

國立臺灣大學生命科學院生命科學系

碩士論文

Department of Life Science

College of Life Science

National Taiwan University

Master Thesis



分析斑馬魚不同 stathmin 基因在胚胎中的表現
Expression analysis of stathmin family genes in zebrafish
embryos

林碩芃

Shuo-Peng Lin

指導教授：李士傑 博士

Advisor: Shyh-Jye Lee, Ph.D.

中華民國 105 年 6 月

June, 2016

國立臺灣大學碩士學位論文
口試委員會審定書

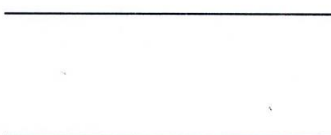
分析斑馬魚不同 Stathmin 基因在胚胎中的表現
Expression analysis of stathmin family genes in
zebrafish embryos

本論文係 林碩芃 君 (R02B21030) 在國立臺灣大學生命科學系 完成之碩士學位論文，於民國 105 年 05 月 25 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

 (簽名)
(指導教授)

生命科學系 系主任  (簽名)

謝

誌



「驚心動魄」這四字，大概是這研究所生活最大的心情寫照，面對著許多煩惱與不安，以及對未來的迷惘，就這樣度過了這些個日子，這過程中雖然有歡笑，有溫馨，有煩惱，有沮喪，有挫折，終於走到了最後，首先，謝謝李士傑老師，面對著總是糊里糊塗報告的我，仍然很有耐心地指導我；謝謝 Ray 學長和 Maggie 學姊，總是幫我解答實驗上的任何疑惑，特別是 Maggie 學姊，不管在大事小事上，都幫我很多；謝謝這一路走來在實驗室遇到的所有人 Vicky、Cathy、LuLu、Tony、Jessie、Murat、Ben、Bruce、Jennie、Tina 以及其他的學弟妹；謝謝我的好飯友思卉及我任何時期的好朋友們，總在我心情煩躁時，聽我歇斯底里；最重要的是謝謝我最親愛的家人，在背後給予我很大的支持，讓我無後顧之憂的在這裡唸書。這一路上，得到了很多的幫助，遇到了一些人，經歷了一些事，讓我更加的成長，謝謝在這裏遇到的所有人，因為有你們，讓我在待人處事上，才能更臻於成熟。

中文摘要

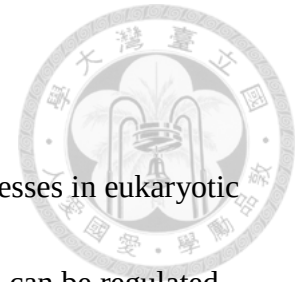


在真核生物，微管在調節細胞生理訊息傳遞的過程中扮演很重要的角色，它能夠穩定細胞骨架以及影響神經發育。有許多不同的微管蛋白能夠調節微管的聚合，其中包含 stathmin。stathmin 蛋白是一種磷酸蛋白，它可調節微管的聚合，stathmin 能夠和兩分子的 α -和 β -微管蛋白形成的二聚體結合，形成一個四聚體，因此 stathmin 能夠干擾微管的聚合化和去聚合化。藉由調節微管的聚合和去聚合，stathmin 參與了許多不同的細胞訊息傳遞的過程，包括神經形成，細胞的分化和增生。在哺乳類的研究中指出 *stmn1* 廣泛的表現在細胞中，而 *stmn2*，*stmn3* 以及 *stmn4* 則主要表現在神經系統，在先前我們實驗室的研究，發現在斑馬魚中的一個 stathmin 蛋白，stathmin 2，在腦部發育中，扮演一個很重要的角色。目前已知斑馬魚中有 7 個不同的 stathmin 基因，為了瞭解 stathmin 在斑馬魚發育中扮演的角色，我選殖出斑馬魚 stathmin 蛋白的基因 *stmn 1a*，*stmn 2b* 和 *stmn 4l*，發現它們和其他脊椎動物的 stathmin 蛋白有很高的相似度，並利用全標本包埋的原位雜交技術觀察斑馬魚 stathmin 蛋白在不同時間點，不同的斑馬魚 stathmin 蛋白的表現情形。在此研究中我觀察到，*stmn 1a* 和 *stmn 2b* 從 16 小時就會表現在脊髓和三叉神經，但是 *stmn 4l* 要從 18 小時才會開始表現，而 *stmn 2b* 和 *stmn 4l* 主要會表現在中樞神經系



統。此外，*stmn 1a* 也會表現在眼睛，鰓弓以及胚胎的尾端。從 24 小時後，*stmn 1a*，*stmn 2b* 和 *stmn 4l* 都會表現在下視丘，頭蓋，間腦以及端腦，在 48 小時後，*stmn 2b* 和 *stmn 4l* 仍然會表現在中樞神經系統，不過 *stmn 1a* 則沒有表現在後腦。*stathmin* 在中樞神經系統發育中的表現情形，顯示 *stathmin* 在斑馬魚中樞神經系統發育上，可能扮演一個很重要的角色。

Abstract



Microtubules are well known to mediate many physiological processes in eukaryotic cell such as stable cell structure and axonal outgrowth. Microtubules can be regulated by different microtubule binding proteins to mediated their assembly and disassembly. Stathmin family proteins are phosphoprotein that regulates microtubules disassembly. Stathmin interacts with microtubules by binding to two moles of alpha and beta tubulin dimer to form a tetramer. Stathmins also interfere with microtubule polymerization by direct binding to microtubules. Via tight regulation of microtubule polymerization and depolymerization, stathmins are involved in different cellular processes, including neurogenesis, cell proliferation, differentiation and activities. In mammals, *stmn 1* is ubiquitous expressed while other stathmin members, *stmn 2*, *stmn 3*, *stmn 4* are mainly expressed in the nervous system. Previously, we have demonstrated that *stmn 2*, one of the zebrafish stathmin proteins, plays a vital role in zebrafish brain development. There are seven different stathmin genes in zebrafish. To understand the role of stathmin in zebrafish, I have cloned *stmn 1a*, *stmn 2b* and *stmn 4l* and demonstrated that they shared high identity with other vertebrate stathmin homologues. I analyzsed their temporal expression patterns using embryos at different stages by whole-mount *in situ* hybridization. I observed that *stmn 1a* and *stmn 2b* were expressed in spinal cord and trigeminal ganglia from 16 h post fertilization (hpf) but *stmn 4l* appeared from 18 hpf.

stmn2b and *stmn4l* were mainly expressed in the central nervous system (CNS).

Furthermore, the *stmn1a* transcripts were found in eyes, pharyngeal arches and tail bud.

stmn 1a, *stmn 2b* and *stmn4l* were all expressed in hypothalamus, tegmentum,

telencephalon and diencephalon after 24 hpf. After 48 hpf, *stmn2b* and *stmn4l* were still

expressed in the CNS while *stmn1a* did not expressed in hindbrain. The dynamic

expression of stathmin family genes in the developing central nervous system suggests a

pivotal roles of those genes in regulating CNS development in zebrafish.



Contents



誌謝.....	I
中文摘要.....	II
Abstract.....	IV
Introduction.....	3
Stathmin family are highly conserved in vertebrates.....	3
Stathmins are involved in neuronal development and differentiation.....	4
Mechanisms of stathmin-mediated microtubule disassembly.....	8
Stathmin-related diseases.....	11
In vivo function of Stathmins.....	12
Materials and methods.....	14
Zebrafish maintenance and embryo yielding.....	14
In silica cloning and sequence analysis.....	14
Whole mount in situ hybridization and RNA probe prepared.....	15
RT-PCR.....	15
Photography of in situ hybridization.....	16
Results.....	17
Sequence analysis and the phylogenetic tree of zebrafish stathmin family proteins.....	17
Expression pattern analysis of stathmin by RT-PCR.....	18
Expression pattern analysis of stathmin by whole-mount in situ hybridization.....	19
Discussion.....	22
References.....	29
Table.....	41
Table 1 Primer pairs used in this study.....	41
Figures.....	42

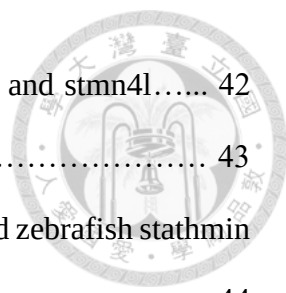


Figure. 1 Zebrafish stathmin sequence analysis of stmn1a, stmn2b and stmn4l.....	42
Figure. 2 Comparison of all zebrafish stathmin family genes.....	43
Figure. 3 The phylogenetic analysis between major model animal and zebrafish stathmin family.....	44
Figure. 4 Expression pattern of stathmin family genes during development by RT-PCR analysis.....	45
Figure. 5 Spatial expression pattern of stathmin1a during segmentation stages.....	46
Figure. 6 Spatial expression pattern of stathmin1a early larval stages.....	47
Figure. 7 Spatial expression pattern of stathmin2b during segmentation stages.....	48
Figure. 8 Spatial expression pattern of stathmin2b early larval stages.....	49
Figure. 9 Spatial expression pattern of stathmin4l during segmentation stages.....	50
Figure. 10 Spatial expression pattern of stathmin4l during early larval stages.....	51

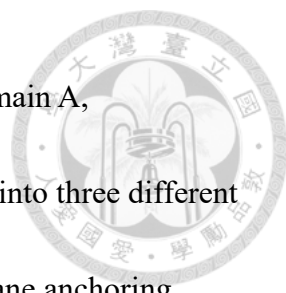
Introduction



During nervous system development, cytoskeleton dynamic plays a key role in controlling cellular processes, such as cell proliferation, migration, differentiation and morphogenesis. Cytoskeleton dynamic is mediated by changes in cytoskeleton proteins, mainly actins and microtubules, to regulate different cellular processes. Stathmin family phosphoproteins are known regulators of microtubule dynamic in neuron development (Charbaut, Curmi et al. 2001). They all possess a phosphorylation - dependent domain that controls intracellular pathways in neurons and they also possess membrane binding extensions which assist their transport to Golgi apparatus and intracellular membrane vesicles (Grenningloh, Soehrman et al. 2004).

