLC (Philomath, OR): *di-ras1a*-MO (5'-GGTAATCGTTACTCTGCTCTGGCAT-3'); *di-ras1a* 5-mismatch MO (1a 5-mis MO: 5'-GCTAATCCTTAGTCTCCTCTCGCAT-3'), *di-ras1b*-MO (5'-AGTCGTTGCTCTGCTCAGGCATCTT-3'); *di-ras1b* 5-mismatch MO (1b 5-mis MO: 5'-AGTCCTTCCTCTCCTCACGGATCTT-3'), where the underlines indicate the mismatched sites; control MO: (5'-CCTCTTACCTCAGTTACAATTTATA-3') corresponding to the human β-globin gene was used as a negative control. MOs were dissolved in 1X Danieau solution and injected into the embryos at the 1-to 4-cell stage. The pCS2-mouse-*di-ras1* plasmid were linearized by NotI, and the mRNA was synthesized by SP6 RNA polymerase using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's recommendation. For the rescue experiments, morpholinos were co-injected with 110 pg of mRNA into 1- to 4-cell embryos.

Whole mount in situ hybridization-Whole mount in situ hybridization (WISH) was performed as previous described[22]. Digoxigenin-labeled antisense riboprobes were generated from a pGEM-T-Easy plasmid containing the full coding sequence and partial 3'-untranslated regions of di-ras1a/1b. The plasmid was linearized using NotI. The RNA was transcribed in vitro by the SP6 RNA polymerase to generate antisense probes using the DIG-RNA labeling kit (Roche, Mannheim, Germany).

Anti-acetylated α-tubulin antibody staining-Briefly, embryos at indicated stages were collected, fixed in 4% paraformaldehyde at 4 °C for overnight, and stored at methanol at -20 °C. After rehydration with PBST (PBS containing 0.1% Tween 20), the embryos were blocked with blocked solution (5% goat serum; 2% bovine serum albumin and 1% DMSO in PBST) for 1 h and then incubated with a monoclonal anti-acetylated α-tubulin at 4 °C for overnight. Samples were washed with PBST and incubated the with goat anti-mouse conjugate-FITC antibodies (1:500) in blocking buffer at 4 °C for overnight in the dark. After washed with washing buffer (2% BSA and 1% DMSO in PBST), the images were captured by an scanning laser confocal microscope (ZEISS 510 META).

Result

We previously identified two rgs7 downstream targets[23-24]. In the present study, we selected another putative rgs7 target (GenBank Accession No. NM-199831.1) for further investigation. The *di-ras1a* gene contains 2142 base pairs; encodes a polypeptide of 195 amino acids residues. Furthermore, a BLASTP search was performed using *di-ras1a* amino acids as the query. One gene (LOC565395; GenBank Accession No.NM-001126421.1), which shared high similarity to human *Di-Ras1*, was found and designated as *di-ras1b*.

The 198 amino acids encodes a di-ras1b protein and shared 90% identity and 95% similarity to *di-ras1a*. The homologies of the deduced amino acid sequences of *di-ras1* and that of other species were analyzed using the CLUSTALW program (Fig. 1A). The amino acid alignment of *di-ras1a* and *di-ras1b* showed identity with those from rats, mice, and humans (range from 80% to 87%). Motif research of the di-ras sequences displayed that they contains a well-conserved guanine-nucleotide binding domain. The G1 domain contained GXXXXGKS motif (corresponding to the Residues 14 to 21 of both genes). The invariant T of G2 domain and DXXG motif of G3 domain were found in residue 39 and residues 61–64, respectively. The conserved motif NKXD in G4 domain and EXSAK in G5 domain were localized at Residues 121–124 and 148–152, respectively. In addition, both *di-ras1a* and *di-ras1b* contain a C-terminal CAAX (A represented aromatic amino acids; X represented any amino acid) motif with the X being a methionine, suggesting that both genes were putative substrate for farnesyl transferase. On the basis of the phylogenetic analysis, zebrafish *di-ras1a* and *di-ras1b* were branched into a cluster (Fig. 1B). To determine whether *di-ras1a* and *di-ras1b* genes shared conserved synteny with mammalian species, we compared the gene order around *di-ras1a* and *di-ras1b* in zebrafish, humans, rats, and mice. The *di-ras1b* gene clustered with *gadd45bb (growth arrest and DNA-damage–induced beta), gng7 (guanine nucleotide binding protein gamma 7),* and *slc1a8b (silute carrier protein family 1, member 8b)* in linking group (LG) 2 had conserved synteny with the genes in human Chromosome 19, in rat Chromosome 7, and in mouse Chromosome 10 (Fig. 1C). The *di-ras1a* was flanked by *apc2* (*adenomatous polyposis coli 2*) and *vtg3* (*vitellogenin 3).* Our results suggest that *di-ras1b* is a true orthologues of mammalian *DI-RAS1*, whereas *di-ras1a* was generated from whole genome duplication.