Stathmin family are highly conserved in vertebrates

In most vertebrates studied so far, the Stathmin family is made up with four orthologs, stathmin 1-4. The mammalian stathmin 4 has four splicing variants, stmn 4a-4d. The Stathmin family genes have a stathmin-like domain (SLD) at C-terminal, which controls microtubule polymerization and depolymerization by binding to $\alpha\beta$ tubulin dimer (Curmi, Gavet et al. 1999). Stathmin 2-4 have a N-terminal palmitoylation-dependent membrane binding domain called Domain A, which can be tethered to Golgi and intracellular membrane vesicle. Domain A is missing from Stathmin 1. Stathmin 3 and



stathmin 4 has 68% and 56% identical sequence to stathmin 2 in Domain A, respectively, in the Domain A. The Domain A can be further divided into three different sub-domains, m, n, c. The sub-domain m is a well conserved membrane anchoring domain, which have two conserved palmitoylation sites. The sub-domain n is a perfectly conserved Golgi-specifying subdomain and the c subdomain is a poorly conserved domain (Charbaut, Chauvin et al. 2005). Furthermore, stathmin 4 also possess two additional N-terminal domains, domain A' and A''. Domain A' is conserved in all stathmin 4 variants, stathmin 4a-4d, but only stathmin 4c and 4d have domain A''. This allows stathmin 4 variants function differently to regulate the subcellular pathway signals (Ozon, Byk et al. 1998, Curmi, Gavet et al. 1999, Chauvin and Sobel 2015).

Stathmins are involved in neuronal development and differentiation.

Neuronal development, including neuronal growth, differentiation, migration and functional activities, depends on appropriate regulation of cellular signals and cytoskeleton dynamic. In cellular studies, stathmins were found to be essential in controlling axonal branching and dendritic development (Poulain and Sobel 2007). It was first reported in *Drosophila* that stathmin specifically appear in the nervous system following the decrease in maternal stathmin (Lachkar, Lebois et al. 2010). Later studies showed that Stathmin are highly expressed in the brain and nervous tissue, and stathmin

1 and 4 are expressed in glial cell or cerebellar white matter (Himi, Okazaki et al. 1994)

while stathmin 2 and 3 are specifically found in brain neurons (Sone, Tsuda et al. 2011).

Phosphorylation of stathmin by c-Jun N-terminal kinases (JNK), an extracellular stress

signal, can enhance neurite outgrowth by negative regulation of stathmin-tubulin

binding. Stathmin 1 has four serine residues, Ser¹⁶, Ser²⁵, Ser³⁸ and Ser⁶³, which are

highly conserved within stathmin family (Di Paolo, Antonsson et al. 1997). Stathmin 1

serves as a relaying intracellular signal to regulate microtubule dynamics. All stathmins

but Stathmin 3 (SCIP) are known to be targeted by JNK. Stathmin 3 has a different

target motif compared with other family members, stathmin 1 (STMN) and stathmin 2

(SCG10) (Ng, Zhao et al. 2010). However, stathmin 3 still shared the highly conserved

SLD sequence with stathmin 1 and stathmin 2. Glycogen synthase kinase directly target

the phosphorylation site of extracellular region of stathmin 3 but it was not

phosphorylated by JNK *in vivo* in neuron. However, stathmin 3 can bind to JNK

pathway with different target domain compared with stathmin 1 and stathmin 2 *in vitro*.

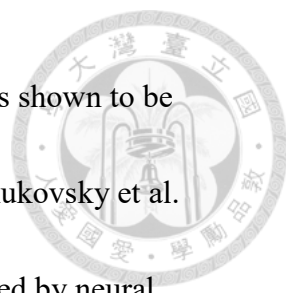
How stathmin 3 regulate neuron development by JNK still unclear.(Yip, Yeap et al.

2014)

Stathmins are ubiquitously expressed in neurons during brain development. stathmin 2

was first recognized as a neuronal differentiation marker in chromaffin cells (Anderson

and Axel 1985). Nerve growth factor (NGF) could induce differentiation of PC12 cells



(the rat adrenal medulla cells) and expression of stathmin 2 and 3 was shown to be stimulated by NGF therein (Doye, Boutterin et al. 1990, Beilharz, Zhukovsky et al. 1998). Stathmin 4 expression was also found to be specifically induced by neural activity and the differentiation of PC12 cells (Anderson and Axel 1985). Manipulation of stathmin 2 expression level can control the neuronal microtubule dynamic. Excess level of Stathmin promotes growth cone collapses while moderate stathmin 2 stimulates neurite outgrowth (Morii, Shiraishi-Yamaguchi et al. 2006). Furthermore, regulation of microtubule dynamics involves the phosphorylation of stathmin. Neuronal protein kinase, cyclin-dependent kinase 5 (Cdk5), is essential cytoskeletal dynamics required for normal neuronal function. Cdk5 control axon guidance and neuronal connectivity by the phosphorylation of MAPK (Ledda, Paratcha et al. 2002, Del Rio, Gonzalez-Billault et al. 2004). Cdk5 is very important for synaptic plasticity and it could phosphorylate stathmin 2 at Ser25 and Ser38 and modulate stathmin 2 activity. The phosphorylation of stathmin lead to synaptic pruning and structure plasticity in neuron development and may involve in microtubule regulatory mechanism that cause neuropsychiatric and neurodegenerative disorders (Hayashi, Pan et al. 2006).

Regulation of stathmin subcellular localization

Palmitoylation is a posttranslational modification by adding a palmitic acid through a

thioester linkage to mainly cysteine residue. By increasing protein hydrophobicity, it can alter the subcellular localization, protein-protein interaction and the binding ability of a protein to cytoplasm and intracellular organelles. With a N-terminal palmitoylation site, stathmins except stathmin1a, are subjected to the regulation of palmitoylation.

Different subcellular localization of stathmins may thus limit where they can exert their microtubule-destabilizing activities with independent and complementary regulatory roles (Poulain and Sobel 2007).

The domain A of stathmins is conserved within stathmin family that they contain two cysteine (Cys²², Cys²⁴ at stathmin 2 and 3; Cys²⁰, Cys²² at stathmin 4) palmitoylation sites. Cooperation of two subdomain within domain A is conceited to the Golgi and stathmin-related protein at stathmin N-terminal domain (Charbaut, Chauvin et al. 2005).

Palmitoylation on domain A of stathmin 2-3 controls the ability of protein to target to Golgi or mitochondria. (Lutjens, Igarashi et al. 2000). Mutation of each or both cysteine residues of stathmin 2 domain A on its palmitoylation level would affect the subcellular targeting between Golgi, vesicles and mitochondria in primary neurons (Di Paolo, Lutjens et al. 1997). Subcellular targeting signals coexist within domain A that one signal leads the pathway depending on the level of protein palmitoylation. When the stathmin-related protein perfectly conserved Golgi-specifying subdomain (α_n) is palmitoylated, it would drive protein to the Golgi and vesicle-like structures, whereas

non-palmitoylation directs the protein to the mitochondria. The palmitoylation of domain A of stathmin protein have the particular ability to control protein targeting between Golgi or mitochondria. (Chauvin, Poulain et al. 2008).



Mechanisms of stathmin-mediated microtubule disassembly

Stathmin is known for catalyzing microtubule disassembly. The N-terminal region of stathmin is essential for microtubule catastrophe whereas its C-terminal end region inhibits microtubule polymerization (Larsson, Segerman et al. 1999). Currently, there are two major mechanisms to explain the action of stathmins. Stathmin can directly promote microtubule catastrophe and sequester tubulin. On the other hand, stathmin can bind two moles of $\alpha\beta$ tubulin dimer to form a complex, thus impedes polymerization of microtubules (Manna, Thrower et al. 2009).

Stathmin catastrophe-promoting activity

Stathmin can bind directly to microtubule and act on the growing ends of tubulin protofilaments via its N-terminal tail to promote microtubule catastrophe. Increasing affinity of stathmin at microtubules minus ends cause more interaction between stathmin N-terminal domain and the microtubules minus end of α tubulin. Evidence suggests that stathmins mainly work on microtubules minus end to increase microtubule

disassembly. Stathmin can also bind to the laterally exposed protofilaments at microtubules growth tips to promote microtubule catastrophe by interfering protofilaments interaction (Gupta, Li et al. 2013).



Binding to free tubulin dimers to reduce tubulin pool.

One stathmin can bind to two microtubules $\alpha\beta$ tubulin dimers via four serine sites, Ser¹⁶, Ser²⁵, Ser³⁸ and Ser⁶³. The binding of stathmin reduces the free available tubulin dimers within the tubulin pool that inhibits the polymerization of growing microtubules (Gupta, Li et al. 2013). Previous studies revealed that the phosphorylation of stathmin on Ser¹⁶ or Ser⁶³ attenuates the binding ability of stathmin to microtubule and thus preventing microtubule from polymerization. Stathmin will reduce affinity of binding to microtubule because of phosphorylated especially on microtubules minus ends. In contrast, phosphorylation of Ser²⁵ and Ser³⁸ has no significant influence on the binding ability of stathmin to sequester soluble tubulin and to impede microtubule polymerization. It suggests that Ser²⁵ and Ser³⁸ in stathmin may not have direct interactions with tubulin. (Manna, Thrower et al. 2009)

Stathmin promotes GTPase activity to increase microtubule catastrophe.

Stathmins increase GTPase rate to promote microtubules catastrophe. (Wang, Cormier



et al. 2007) The tubulin GTP-binding site is located at β tubulin. Microtubule polymerization and depolymerization can be controlled by bound to different form of tubulin. The binding GTP-bound tubulins to microtubules favors microtubule polymerization. In contrast, microtubule depolymerization occurs upon the hydrolysis of GTP-bound tubulin to become GDP-bound form (Howard and Hyman 2009).

Stathmin stimulate GTP hydrolysis at a low and indistinguishable rate. The GTP-GDP exchange is blocked at both GTP-binding site in stathmin-tubulin complex, whereas GTP hydrolysis occurs only at one of the GTP-binding sites. The N-terminus of Stathmin acts on GTP-bound tubulins at the end of microtubule cap to stimulate GTP hydrolysis and promote microtubule dissociation from the growing microtubule ends (Nakao, Itoh et al. 2004). Stathmin also slows down GDP dissociation from tubulin and the GTP-tubulin can reduce the tubulin pool (Amayed, Carlier et al. 2000). Mutation at the first helical repeat of stathmin indicated that the GTP hydrolysis occurs between the head-to-tail interface of heterodimer. The GTPase can interact between heterodimer. GTP-tubulin can thus be protected from dissociation when GTP-binding site is located between the head-to-tail tubulin heterodimer. Hence, stathmin and tubulin complex are deferred to have an allosteric effect that prevent useless cycle of GTP hydrolysis.(Brannstrom, Segerman et al. 2003)

Stathmin-related diseases

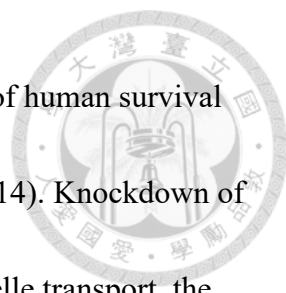
Alzheimer's disease



Stathmins are linked to Alzheimer's disease. Amyloid precursor protein (APP) proteolytic product β -amyloid peptide ($A\beta$) aggregates and causes neurotoxicity in the brain of Alzheimer's patients (Chauvin and Sobel 2015). Stathmin 2 is co-localized with APP at Golgi complex and transported to the axon of hippocampal neurons (Wang, Shan et al. 2013, Yang, Zhou et al. 2013). APP interacts with stathmin 2 through its intracellular domain (AICD) upon stathmin 2 palmitoylation. Knockdown of stathmin 2 promotes APP processing in neuronal cell that increases the distribution of APP on the cell surface and elevates α cleavage (Yang, Zhou et al. 2013). In human AD brain showed that stathmin 2 localization was abnormal in the hippocampus or in the cortex (Okazaki, Wang et al. 1995). However, APP processing induce stathmin 1 deregulation that causes microtubule destabilization that is important for the pathophysiology of Alzheimer's disease. Abnormal expression of stathmin 1-2 may occurred in AD-affected brain regions in hippocampus, cerebral cortex, temporal cortex (Okazaki, Wang et al. 1995).

Spinal muscular atrophy (SMA)

Spinal muscular atrophy (SMA) is a spinal cord motor neuron disorder that causes



muscle atrophy and paralysis. Deletion or loss-of function mutation of human survival of motor neuron gene (*SMN*) would cause SMA (Li, Tisdale et al. 2014). Knockdown of stathmin 1 expression in SMA-like cell reduced the defects of organelle transport, the outgrowth of axons, and microtubules that induces SMA occurrence. It was demonstrated that stathmin 1 plays a vital role in the axonal pathogenesis of SMA (Wen, Lin et al. 2010).

In vivo function of Stathmins

Nervous system development and plasticity are regulated by cellular signal process. One of important regulators is the change of microtubule dynamics and stathmin family phosphoprotein play a key regulators involving cellular localization which controls neuronal development. Knockout of stathmin 2 in mice presented lethal deficit after birth however knockout in stathmin 1, 3 or 4 did not cause life-threatening mutants (Ringhoff and Cassimeris 2009). Knockout stathmin also did not cause significant structural or neurological defect due to the compensatory mechanisms (Ringhoff and Cassimeris 2009). Although stathmin family have been revealed that they are specific involved in neuronal differentiation and regeneration and they mediated biological system by interact with microtubule, the biological roles of stathmin still not fully clear in vertebrates.



Zebrafish, *Danio rerio*, is an excellent model system for understanding vertebrate development. Their embryos are transparent and easy for direct observation under microscopy. Zebrafish embryos develop rapidly after fertilization that primary nervous system begins to form within 24 hours. In order to investigate zebrafish stathmin family protein function during development, I cloned zebrafish stathmin gene, stmn 1a, stmn 2b and stmn4l and examined the temporal and spatial gene expression patterns. It showed that all three stathmin expressed in different way in neuronal tissues. These results indicate that zebrafish stathmin genes may have individual role during zebrafish neuronal development.

Materials and Methods



Zebrafish maintenance and embryo yielding

Wild-type zebrafish, *D. rerio*, maintain under 14hrs light/10hrs dark cycle at 28.5 °C. Embryos were collected after fish mating at 15-20mins and incubated at 28 °C incubator in embryo medium (13.7mM NaCl, 0.54 mM KCl, 0.025 mM Na₂HPO₄, 0.044mM KH₂PO₄ and 0.42 mM NaHCO₃) until use. Embryos collected for whole-mount in situ hybridization were grown in 0.2 mM 1-phenyl-2-thiourea (Sigma) to inhibit pigment formation before 24hrs for stage 24hpf, 36hpf, 48hpf and 72hpf. Animal maintain procedures were followed the guidelines of laboratory at National Taiwan University, Taipei, Taiwan.

In silico cloning and sequence analysis

The stathmin gene stmn1a, stmn2b and stmn4l were selected from NCBI gene database. The complete coding sequences of selected stathmin gene were isolated by the primer that showed at table 1. The amino acid sequence of zebrafish stathmins and the orthologs of other species, human, mice, rat and fugu were collect from NCBI (<http://www.ncbi.nlm.nih.gov/>) or Ensemble (http://www.ensembl.org/Danio_rerio/) and Uniprot (<http://www.uniprot.org/>) website. Sequences alignment of stathmin amino acid was demonstrated by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)

website and motif analysis of stathmin protein were demonstrated by SMART web (<http://smart.embl-heidelberg.de/>). The phylogenetic tree were analyzed by using software MEGA6.



Whole mount in situ hybridization and RNA probe prepared

PCR product with the primer designed by Vector-NTI with different sequence of each stathmin gene were cloned into pGEMT-Easy Vector. (Promega) Antisense RNA riboprobes were synthesized using the digoxigenin RNA Labeling Kit. (Roche Applied Science, Penzberg, Germany) Collected embryo with stage according to our lab previous study and fixed in 4% paraformaldehyde in phosphate-buffered saline(PBS) at 4°C overnight and then replaced by methanol and stored at -20°C until used for Whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization was performed with antisense riboprobes follow the protocol from Nobuhiko Miyasaka lab, RIKEN BSI.

RT-PCR

Total RNAs extracted from early embryos to 72hpf by using TRIzol reagent (Invitrogen). RNAs synthesize single-stranded cDNA by M-MLV Reverse Transcriptase (Promega, Madison, WI) with 3 µg of total RNA and oligo dT primer. PCRs were performed with respective primer for 25 cycles by using KOD DNA polymerase in PCR

machine (PTC-200, MJ Research, Waltham, MA). The protocol as following described:

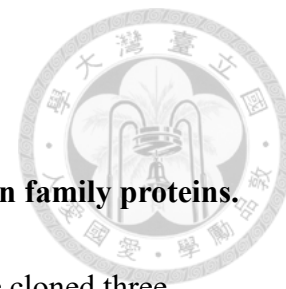
pre-denaturation at 94°C for 2mins, denaturation at 98°C for 20s, annealing at 58°C for 30s and elongation at 68°C for 2mins, with *ef1α* as an internal control. PCR product were analyzed on 1% agarose gels stained by ethidium bromide.

Photography of in situ hybridization

Whole-mount *in situ* hybridization was observed under Leica S8AP0 stereomicroscope.

The photograph data were made by CANON digital camera.

Results



Sequence analysis and the phylogenetic tree of zebrafish stathmin family proteins.

To investigate the diversity of the zebrafish stathmin family, we cloned three orthologs gene of the stathmin family, stmn1a, stmn2b, stmn4l by PCR. PCR primer were designed by software Vector-NTI based on zebrafish stathmin sequence from NCBI database. (NM_001040375.1 for stmn1a, NM_001024222.1 for stmn2b, NM_001002563.1 for stmn4l). The size of PCR product and the primer designed were summarized on table 1. Stmn1a, stmn2b and stmn4l contained a conserve stathmin-like domain that stmn2b and stmn4l amino acid sequence shared 59% and 62% identity to stmn1a (Fig. 1B). Zebrafish stmn2b and stmn4l also have N-terminal membrane-anchoring domain as in mammals that help stathmin attach to vesicle in cell by palmitoylation of two cysteine residues (diamond, Fig.1A). Moreover, stathmin have a conserved coiled-coil domain that would form α -helix structure to interact with other protein (Fig.1A, Fig.2A) such as KIS, a kinase involved in microtubule regulation in RNA-related process (Pedraza, Ortiz et al. 2014), the Hsc70 chaperone family (Manceau, Gavet et al. 1999) and the tubulin (Wang, Cormier et al. 2007). Moreover, zebrafish stathmin contain multiple phosphorylation sites ; Ser¹⁶, Ser²⁵, Ser³⁸ and Ser⁶³ of stmn1 and Ser⁵⁰, Ser⁶², Ser⁷³, Ser⁹⁷ of stmn2 as labeled on Fig.1A. (Beretta, Dobransky et al. 1993, Honnappa, Jahnke et al. 2006) We further analysis the similarity

within all zebrafish stathmin protein. Stmn1a, 1b and stmn2a, 2b share 90% and 93% similarities, respectively. Stmn4 and stmn4l share 84% similarity. Pairwise comparison between stathmin showed 76% -78% similarities except stmn3 that only share 68% - 75% similarities between stmn proteins with each other (Fig.2B).

The phylogenetic tree analysis showed that zebrafish stmn2a and 2b are highly homologous to the *Fugu* single stmn2, however zebrafish stmn2a and stmn2b were 53% probability from the same ancestor to human, rat and mouse STMN2. Zebrafish stmn1b and stmn1b were highly homologous to mammalian STMN1. Zebrafish stmn1b showed highly homologous to the *Fugu* however zebrafish stmn1a and stmn1b and the *Fugu* stmn1 were from the same ancestor. Zebrafish stmn3 showed 80% homologous to mammalian STMN3. Zebrafish stmn4l showed 90% homologous to the *Fugu* stmn4l and zebrafish stmn4 showed only 69% homologous to the *Fugu* stmn4. Zebrafish stmn4 showed 79% homologous to mammalian STMN4. Zebrafish stmn4l and mammalian stmn4 were derived from the same ancestor but their evolution divided that zebrafish stmn4l and mammalian STMN4 were less related than zebrafish stmn4 and mammalian STMN4. (Fig.3).