To determine the expression pattern of these two genes during zebrafish development, RT-PCR and whole mount *in situ* hybridization were conducted. The RNA transcripts of *di-ras1a* and *di-ras1b* were rarely detectable before 12 hour post-fertilization (HPF), its expression was first appeared at 24 HPF, and then they were persistently expressed thereafter (Fig. 2A). Moreover, *di-Ras1a* and *di-Ras1b* mRNA were relative enriched in brain. Other tissues expressed slight levels of both transcripts (Fig. 2B). Whole mount *in situ* hybridization analysis revealed that no RNA transcripts of *di-ras1a* and *di-ras1b* were found at 32 cells stage. At 24 HPF, *di-ras1b* was very weakly expressed in the brain region. *di-ras1a* RNA was diffusedly expressed in the brain region with higher expression in hindbrain region at 24 HPF. The *di-ras1a* was abundantly expressed in olfactory bulb, ventral rostral cluster of ventral telencephalon, hypothalamus, hindbrain, otic vesicle, and neurons in dorsal spinal cord at 36 HPF. At 48 HPF, *di-ras1a* expression was strongly observed in the olfactory bulb, thalamus, diencephalons, otic vesicle, and midbrain, as well as in the GCL of the retina. Low levels of *di-ras1a* were also found in dorsal spinal cord neurons. At 72 HPF, *di-ras1a* expression in the neurons was enhanced in the whole brain region, such as the forebrain, midbrain, and hindbrain. RNA transcripts also appeared in the ganglion cell layer and inner nuclear layer of retina (Fig. 2C). On the other hand, the transcript of *di-ras1b* was weakly expressed in the telencephalon, diencephalon, and hindbrain at 36 HPF. At 48 and 72 HPF, *di-ras1b* appeared in telencephalon, diencephalon, hindbrain, and ganglion cell layer (GCL) in the retina (Fig. 2D).

To extend the function of *di-ras1* in zebrafish embryogenesis, we injected antisense morpholino oligonucleotides (MO) into one-cell stage embryos to knockdown of *di-ras1* and generated loss-of-function morphants. Because of the effectiveness and specificity of the certain MO have become the major concerns in MO-mediated knockdown studies, an expression construct that included the sequence of di-ras1 translation start codon targeted by the MO (di-ras1a ATG-MO and di-ras1b ATG-MO), and GFP-tags at the C-terminus was used to determine effectiveness of di-ras1 MO. In addition, 5-mismatch MO corresponding to the di-ras1

coding sequence targeting MO but with a 5-bp mismatch was used as negative control. Expression of *di-ras1-GFP* hybrid gene (100 pg/embryo) was detectable in injected embryos at 24 hpf without developmental malformation. To further gauge the efficacy of di-ras1 MO that capable of inhibiting *di-ras1* expression, *di-ras1-GFP* and MO (4 ng) were co-injected, and GFP-tagged *di-ras1* expression determined by immunofluorescence. The fluorescence signal due to the hybrid protein could not be detected in essentially any injected embryos at 24 hpf. By contrast, when 5-mis MO was co-injected into embryos with *di-ras1-GFP* fusion gene, the fluorescence was detectable of most injected embryos, which confirm the effectiveness of di-ras1a and di-ras1b MO (Fig. 3).

To determine the role of *di-ras1a/1b* during neurodevelopment, we conducted acetylated α-tubulin (AcTub) staining to determine whether disruption of *di-ras1a/1b* affects neuronal differentiation. At 24 HPF, AcTub staining revealed the presence of anterior commissure At 24 HPF, AcTub staining revealed the presence of anterior commissure (AC), post-optic commissure, supra-optic tract, nucleus of the tract of AC, dorsal–ventral diencephalic tract, epiphyseal cluster, and tract of the posterior commissure in the forebrain and the midbrain in the embryos injected with control MOs (Fig. 4). Axonal scaffolds in *di-ras1a* and *di-ras1b* morphants visibly deteriorated. Embryos co-injected with full-length but not truncated mRNA resembled those of the control group. We also used the *Tg (huC:gfp)* model to track the generation of terminally differentiated neurons. A lateral view of the embryos at 24 HPF revealed that the control and MO plus full-length mRNA groups exhibited high levels of *huC* expression in the telencephalic cluster (TC). We observed that *di-ras1a/1b* morphants exhibited abrogated *huC* expression in the forebrain region (Fig. 5A). At 48 HPF, only a small number of *huC*-positive neurons were present in the *di-ras1a/1b* morphants (Fig. 5B). Thus, *di-ras1a/1b* knockdown in zebrafish embryos affects neuronal development during the development. Due to the defect of trigeminal ganglion in AcTub staining, we sought to investigate whether *di-ras1a/1b* may contribute the trigeminal ganglion development during embryogenesis. Anti-Islet2 staining at 24HPF showed that amount of trigeminal ganglia significantly decreased in *di-ras1a/1b* morphants (Fig. 6). Hence, *di-ras1a* and *di-ras1b* may participate in neurogenesis during zebrafish development.

Discussion

The trigeminal ganglion is constructed by neural crest and placodal cells that ultimately composed a compact cluster of neurons on either side of the head between eye and ear[25]. These sensory neurons are born at around 11 HPF and rapidly converge to form compact ganglia by 14 HPF. At later stages trigeminal ganglion grow continuously to form ~30 neurons by 24 HPF[26]. Furthermore, trigeminal ganglion extend peripheral axons underneath the skin of the head, it can detect various stimuli and communicate these inputs to the central nervous system[27].

In this study, we indicated unequivocal defects in trigeminal neuron numbers in the peripheral nervous systems of the MO-mediated *di-ras1a/1b* knockdown embryos. Nevertheless, we need more evidence to pinpoint the reduction of the trigeminal ganglion neuron could be attributed to a role of *di-ras1a/1b* in neuronal cell development like neurogenesis, differentiation and apoptosis. Whether *di-ras* regulates the expression of proneural genes, *ngn1*, remains to be explored. Likewise, several signaling cascade regulate the function of trigeminal ganglion neuron for axon outgrowth, such as Sdf1/Cxcr4-dependent chemotaxis and Notch and/or Her4 pathway[28-29]. Whether *di-ras* may participate in these cascade is an important issue that we must to realize.