Expression pattern analysis of stathmin by RT-PCR

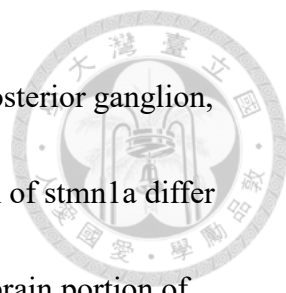
To investigate the expression pattern of stathmin at early development stage, I

performed RT-PCR analysis on zebrafish embryos from the 1-cell stage to 72hpf at different stages. Stathmin 1a and stathmin 2b transcripts expressed in all stage and stathmin 4l transcripts expressed from 12hpf to 72hpf. Stathmin2b transcripts were expressed at a higher level than stathmin 1a or stathmin 4l transcripts. Stathmin 4l transcripts were expressed from 12hpf and further upregulated after 36hpf (Fig.4).



Expression pattern analysis of stathmin by whole-mount *in situ* hybridization

To understand the spatial and temporal expression of stathmin family, we used whole-mount *in situ* hybridization to examine the expression pattern. The expression of stathmin were mainly localized to the central nervous system (CNS). The zebrafish stathmin were mostly perform at around 16hpf because it approximately beginning of neurogenesis in zebrafish. I collected embryos from 16hpf to 72hpf examined the spatial expressions of stathmin by whole-mount *in situ* hybridization. In brain segmentation stage, stmn 1a and stmn 2b, but not stmn4l, were expressed in spinal cord and trigeminal ganglia. In segmentation stage, stmn1a was expressed in eye, ventral mesoderm, midbrain, tail bud and spinal cord (Fig.5A, B), stmn2b were expressed in trigeminal ganglion, tegmentum, diencephalon, telencephalon, anterior and posterior ganglion, rhombomeres and spinal cord (Fig.7A, B). Stmn4l was expressed in trigeminal ganglion only (Fig.9A, B). In brain regionalization stage (~18hpf), stmn2b



and stmn4l were all expressed in trigeminal ganglion, anterior and posterior ganglion, tegmentum, rhombomeres and spinal cord, but the expression pattern of stmn1a differ from that of stmn2b and stmn4l. Stmn1a was strongly expressed on brain portion of embryos especially in the eyes, midbrain, hindbrain and spinal cord. Stmn1a was also expressed in ventral mesoderm, posterior somite, branchial arches and tail bud (Fig. 5C, D, E, F, G, H). Stmn 2b was highly expressed in midbrain, spinal cord neuron and trigeminal ganglia, anterior and posterior ganglion, tegmentum and rhombomeres at 16hpf to 24hpf (Fig.7C, D, E, F, G, H). The expression of stmn 4l appeared in spinal cord neuron, midbrain and hindbrain including cranial ganglia and rhombomeres (Fig.9C, D, E, F, G, H). At early embryonic development, stmn1a, stmn2b and stmn4l all expressed in hypothalamus, tegmentum, telencephalon and diencephalon. Stmn1a is expressed in midbrain, hindbrain, forebrain and spinal cord neuron between 24hpf and 36hpf, and hindbrain expression disappeared after 48hpf (Fig.6A, B, C, D, E, F). The expression of stmn2b was also expressed in forebrain, hindbrain and spinal cord neuron but not detected in tectum opticum and hypothalamus of midbrain (Fig.8A, B, C, D, E, F). The expression of stmn 4l was detected in forebrain, midbrain, hindbrain and spinal cord neurons but not in cerebellum and tectum opticum at 24hpf to 72hpf. The spinal cord neurons expression of stmn 4l was disappeared after 48hpf (Fig.10A, B, C, D, E, F). In summary, in early embryonic development from 36hpf to 72hpf, stmn1a, stmn2b

and stmn4l were expressed in brain and spinal cord and stmn1a was not expressed hindbrain after 48hpf. The tectum and cerebellum did not express stmn4l and spinal cord expression of stmn4l disappear after 48hpf.

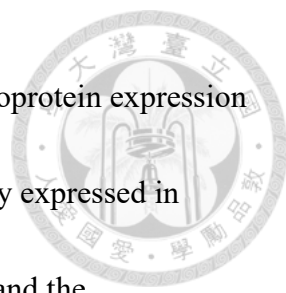


Discussion



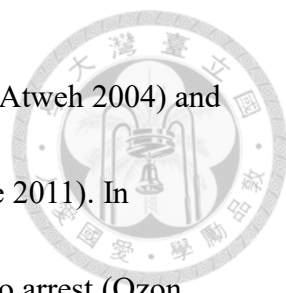
Stathmins are involved in neuronal development. They are known to regulate neuronal differentiation, plasticity and regeneration (Poulain and Sobel 2010). In this study, I cloned and identified three stathmin gene: stathmin1a (*stmn1a*), stathmin2b (*stmn2b*), stathmin-like 4, like (*stmn4l*) from the zebrafish genome. These three stathmin phosphoprotein are highly conserved within the zebrafish stathmin family, as well as share similar gene structure such as putative phosphorylation sites and the coiled-coil region as shown in Fig. 1. According to previous studies, stathmin family genes are also conserved in vertebrates with similar intron/exon structure organization (Okazaki, Yoshida et al. 1993). The phylogenetic analysis showed that the zebrafish stathmin family genes have different degree of homology compared to respective counter genes with other vertebrates. The zebrafish stathmin amino acid sequences are more distinct from mammals than those in *Fugu* except *stmn3*. The amino acid sequence of *Stmn3* is closer to its mammalian counter parts. Furthermore, although *stmn1a* shares similar amino acid sequence with *stmn1b*, *stmn1a* and *stmn1b* are classified into different subgroup.

Stathmins are highly expressed in developing nervous system in vertebrates. During neural development, stathmins are involved in cell proliferation, differentiation and maturation. In adults, stathmin may also regulate neuronal functions (Koppel,



Boutterin et al. 1990). Previous studies showed that stathmin phosphoprotein expression patterns differ in different species. In *Drosophila*, stathmin are mainly expressed in peripheral nervous system to support long-distance axonal transport and the development of reproductive organs (Ozon, Guichet et al. 2002). In mammals, stathmins express ubiquitously in the neuronal system except for that human STMN3 is also expressed in other tissues (Bieche, Maucuer et al. 2003). Here, I showed that zebrafish stathmin are highly expressed in embryos according to the RT-PCR analysis. The zebrafish stmn4l begins to express from 12hpf-16hpf, maching the beginning stage of neurogenesis, while stmn1a and stmn2b are continuously expressed throughout all stages. Compared with our lab previous study, zebrafish stathmin family of stmn1a, stmn2a and stmn2b are expressed throughout all stage however stmn1b, stmn3, stmn4 and stmn4l perform from 12hpf-16hpf. This indicatesn that zebrafish stmn1a, stmn2a and stmn2b mRNA are maternal expressed and they may play important roles during embryo development. Previously studies in other vertebrates like mammals, *Drosophila*, and *Xenopus*, stathmin were found to express in blastula and fertilized oocytes (Maucuer, Moreau et al. 1993, Koppel, Rehak et al. 1999, Lachkar, Lebois et al. 2010). Compared with zebrafish, the different expression patterns of zebrafish and other vertebrate stathmin may be due to evolutionary diversity (Fig.4).

Stathmins are known to participate in different cellular processes such as cellular



proliferation, G2/M phase accumulation of the cell cycle (Rubin and Atweh 2004) and increased cancer cell to become malignancy (Belletti and Baldassarre 2011). In *Drosophila*, knockdown of stathmin may cause germ cell migration to arrest (Ozon, Guichet et al. 2002). In mammals, stathmins are involved in the migration of T cells (Verma, Dourlat et al. 2009), mesenchymal sarcoma cells (Baldassarre, Belletti et al. 2005), radial cells in the developing cortex (Westerlund, Zdrojewska et al. 2011), neuron outgrowth (Poulain, Chauvin et al. 2008) and axon specification (Watabe-Uchida, John et al. 2006). Stathmin is widely expressed in embryonic neuron brain development and declined during development (Amat, Fields et al. 1990). In zebrafish, stathmins are mainly in nervous system especially on central nervous system. I demonstrated that in zebrafish, the expression of *stmn1a*, *stmn2b* and *stmn4l* were found in nervous systems (Fig.5, 7, 9) and restricted to the brain region after 24 hpf (Fig. 6, 8, 10). Stathmin family proteins are participated in neuron development such as stathmin 1 and stathmin 2 were important in neurite elongation though stathmin 1 cytosolic distribute through the embryo neuron and stathmin 2 is strongly expressed in the growth cones of axons and dendrites (Di Paolo, Lutjens et al. 1997), controlling growth cones expansion (Di Paolo, Lutjens et al. 1997, Morii, Yamada et al. 2006) and axonal elongation (Grenningloh, Soehrman et al. 2004, Poulain and Sobel 2007). However, unlike stathmin 2, stathmin 3 was involved in growth cones of axonal and dendrites by

controlling axonal branching and dendritic formation or maturation (Poulain and Sobel 2007, Poulain, Chauvin et al. 2008). Stathmin 4 was expressed on diencephalon and olfactory bulb in early embryo development but declined after pharyngula period (Li, Yin et al. 2010).

Stathmins are important in neuronal differentiation due to their function of destabilizing microtubule in zebrafish and mammals. Stathmin family gene are highly conserved in vertebrate. Different types of stathmin are encoded by distinct genes and in mammalian stathmin 4 contain further initiation and splice variant domain (Ozon, Byk et al. 1998). In mammals, stathmin were related to some neurological disorder disease such as Alzheimer's disease (Okazaki, Wang et al. 1995, Jin, Masliah et al. 1996), Parkinson's disease (Liu, Stadelmann et al. 2005) and some motor neuron disease. Stathmin 1 and stathmin 2 mRNA and protein expression are controversial in hippocampus and frontal cortex in Alzheimer's disease. When stathmin 1 mRNA was up, their protein level was down whereas the expression of stathmin 2 was contrary (Okazaki, Wang et al. 1995, Jin, Masliah et al. 1996). Inflammation, abnormal tremble and movement were regarded as symptom in Parkinson's disease that patient's brain were injured and the expression level of stathmin increase (Liu, Stadelmann et al. 2005). Thus, abnormal expression of stathmin would cause brain and neuron defect. According to the *in situ* hybridization data in zebrafish (Fig. 5, 6, 7, 8, 9, 10) that stathmin showed

highly expressed in the developing nervous system and restricted to brain. These results were similar to the previous studies (Burzynski, Delalande et al. 2009, Li, Yin et al. 2010) and suggested that stathmin play an important role in the early development of zebrafish brain.

Stathmin also related to human age-dependent disease (Liedtke, Leman et al. 2002) and cancer metastasis, such as endometrial cancer (Vandenput, Trovik et al. 2011, Wojcechowskyj, Lee et al. 2011). It had been demonstrated that stathmin showed expression in rat brain (Cheng, Jiang et al. 1997) and retina (Bohm, Mertsch et al. 2013) at younger ages but not found in older one. Stathmin expression is related to age and would reduce expression with age. It had evidenced that stathmins are involved in tumor progression. Overexpression stathmin was associated with low survival rate of the patients and caused the metastasis within lymph nodes (Trovik, Wik et al. 2011). In human cancer, stathmin also influenced the treatment of cytotoxic drugs. High expression of stathmin negatively affected the cell to response to microtubule-drugs in tumor derived cell line *in vitro* (Mistry and Atweh 2006, Belletti and Baldassarre 2011) and it had evidenced that high expression of stathmin were found to associated with low effective of therapy in ovarian cancer (Su, Smith et al. 2009). It had been demonstrated that loss of stathmin would cause cell cycle arrest and induce apoptosis in some cancer cell line including prostate cancer (Mistry, Bank et al. 2005) and endometrial cancer

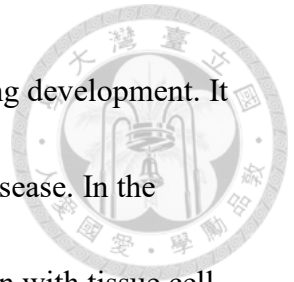
(Zhang, Wang et al. 2006), so stathmin would control cell death either. Thus, unusual stathmin expression not only causes the neuron and brain developing defect but also affects tumor progression in cell.



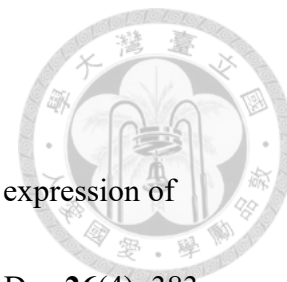
In the mammalian stathmin research, it mainly focuses on human disease studies. Zebrafish is a powerful model for cancer (Zhang, Xuan et al. 2015) and neurological disorder research (Li, Yin et al. 2010). Zebrafish shares homology protein domain with mammalian signal pathway and stathmin express highly conserved within vertebrate that make zebrafish become an excellent model for research human disease. Following zebrafish stathmin *in situ* hybridization data, it showed that stathmin1a express in neuronal system and other tissues such as gut. These result suggested that stathmin1a may play a role in other body tissue and zebrafish stathmin1a may become a great material to investigate intestinal cancer.

Taken together, according to the results, zebrafish stathmin, stathmin1a, stathmin2b and stathmin4l, were mainly expressed in neuronal system but showed different spatial and temporal expression pattern during the development of zebrafish. Stathmin defects related to some neuron disease and some cancer cell progression in mammal and their amino acid sequence were highly conserved within vertebrate that zebrafish can substitute as a good model for investigation human disease. Different stathmin isoform expresses variable mRNA patterns during development stage that specific expression

pattern of each stathmin makes stathmin have their distinct role during development. It still limited to understand the biological role of stathmin in human disease. In the further studies, we can investigate the interaction of different stathmin with tissue cell line to found the potential target for therapies on human diseases.



References



- Amat, J. A., K. L. Fields and U. K. Schubart (1990). "Stage-specific expression of phosphoprotein p19 during spermatogenesis in the rat." Mol Reprod Dev **26**(4): 383-390.
- Amayed, P., M. F. Carlier and D. Pantaloni (2000). "Stathmin slows down guanosine diphosphate dissociation from tubulin in a phosphorylation-controlled fashion." Biochemistry **39**(40): 12295-12302.
- Anderson, D. J. and R. Axel (1985). "Molecular probes for the development and plasticity of neural crest derivatives." Cell **42**(2): 649-662.
- Baldassarre, G., B. Belletti, M. S. Nicoloso, M. Schiappacassi, A. Vecchione, P. Spessotto, A. Morrione, V. Canzonieri and A. Colombatti (2005). "p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion." Cancer Cell **7**(1): 51-63.
- Beilharz, E. J., E. Zhukovsky, A. A. Lanahan, P. F. Worley, K. Nikolich and L. J. Goodman (1998). "Neuronal activity induction of the stathmin-like gene RB3 in the rat hippocampus: possible role in neuronal plasticity." J Neurosci **18**(23): 9780-9789.
- Belletti, B. and G. Baldassarre (2011). "Stathmin: a protein with many tasks. New biomarker and potential target in cancer." Expert Opin Ther Targets **15**(11): 1249-1266.
- Beretta, L., T. Dobransky and A. Sobel (1993). "Multiple phosphorylation of stathmin. Identification of four sites phosphorylated in intact cells and in vitro by cyclic AMP-

dependent protein kinase and p34cdc2." J Biol Chem **268**(27): 20076-20084.

Bieche, I., A. Maucuer, I. Laurendeau, S. Lachkar, A. J. Spano, A. Frankfurter, P. Levy,

V. Manceau, A. Sobel, M. Vidaud and P. A. Curmi (2003). "Expression of stathmin

family genes in human tissues: non-neural-restricted expression for SCLIP." Genomics

81(4): 400-410.

Boekhoorn, K., V. van Dis, E. Goedknecht, A. Sobel, P. J. Lucassen and C. C.

Hoogenraad (2014). "The microtubule destabilizing protein stathmin controls the

transition from dividing neuronal precursors to postmitotic neurons during adult

hippocampal neurogenesis." Dev Neurobiol **74**(12): 1226-1242.

Bohm, M. R., S. Mertsch, S. Konig, T. Spieker and S. Thanos (2013). "Macula-less rat

and macula-bearing monkey retinas exhibit common lifelong proteomic changes."

Neurobiol Aging **34**(11): 2659-2675.

Brannstrom, K., B. Segerman and M. Gullberg (2003). "Molecular dissection of GTP

exchange and hydrolysis within the ternary complex of tubulin heterodimers and

Op18/stathmin family members." J Biol Chem **278**(19): 16651-16657.

Burzynski, G. M., J. M. Delalande and I. Shepherd (2009). "Characterization of spatial

and temporal expression pattern of SCG10 during zebrafish development." Gene Expr

Patterns **9**(4): 231-237.

Charbaut, E., S. Chauvin, H. Enslin, S. Zamaroczy and A. Sobel (2005). "Two separate

motifs cooperate to target stathmin-related proteins to the Golgi complex." J Cell Sci

118(Pt 10): 2313-2323.

Charbaut, E., P. A. Curmi, S. Ozon, S. Lachkar, V. Redeker and A. Sobel (2001).

"Stathmin family proteins display specific molecular and tubulin binding properties." J

Biol Chem **276**(19): 16146-16154.

Chauvin, S., F. E. Poulain, S. Ozon and A. Sobel (2008). "Palmitoylation of stathmin

family proteins domain A controls Golgi versus mitochondrial subcellular targeting."

Biol Cell **100**(10): 577-589.

Chauvin, S. and A. Sobel (2015). "Neuronal stathmins: A family of phosphoproteins

cooperating for neuronal development, plasticity and regeneration." Prog Neurobiol

126: 1-18.

Cheng, H. W., T. Jiang, N. Mori and T. H. McNeill (1997). "Upregulation of stathmin

(p19) gene expression in adult rat brain during injury-induced synapse formation."

Neuroreport **8**(17): 3691-3695.

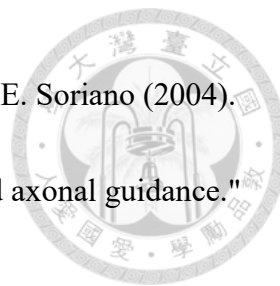
Curmi, P. A., O. Gavet, E. Charbaut, S. Ozon, S. Lachkar-Colmerauer, V. Manceau, S.

Siavoshian, A. Maucuer and A. Sobel (1999). "Stathmin and its phosphoprotein family:

general properties, biochemical and functional interaction with tubulin." Cell Struct

Funct **24**(5): 345-357.

Del Rio, J. A., C. Gonzalez-Billault, J. M. Urena, E. M. Jimenez, M. J. Barallobre, M.



Pascual, L. Pujadas, S. Simo, A. La Torre, F. Wandosell, J. Avila and E. Soriano (2004).

"MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance."

Curr Biol **14**(10): 840-850.

Di Paolo, G., B. Antonsson, D. Kassel, B. M. Riederer and G. Grenningloh (1997).

"Phosphorylation regulates the microtubule-destabilizing activity of stathmin and its interaction with tubulin." FEBS Lett **416**(2): 149-152.

Di Paolo, G., R. Lutjens, A. Osen-Sand, A. Sobel, S. Catsicas and G. Grenningloh

(1997). "Differential distribution of stathmin and SCG10 in developing neurons in culture." J Neurosci Res **50**(6): 1000-1009.

Di Paolo, G., R. Lutjens, V. Pellier, S. A. Stimpson, M. H. Beuchat, S. Catsicas and G.