In summary, we suggest that *di-ras* plays a critical role in the peripheral nervous system development in

zebrafish.

Reference

1 Colicelli, J. Human RAS superfamily proteins and related GTPases. *Sci STKE*, RE13. (2004).

2 Mitin, N., Rossman, K. L. & Der, C. J. Signaling interplay in Ras superfamily function. *Curr Biol* **15**, R563-574. (2005).

3 Wennerberg, K., Rossman, K. L. & Der, C. J. The Ras superfamily at a glance. *J Cell Sci* **118**, 843-846. (2005).

4 Fukata, M. & Kaibuchi, K. Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nat Rev Mol Cell Biol* **2**, 887-897. (2001).

5 Hall, A. Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514 (1998).

6 Luo, L. Rho GTPases in neuronal morphogenesis. *Nat Rev Neurosci* **1**, 173-180. (2000).

7 Barbacid, M. ras genes. *Annu Rev Biochem* **56**, 779-827. (1987)

8 Konstantinopoulos, P. A., Karamouzis, M. V. & Papavassiliou, A. G. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov* **6**, 541-555. (2007).

9 Martin, S. J., Grimwood, P. D. & Morris, R. G. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci* **23**, 649-711. (2000).

10 Mozzachiodi, R. & Byrne, J. H. More than synaptic plasticity: role of nonsynaptic plasticity in learning and memory. *Trends Neurosci* **33**, 17-26. (2010).

11 Neves, G., Cooke, S. F. & Bliss, T. V. Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nat Rev Neurosci* **9**, 65-75. (2008).

12 Ellis, C. A. *et al.* Rig is a novel Ras-related protein and potential neural tumor suppressor. *Proc Natl Acad Sci U S A* **99**, 9876-9881. (2002).

13 Kontani, K. *et al.* Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. *J Biol Chem* **277**, 41070-41078. (2002).

14 Gasper, R., Sot, B. & Wittinghofer, A. GTPase activity of Di-Ras proteins is stimulated by Rap1GAP proteins. *Small GTPases* **1**, 133-141. (2010).

15 Tada, M. *et al.* Neuronally expressed Ras-family GTPase Di-Ras modulates synaptic activity in Caenorhabditis elegans. *Genes Cells* **17**, 778-789. (2012).

16 Chen, E. & Ekker, S. C. Zebrafish as a genomics research model. *Curr Pharm Biotechnol* **5**, 409-413 (2004).

17 Gerlai, R. Using zebrafish to unravel the genetics of complex brain disorders. *Curr Top Behav Neurosci* **12**, 3-24. (2012).

18 Reimers, M. J., Hahn, M. E. & Tanguay, R. L. Two zebrafish alcohol dehydrogenases share common ancestry with mammalian class I, II, IV, and V alcohol dehydrogenase genes but have distinct functional characteristics. *J Biol Chem* **279**, 38303-38312, (2004).

19 Renier, C. *et al.* Genomic and functional conservation of sedative-hypnotic targets in the zebrafish. *Pharmacogenet Genomics* **17**, 237-253, (2007).

20 Westerfield, M., Doerry, E., Kirkpatrick, A. E., Driever, W. & Douglas, S. A. An on-line database for zebrafish development and genetics research. *Semin Cell Dev Biol* **8**, 477-488 (1997).

21 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic

development of the zebrafish. *Dev Dyn* **203**, 253-310 (1995).

22 Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* **3**, 59-69, (2008).

23 Hsu, L. S., Liang, C. J., Tseng, C. Y., Yeh, C. W. & Tsai, J. N. Zebrafish Cyclin-Dependent Protein Kinase-Like 1 (zcdkl1): Identification and Functional Characterization. *Int J Mol Sci* **12**, 3606-3617, (2011).

24 Hsu, L. S. & Tseng, C. Y. Zebrafish calcium/calmodulin-dependent protein kinase II (cam-kii) inhibitors: expression patterns and their roles in zebrafish brain development. *Dev Dyn* **239**, 3098-3105, (2010).

25 Davies, A. M. The trigeminal system: an advantageous experimental model for studying neuronal development. *Development* **103 Suppl**, 175-183 (1988).

26 Caron, S. J., Prober, D., Choy, M. & Schier, A. F. In vivo birthdating by BAPTISM reveals that trigeminal sensory neuron diversity depends on early neurogenesis. *Development* **135**, 3259-3269, (2008).

27 Baker, C. V. & Bronner-Fraser, M. Vertebrate cranial placodes I. Embryonic induction. *Dev Biol* **232**, 1-61, (2001).

28 Knaut, H., Blader, P., Strahle, U. & Schier, A. F. Assembly of trigeminal sensory ganglia by chemokine signaling. *Neuron* **47**, 653-666, (2005).

29 So, J. H. *et al.* Her4 is necessary for establishing peripheral projections of the trigeminal ganglia in zebrafish. *Biochem Biophys Res Commun* **379**, 22-26, (2009).

FIGURE LEGENDS

FIGURE 1. Protein sequence alignments and phylogenetic tree. (A) Sequence alignment of zebrafish *di-ras1a* and *di-ras1b* with human, rat, mouse, and Xenopus. Identical amino acids in all or three species are shaded in black and light gray, respectively. (B) Phylogenetic tree of zebrafish *di-ras1a* and *di-ras1b*. The phylogenetic tree was created using the MegAlign program in DNASTAR using the neighbor-joining algorithm. (C)….