Grenningloh (1997). "Targeting of SCG10 to the area of the Golgi complex is mediated by its NH2-terminal region." J Biol Chem **272**(8): 5175-5182.

Doye, V., M. C. Bouterin and A. Sobel (1990). "Phosphorylation of stathmin and other proteins related to nerve growth factor-induced regulation of PC12 cells." J Biol Chem **265**(20): 11650-11655.

Grenningloh, G., S. Sohrman, P. Bondallaz, E. Ruchti and H. Cadas (2004). "Role of the microtubule destabilizing proteins SCG10 and stathmin in neuronal growth." J Neurobiol **58**(1): 60-69.

Gupta, K. K., C. Li, A. Duan, E. O. Alberico, O. V. Kim, M. S. Alber and H. V. Goodson

(2013). "Mechanism for the catastrophe-promoting activity of the microtubule destabilizer Op18/stathmin." Proc Natl Acad Sci U S A **110**(51): 20449-20454.

Hayashi, K., Y. Pan, H. Shu, T. Ohshima, J. W. Kansy, C. L. White, 3rd, C. A.

Tamminga, A. Sobel, P. A. Curmi, K. Mikoshiba and J. A. Bibb (2006).

"Phosphorylation of the tubulin-binding protein, stathmin, by Cdk5 and MAP kinases in the brain." J Neurochem **99**(1): 237-250.

Himi, T., T. Okazaki, H. Wang, T. H. McNeill and N. Mori (1994). "Differential localization of SCG10 and p19/stathmin messenger RNAs in adult rat brain indicates distinct roles for these growth-associated proteins." Neuroscience **60**(4): 907-926.

Honnappa, S., W. Jahnke, J. Seelig and M. O. Steinmetz (2006). "Control of intrinsically disordered stathmin by multisite phosphorylation." J Biol Chem **281**(23): 16078-16083.

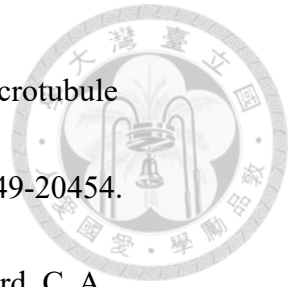
Howard, J. and A. A. Hyman (2009). "Growth, fluctuation and switching at microtubule plus ends." Nat Rev Mol Cell Biol **10**(8): 569-574.

Jin, L. W., E. Masliah, D. Iimoto, R. Deteresa, M. Mallory, M. Sundsmo, N. Mori, A.

Sobel and T. Saitoh (1996). "Neurofibrillary tangle-associated alteration of stathmin in Alzheimer's disease." Neurobiol Aging **17**(3): 331-341.

Koppel, J., M. C. Bouterin, V. Doye, H. Peyro-Saint-Paul and A. Sobel (1990).

"Developmental tissue expression and phylogenetic conservation of stathmin, a



phosphoprotein associated with cell regulations." J Biol Chem **265**(7): 3703-3707.

Koppel, J., P. Rehak, V. Baran, J. Vesela, D. Hlinka, V. Manceau and A. Sobel (1999).

"Cellular and subcellular localization of stathmin during oocyte and preimplantation

embryo development." Mol Reprod Dev **53**(3): 306-317.

Lachkar, S., M. Lebois, M. O. Steinmetz, A. Guichet, N. Lal, P. A. Curmi, A. Sobel and

S. Ozon (2010). "Drosophila stathmins bind tubulin heterodimers with high and variable

stoichiometries." J Biol Chem **285**(15): 11667-11680.

Larsson, N., B. Segerman, B. Howell, K. Fridell, L. Cassimeris and M. Gullberg (1999).

"Op18/stathmin mediates multiple region-specific tubulin and microtubule-regulating

activities." J Cell Biol **146**(6): 1289-1302.

Ledda, F., G. Paratcha and C. F. Ibanez (2002). "Target-derived GFRalpha1 as an

attractive guidance signal for developing sensory and sympathetic axons via activation

of Cdk5." Neuron **36**(3): 387-401.

Li, D. K., S. Tisdale, F. Lotti and L. Pellizzoni (2014). "SMN control of RNP assembly:

from post-transcriptional gene regulation to motor neuron disease." Semin Cell Dev

Biol **32**: 22-29.

Li, S., M. Yin, S. Liu, Y. Chen, Y. Yin, T. Liu and J. Zhou (2010). "Expression of ventral

diencephalon-enriched genes in zebrafish." Dev Dyn **239**(12): 3368-3379.

Liedtke, W., E. E. Leman, R. E. Fyffe, C. S. Raine and U. K. Schubart (2002).



"Stathmin-deficient mice develop an age-dependent axonopathy of the central and peripheral nervous systems." Am J Pathol **160**(2): 469-480.

Liu, A., C. Stadelmann, M. Moscarello, W. Bruck, A. Sobel, F. G. Mastronardi and P.

Casaccia-Bonnet (2005). "Expression of stathmin, a developmentally controlled cytoskeleton-regulating molecule, in demyelinating disorders." J Neurosci **25**(3): 737-747.

Lutjens, R., M. Igarashi, V. Pelletier, H. Blasey, G. Di Paolo, E. Ruchti, C. Pfulg, J. K.

Staple, S. Catsicas and G. Grenningloh (2000). "Localization and targeting of SCG10 to the trans-Golgi apparatus and growth cone vesicles." Eur J Neurosci **12**(7): 2224-2234.

Manceau, V., O. Gavet, P. Curmi and A. Sobel (1999). "Stathmin interaction with HSC70 family proteins." Electrophoresis **20**(2): 409-417.

Manna, T., D. A. Thrower, S. Honnappa, M. O. Steinmetz and L. Wilson (2009).

"Regulation of microtubule dynamic instability in vitro by differentially phosphorylated stathmin." J Biol Chem **284**(23): 15640-15649.

Maucuer, A., J. Moreau, M. Mechali and A. Sobel (1993). "Stathmin gene family: phylogenetic conservation and developmental regulation in *Xenopus*." J Biol Chem **268**(22): 16420-16429.

Mistry, S. J. and G. F. Atweh (2006). "Therapeutic interactions between stathmin inhibition and chemotherapeutic agents in prostate cancer." Mol Cancer Ther **5**(12):



3248-3257.

Mistry, S. J., A. Bank and G. F. Atweh (2005). "Targeting stathmin in prostate cancer."

Mol Cancer Ther **4**(12): 1821-1829.

Morii, H., Y. Shiraishi-Yamaguchi and N. Mori (2006). "SCG10, a microtubule

destabilizing factor, stimulates the neurite outgrowth by modulating microtubule

dynamics in rat hippocampal primary cultured neurons." J Neurobiol **66**(10): 1101-

1114.

Morii, H., T. Yamada, I. Nakano, J. M. Coulson and N. Mori (2006). "Site-specific

phosphorylation of SCG10 in neuronal plasticity: role of Ser73 phosphorylation by N-

methyl D-aspartic acid receptor activation in rat hippocampus." Neurosci Lett **396**(3):

241-246.

Nakao, C., T. J. Itoh, H. Hotani and N. Mori (2004). "Modulation of the stathmin-like

microtubule destabilizing activity of RB3, a neuron-specific member of the SCG10

family, by its N-terminal domain." J Biol Chem **279**(22): 23014-23021.

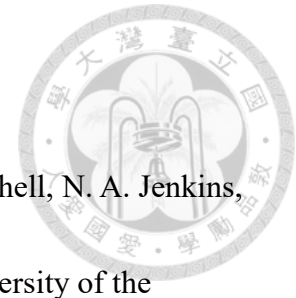
Ng, D. C., T. T. Zhao, Y. Y. Yeap, K. R. Ngoei and M. A. Bogoyevitch (2010). "c-Jun N-

terminal kinase phosphorylation of stathmin confers protection against cellular stress." J

Biol Chem **285**(37): 29001-29013.

Okazaki, T., H. Wang, E. Masliah, M. Cao, S. A. Johnson, M. Sundsmo, T. Saitoh and

N. Mori (1995). "SCG10, a neuron-specific growth-associated protein in Alzheimer's



disease." Neurobiol Aging **16**(6): 883-894.

Okazaki, T., B. N. Yoshida, K. B. Avraham, H. Wang, C. W. Wuenschell, N. A. Jenkins,

N. G. Copeland, D. J. Anderson and N. Mori (1993). "Molecular diversity of the

SCG10/stathmin gene family in the mouse." Genomics **18**(2): 360-373.

Ozon, S., T. Byk and A. Sobel (1998). "SCLIP: a novel SCG10-like protein of the

stathmin family expressed in the nervous system." J Neurochem **70**(6): 2386-2396.

Ozon, S., A. Guichet, O. Gavet, S. Roth and A. Sobel (2002). "Drosophila stathmin: a

microtubule-destabilizing factor involved in nervous system formation." Mol Biol Cell

13(2): 698-710.

Pedraza, N., R. Ortiz, A. Cornado, A. Llobet, M. Aldea and C. Gallego (2014). "KIS, a

kinase associated with microtubule regulators, enhances translation of AMPA receptors

and stimulates dendritic spine remodeling." J Neurosci **34**(42): 13988-13997.

Poulain, F. E., S. Chauvin, R. Wehrle, M. Desclaux, J. Mallet, G. Vojdani, I. Dusart

and A. Sobel (2008). "SCLIP is crucial for the formation and development of the

Purkinje cell dendritic arbor." J Neurosci **28**(29): 7387-7398.

Poulain, F. E. and A. Sobel (2007). "The "SCG10-Like Protein" SCLIP is a novel

regulator of axonal branching in hippocampal neurons, unlike SCG10." Mol Cell

Neurosci **34**(2): 137-146.

Poulain, F. E. and A. Sobel (2010). "The microtubule network and neuronal



morphogenesis: Dynamic and coordinated orchestration through multiple players." Mol Cell Neurosci **43**(1): 15-32.

Ringhoff, D. N. and L. Cassimeris (2009). "Gene expression profiles in mouse embryo fibroblasts lacking stathmin, a microtubule regulatory protein, reveal changes in the expression of genes contributing to cell motility." BMC Genomics **10**: 343.

Rubin, C. I. and G. F. Atweh (2004). "The role of stathmin in the regulation of the cell cycle." J Cell Biochem **93**(2): 242-250.

Sone, K., M. Tsuda and N. Mori (2011). "Position-dependent effect of a neural-restrictive silencer-like element present in the promoter downstream of the SCG10-like protein gene." J Biochem **150**(4): 451-460.

Su, D., S. M. Smith, M. Preti, P. Schwartz, T. J. Rutherford, G. Menato, S. Danese, S.

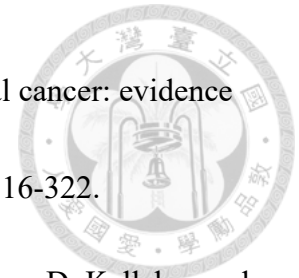
Ma, H. Yu and D. Katsaros (2009). "Stathmin and tubulin expression and survival of ovarian cancer patients receiving platinum treatment with and without paclitaxel."

Cancer **115**(11): 2453-2463.

Trovik, J., E. Wik, I. M. Stefansson, J. Marcickiewicz, S. Tingulstad, A. C. Staff, T. S.

Njolstad, G. MoMaTec Study, I. Vandenput, F. Amant, L. A. Akslen and H. B. Salvesen (2011). "Stathmin overexpression identifies high-risk patients and lymph node metastasis in endometrial cancer." Clin Cancer Res **17**(10): 3368-3377.

Vandenput, I., J. Trovik, K. Leunen, E. Wik, I. Stefansson, L. Akslen, P. Moerman, I.



Vergote, H. Salvesen and F. Amant (2011). "Evolution in endometrial cancer: evidence from an immunohistochemical study." Int J Gynecol Cancer **21**(2): 316-322.

Verma, N. K., J. Dourlat, A. M. Davies, A. Long, W. Q. Liu, C. Garbay, D. Kelleher and

Y. Volkov (2009). "STAT3-stathmin interactions control microtubule dynamics in migrating T-cells." J Biol Chem **284**(18): 12349-12362.

Wang, C., A. Cormier, B. Gigant and M. Knossow (2007). "Insight into the GTPase activity of tubulin from complexes with stathmin-like domains." Biochemistry **46**(37): 10595-10602.

Wang, J., C. Shan, W. Cao, C. Zhang, J. Teng and J. Chen (2013). "SCG10 promotes non-amyloidogenic processing of amyloid precursor protein by facilitating its trafficking to the cell surface." Hum Mol Genet **22**(24): 4888-4900.

Watabe-Uchida, M., K. A. John, J. A. Janas, S. E. Newey and L. Van Aelst (2006). "The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18." Neuron **51**(6): 727-739.

Wen, H. L., Y. T. Lin, C. H. Ting, S. Lin-Chao, H. Li and H. M. Hsieh-Li (2010). "Stathmin, a microtubule-destabilizing protein, is dysregulated in spinal muscular atrophy." Hum Mol Genet **19**(9): 1766-1778.

Westerlund, N., J. Zdrojewska, A. Padzik, E. Komulainen, B. Bjorkblom, E. Rannikko, T. Tararuk, C. Garcia-Frigola, J. Sandholm, L. Nguyen, T. Kallunki, M. J. Courtney and



E. T. Coffey (2011). "Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate." Nat Neurosci **14**(3): 305-313.

Wojcechowskyj, J. A., J. Y. Lee, S. H. Seeholzer and R. W. Doms (2011). "Quantitative phosphoproteomics of CXCL12 (SDF-1) signaling." PLoS One **6**(9): e24918.

Yang, H., Y. Zhou, J. Gu, S. Xie, Y. Xu, G. Zhu, L. Wang, J. Huang, H. Ma and J. Yao (2013). "Deep mRNA sequencing analysis to capture the transcriptome landscape of zebrafish embryos and larvae." PLoS One **8**(5): e64058.

Yip, Y. Y., Y. Y. Yeap, M. A. Bogoyevitch and D. C. Ng (2014). "Differences in c-Jun N-terminal kinase recognition and phosphorylation of closely related stathmin-family members." Biochem Biophys Res Commun **446**(1): 248-254.

Zhang, B., C. Xuan, Y. Ji, W. Zhang and D. Wang (2015). "Zebrafish xenotransplantation as a tool for in vivo cancer study." Fam Cancer.

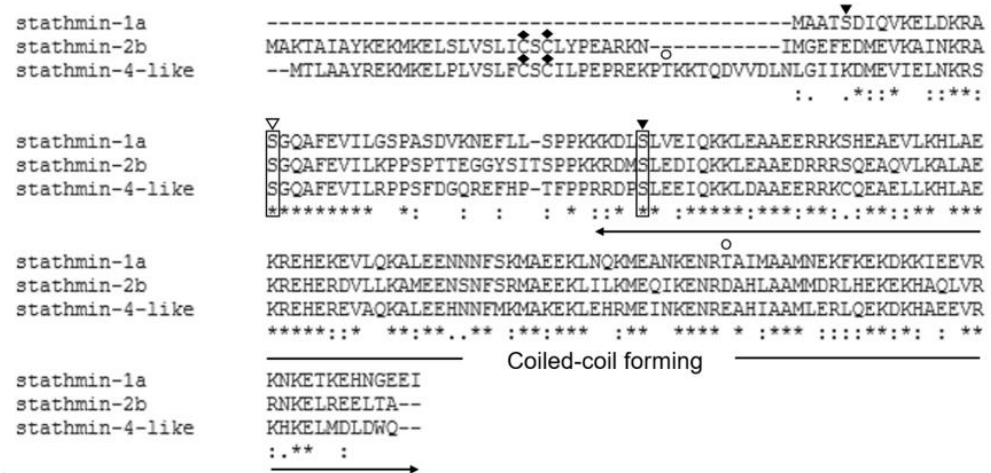
Zhang, H. Z., Y. Wang, P. Gao, F. Lin, L. Liu, B. Yu, J. H. Ren, H. Zhao and R. Wang (2006). "Silencing stathmin gene expression by survivin promoter-driven siRNA vector to reverse malignant phenotype of tumor cells." Cancer Biol Ther **5**(11): 1457-1461.

Table 1. Primer pairs used in this study

Gene	Accession no.	Sequence	Amplicon (bp)
stmn1a	NM_001040375.1	F: 5'-AGCTTTGTTACCAAGCCTGG-3' R: 5'-TACATAATTTTTTATTTTGTACAG-3'	927
stmn2b	NM_001024222.1	F: 5'-AGAGGCACGCAAGAACATCATGG-3' R: 5'-CACCCGCACTGTTCTCGAAA-3'	1194
stmn4l	NM_001002563.1	F: 5'-TCGAAGGAAAGATGACTCTCG-3' R: 5'-GAGATGCAAGTCAACTGTGG-3'	1047
<i>eflα</i>	NM_131263	F: 5'-CAAGGAAGTCAGCGCA TACA-3' R: 5'-TGATGACCTGAG CGTTGAAG-3'	542

Note: F: forward primer; R: reverse primer

A



B

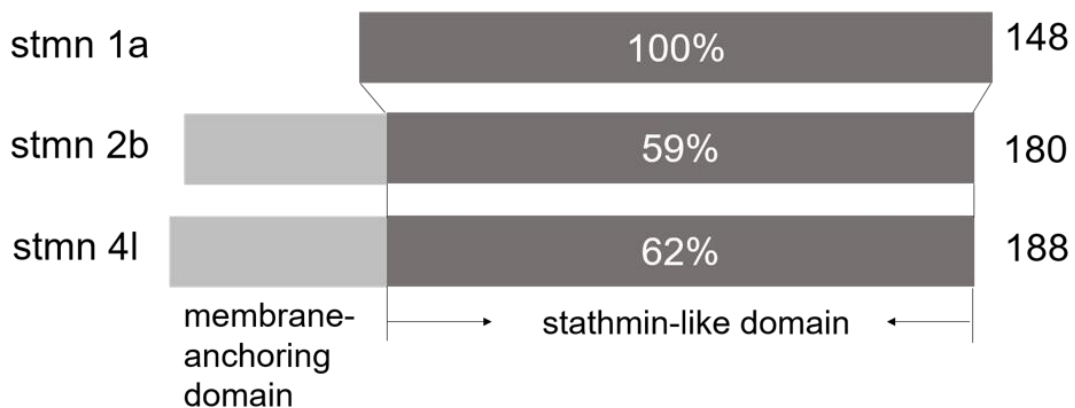
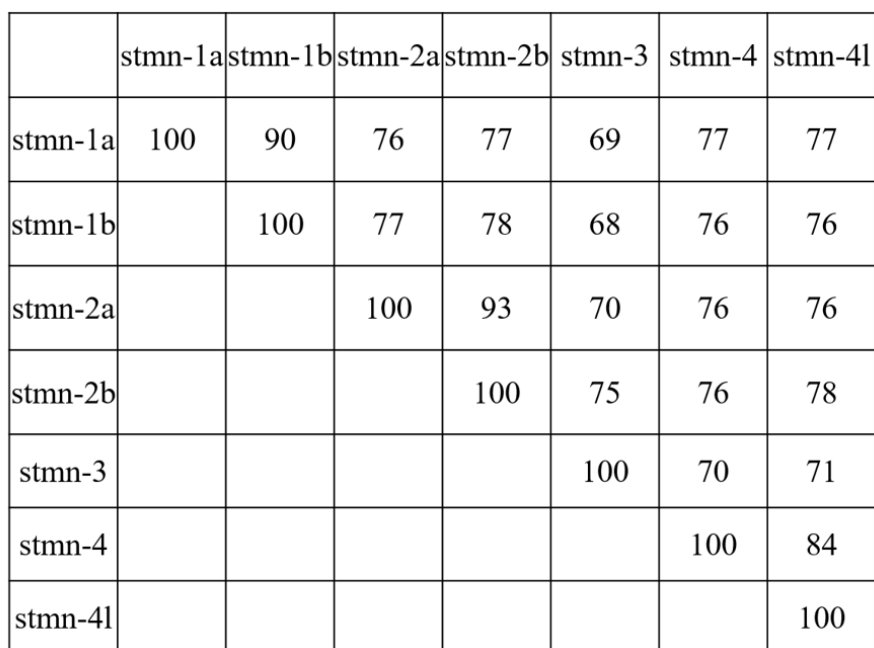
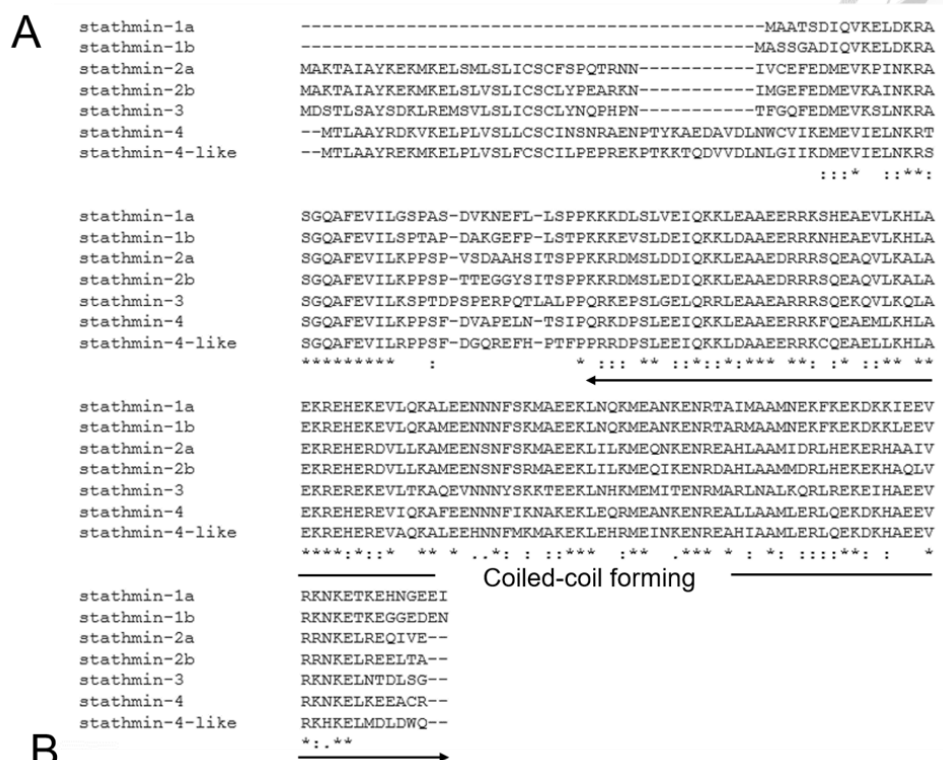