FIGURE 2. Spatial and temporal expression pattern of zebrafish *di-ras1a/1b.* (A) RT-PCR analyses of *di-ras1a* and *di-ras1b* mRNA in different stages (upper panel) and (B) different adult tissues (lower panel). Elongation factor-1α is used as internal control. Whole-mount *in situ* hybridization analyses of (C) *di-ras1a* and (D) *di-ras1b* mRNA during zebrafish development. Zebrafish *di-ras1a* and *di-ras1b* is expressed zygotically throughout the development, with relative enrichment in the brain.

FIGURE 3. Analysis of *di-ras1a* and *di-ras1b* knockdown efficiency. The effectiveness of morpholino oligonucleotides was tested using the corresponding di-ras1-GFP expression constructs. 100 ng of the di-ras1a-GFP or di-ras1b-GFP plasmid DNA was injected alone or with 4 ng of a morpholino oligonucleotides at the one-cell stage, and a group of embryos were photographed during 24 HPF.

FIGURE 4. Malformation of axons under *di-ras1a/1b* knock down conditions. Effects of *di-ras1a* and *di-ras1b* depletion on neurogenesis, as visualized by acetylated α-tubulin (AcTub) staining. AcTub expression (an axonal marker) was examined using confocal microscopy at 24 HPF. Axonal scaffolds in *di-ras1a* and *di-ras1b* morphants were visibly deteriorated. All images are shown in the lateral view.

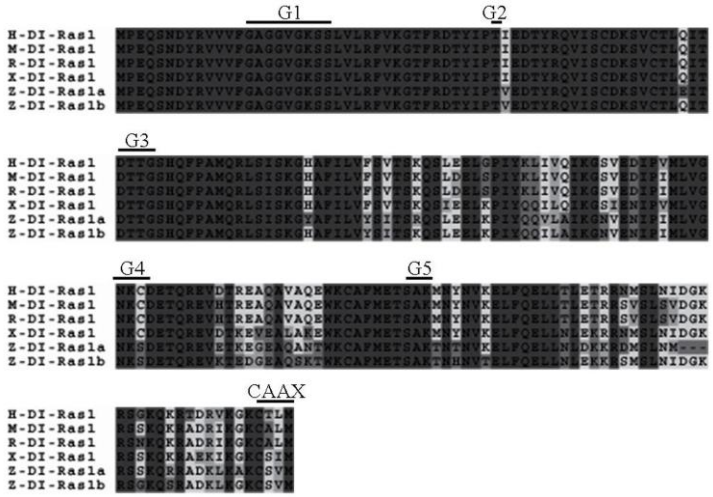
FIGURE 5. Effects of *di-ras1a* and *di-ras1b* depletion on neurogenesis on the basis of the *Tg (huC:gfp)*

model. Embryos that received the indicated MO were collected at (A) 24 and (B) 48 HPF. (A) The *huC* expression in the telencephalon decreased in *di-ras1a* and *di-ras1b* morphants. (B) The *di-ras1a* and *di-ras1b* morphants exhibited abrogated *huC* expression in the forebrain region and significantly decreased *huC* population throughout the neurogenesis.

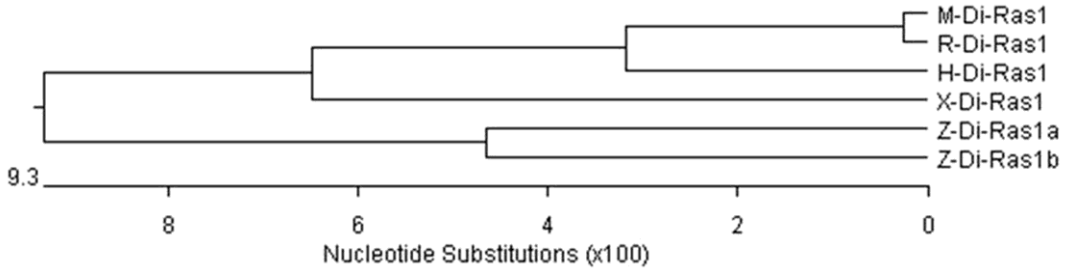
FIGURE 6. *Di-ras1a/1b* are required for trigeminal ganglion neurogenesis. Immunostaining of 24 HPF embryos with an antibody against islet-1 marks (lateral views of the anterior regions of the embryos) primary neurons in corresponding condition.

Figure 1.

(A)


G1
G2
G3
G4
G5
CAAX
H-DI-Ras1
M-DI-Ras1
R-DI-Ras1
X-DI-Ras1
Z-DI-Rasla
Z-DI-Ras1b

(B)


M-Di-Ras1
R-Di-Ras1
H-Di-Ras1
X-Di-Ras1
Z-Di-Ras1a
Z-Di-Ras1b
9.3
8
6
4
2
0
Nucleotide Substitutions (x100)

(C)

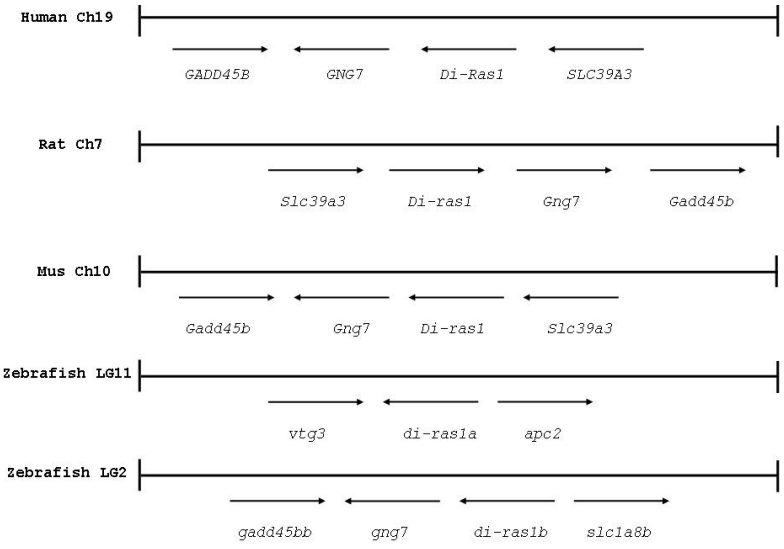
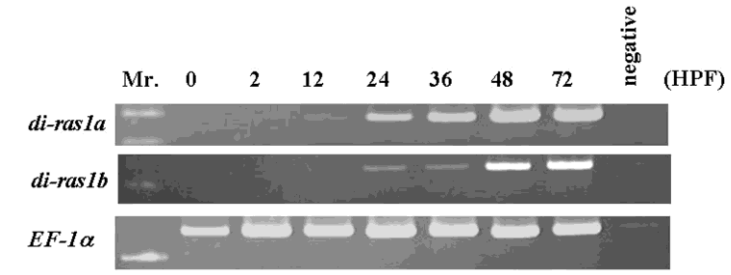
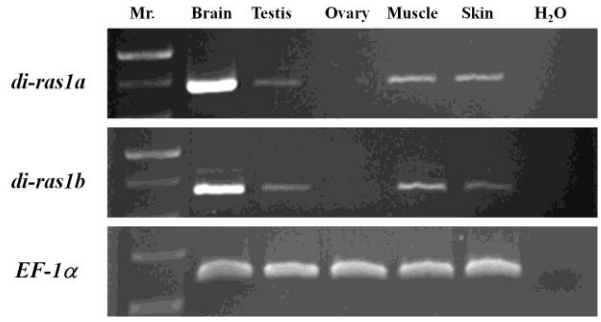

Human Ch19
GADD45B
GNG7
Di-Ras1
SLC39A3
Rat Ch7
Slc39a3
Di-ras1
Gng7
Gadd45b
Mus Ch10
Gadd45b
Gng7
Di-ras1
Slc39a3
Zebrafish LG11
vtg3
di-ras1a
apc2
Zebrafish LG2
gadd45bb
gng7
di-ras1b
slc1a8b

Fig. 2

(A)


Mr.
0
2
12
24
36
48
72
negative
(HPF)
di-ras1a
di-ras1b
EF-1α

(B)


Mr.
Brain
Testis
Ovary
Muscle
Skin
H2O
di-ras1a
di-ras1b
EF-1α

(C) **(D)**

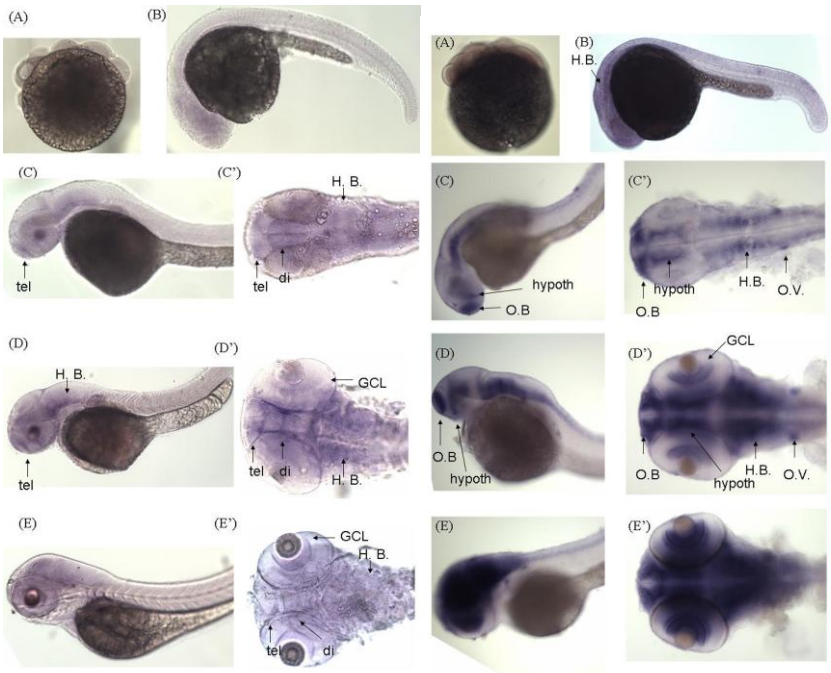

(A)
(B)
(C)
(C')
(D)
(D')
(E)
(E')
H. B.
tel
di
GCL
hypoth
O.B
H.B.
O.V.

Fig. 3

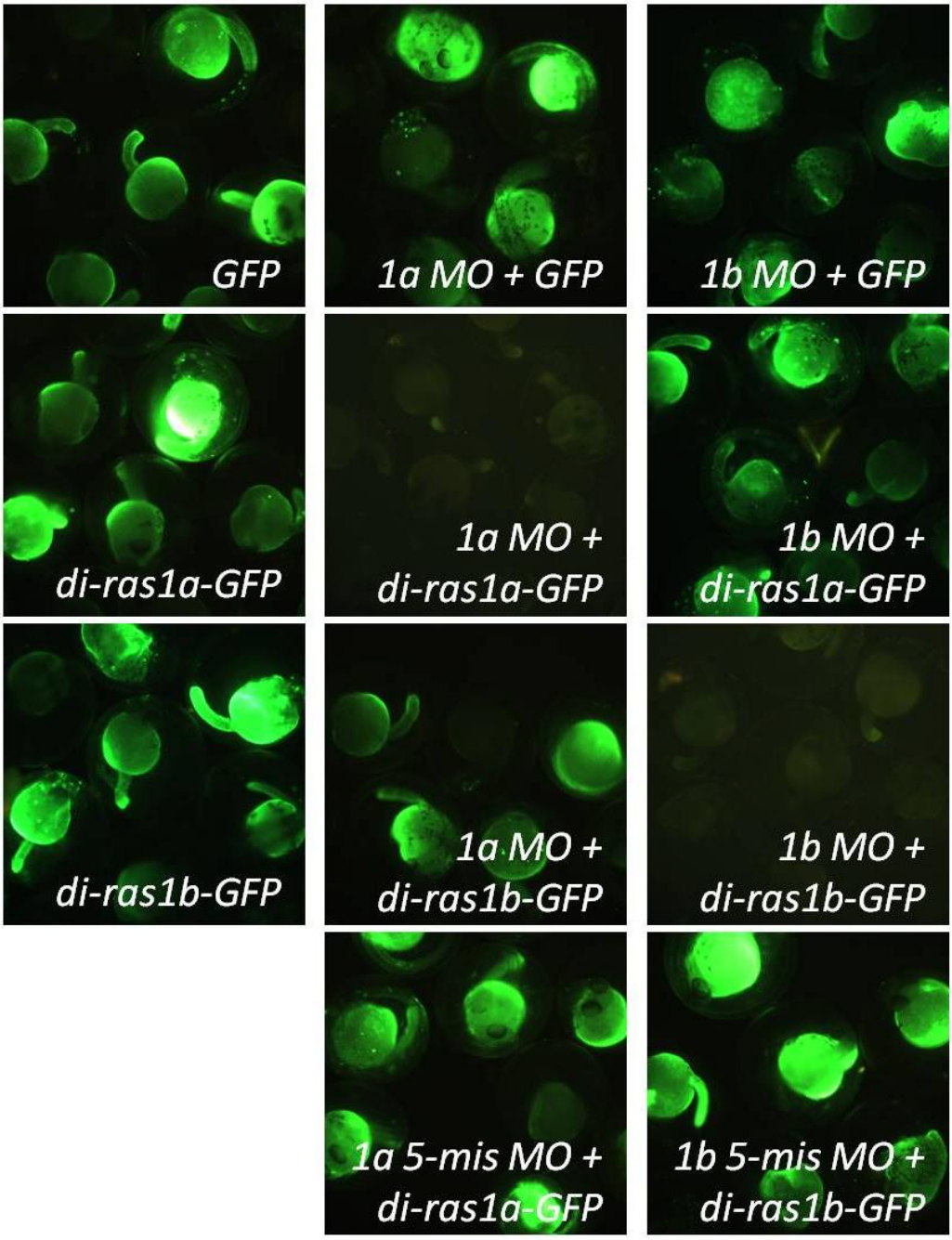

GFP
1a MO + GFP
1b MO + GFP
di-ras1a-GFP
1a MO + di-ras1a-GFP
1b MO + di-ras1a-GFP
di-ras1b-GFP
1a MO + di-ras1b-GFP
1b MO + di-ras1b-GFP
1a 5-mis MO + di-ras1a-GFP
1b 5-mis MO + di-ras1b-GFP

Fig. 4

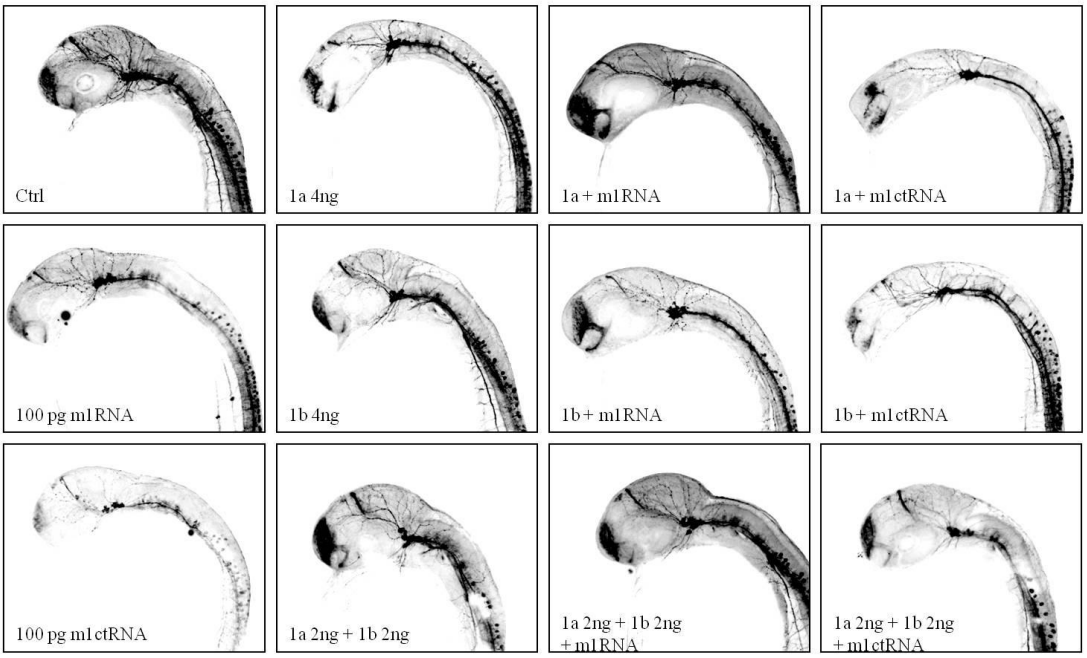
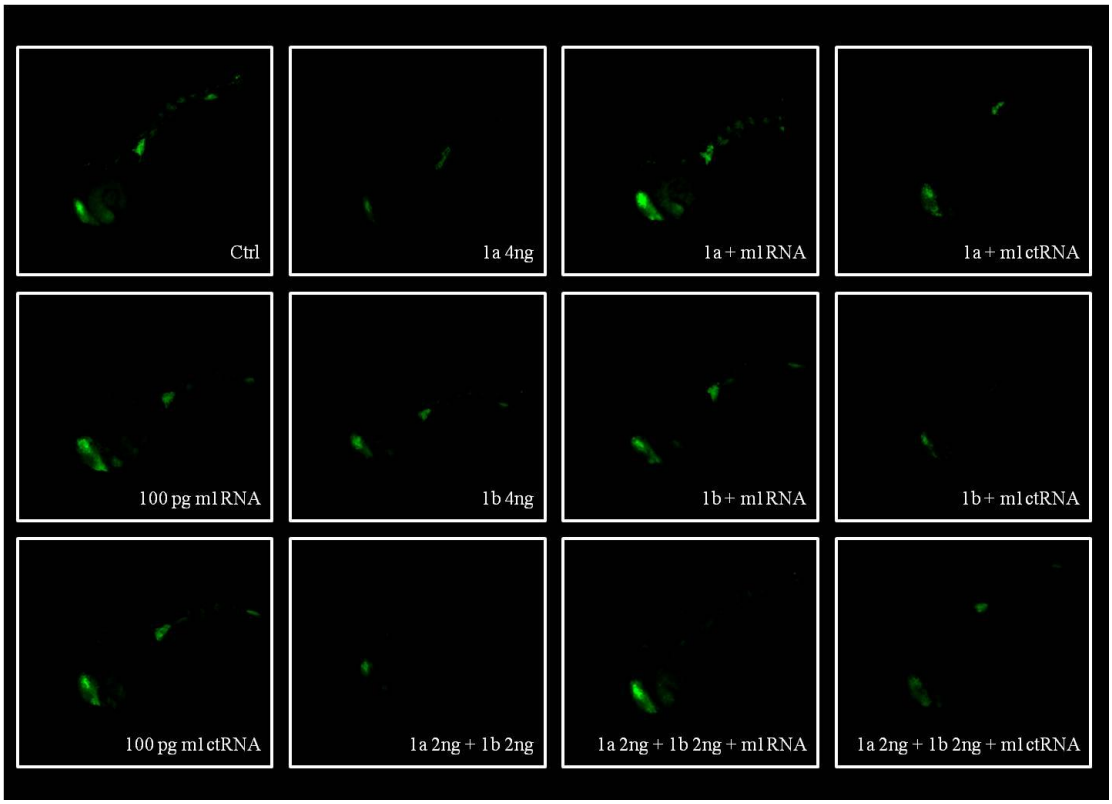

Ctrl
1a 4ng
1a + m1RNA
1a + m1ctRNA
100 pg m1RNA
1b 4ng
1b + m1RNA
1b + m1ctRNA
100 pg m1ctRNA
1a 2ng + 1b 2ng
1a 2ng + 1b 2ng + m1RNA
1a 2ng + 1b 2ng + m1ctRNA

Fig. 5

(A)


Ctrl
1a 4ng
1a + m1RNA
1a + m1ctRNA
100 pg m1RNA
1b 4ng
1b + m1RNA
1b + m1ctRNA
100 pg m1ctRNA
1a 2ng + 1b 2ng
1a 2ng + 1b 2ng + m1RNA
1a 2ng + 1b 2ng + m1ctRNA

(B)

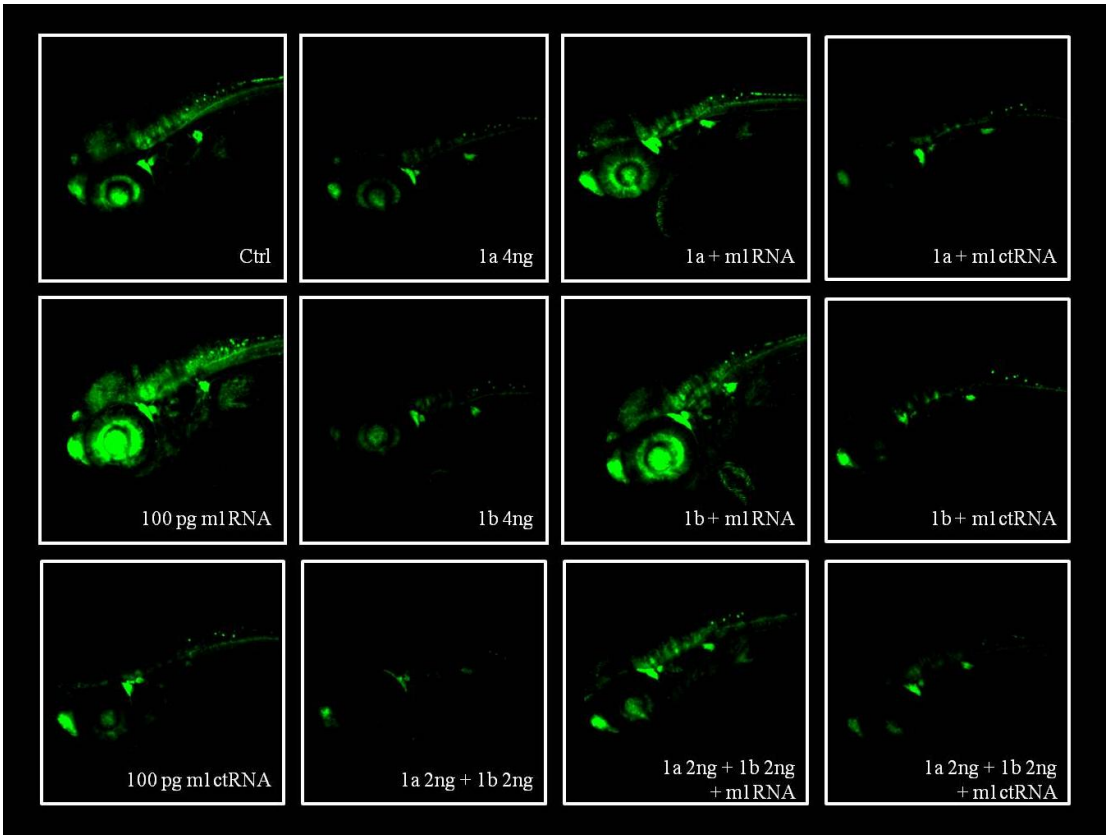
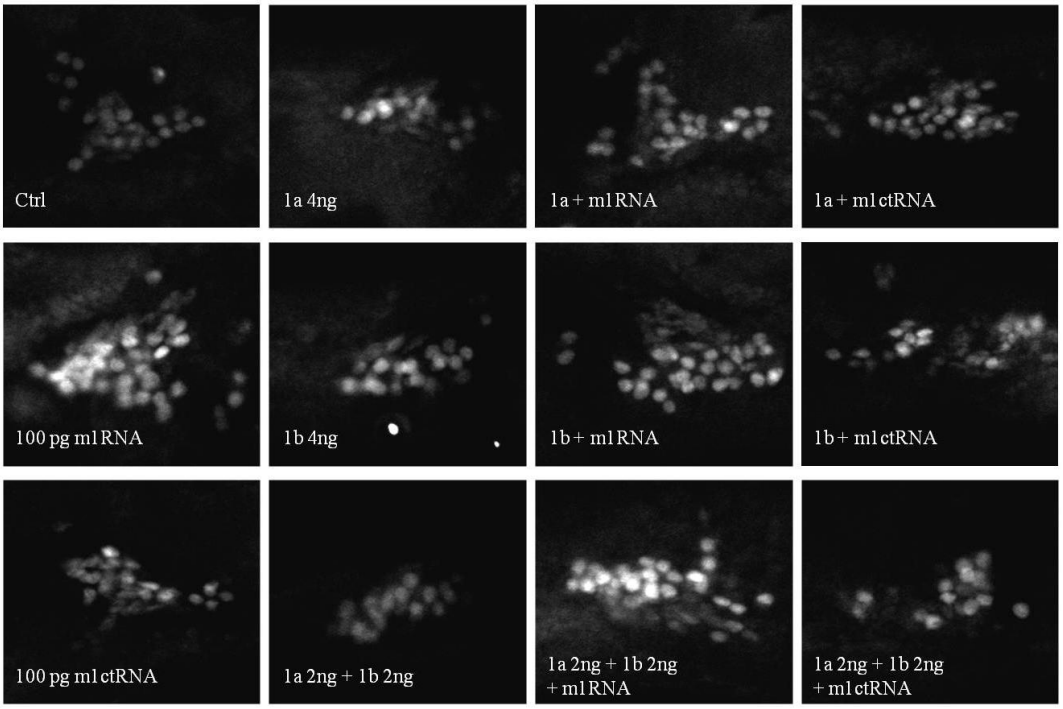

Ctrl
1a 4ng
1a + m1RNA
1a + m1ctRNA
100 pg m1RNA
1b 4ng
1b + m1RNA
1b + m1ctRNA
100 pg m1ctRNA
1a 2ng + 1b 2ng
1a 2ng + 1b 2ng
+ m1RNA
1a 2ng + 1b 2ng
+ m1ctRNA

Fig. 6


Ctrl
1a 4ng
1a + m1 RNA
1a + m1 ctRNA
100 pg m1 RNA
1b 4ng
1b + m1 RNA
1b + m1 ctRNA
100 pg m1 ctRNA
1a 2ng + 1b 2ng
1a 2ng + 1b 2ng
+ m1 RNA
1a 2ng + 1b 2ng
+ m1 ctRNA

國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/01

國科會補助計畫	計畫名稱: 探討diras家族蛋白及其相關基因在斑馬魚胚胎發育所扮演的解色
	計畫主持人: 許立松
	計畫編號: 101-2311-B-040-001- 學門領域: 生物學之生化及分子生物

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：許立松	計畫編號：101-2311-B-040-001-
計畫名稱：探討 diras 家族蛋白及其相關基因在斑馬魚胚胎發育所扮演的解色	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	3	0	100%	人次	
		博士生	1	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	本研究計畫雖未有論文發表, 但是有兩篇論文已被接受或發表有註明此國科會計畫補助編號.

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

我們研究首先發現了班馬於中 di-rasl 基因的表現模式，並且利用反譯股寡核苷酸制基因的表達進而發現 di-rasl 基因與神經細胞的發育有密切關聯，此研究結果顯示當斑馬魚的 dirasl 被抑制後會影響到 trigeminal ganglion 神經的發育，此成果可以當為日後研究此 trigeminal ganglion 發育的重要指標。