Fig. 1. Zebrafish stathmin sequence analysis of stmn1a, stmn2b and stmn4l.

(A) Sequence alignment of stathmin by Clustal Omega. Each symbol represents as following: asterisk (*) identical amino acid; colon (:) a strong conserved substitution; period (.) similar domain; diamond (♦) predicted palmitoylated cysteine amino acid; open arrow head (∇) predicted cAMP- and cGMP- dependent protein kinase phosphorylation site; open circles (○) predicted protein kinase C phosphorylation site; arrowhead (▼) predicted casein kinase II phosphorylation site. (B) The domain composition of the stathmin protein. The number of amino acid for each stathmin protein is shown on the right of the map. Conserved C-terminal stathmin-like domains with indicated identity to Stmn1a are shown. Variable N-terminal membrane anchoring domain domains exist in Stmn2b and Stmn4l, but not Stmn1a.



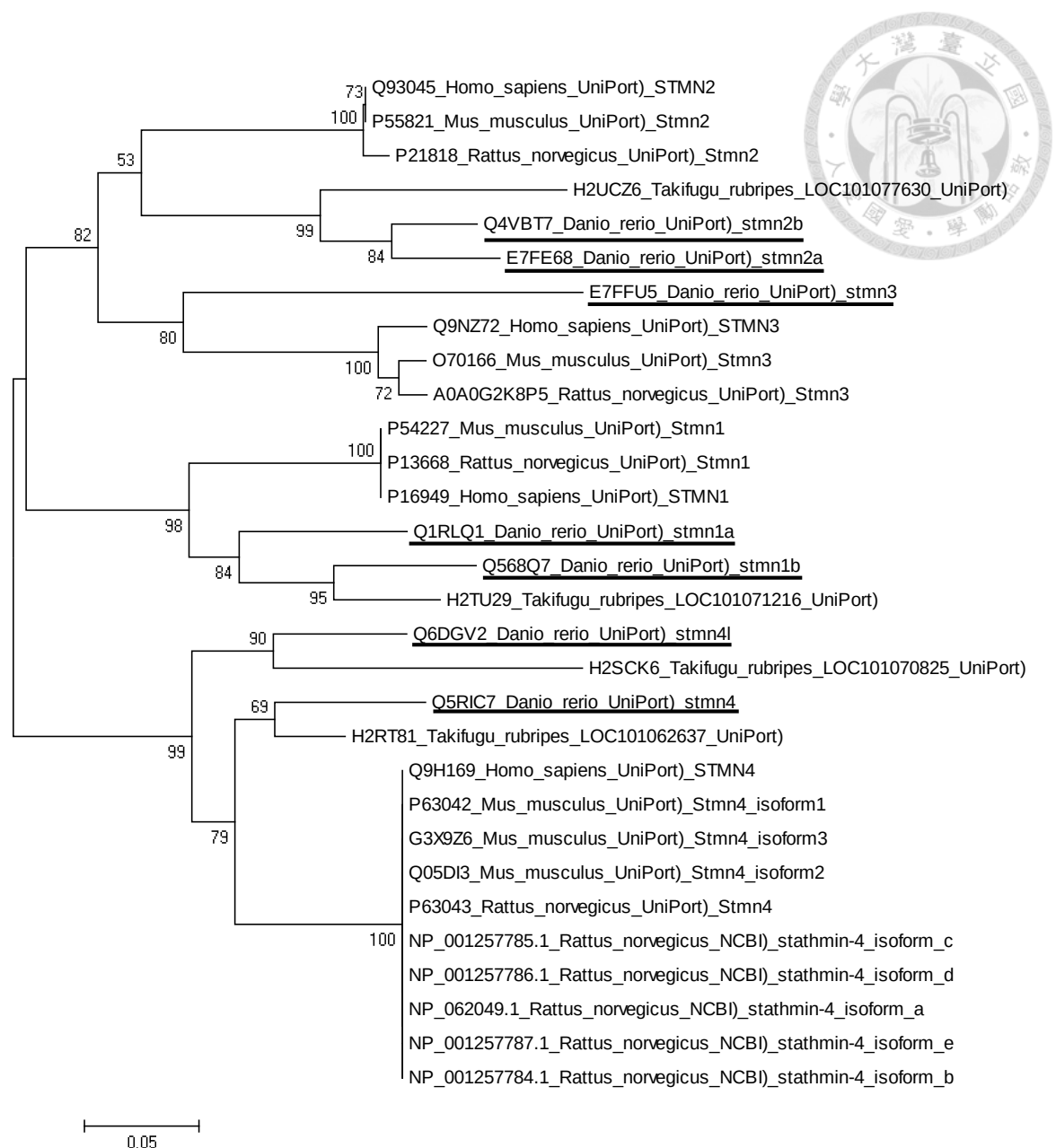


Fig. 3. The phylogenetic analysis between major model animal and zebrafish stathmin family. The bootstrap neighbor-joining phylogenetic tree was built up by ClustalW alignment involved 1000 replications analysis by software MEGA6. The zebrafish family genes are underlined.

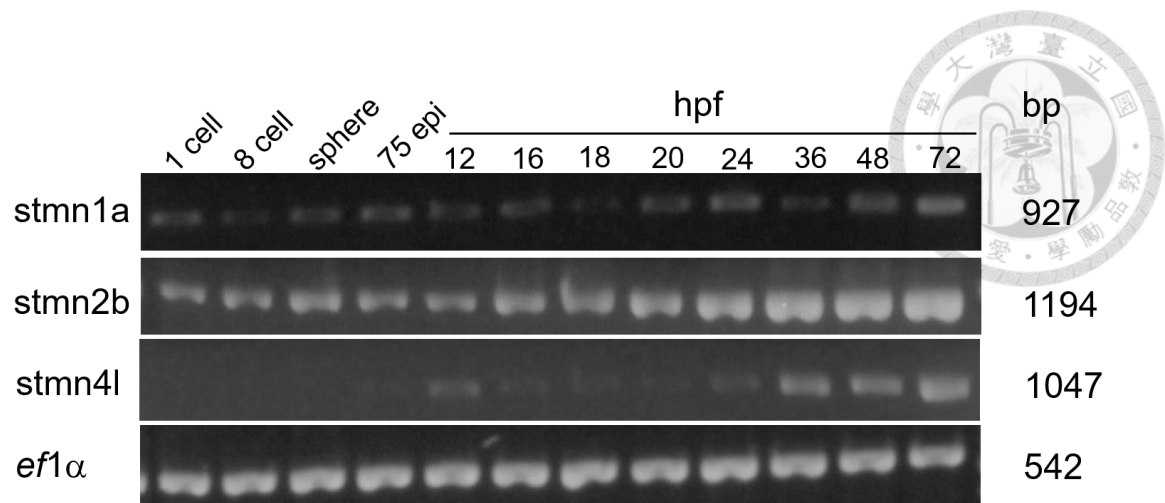


Fig. 4. Expression pattern of stathmin family genes during development by RT-PCR analysis. Zebrafish embryos are collected at designated stages and subjected to RT-PCR analysis for *stmn1a*, *stmn2b* and *stmn4l*. The primer pairs used for each gene are listed on Table 1 and the amplicon size is labeled on the right of each panel. *ef1α* is an internal control.

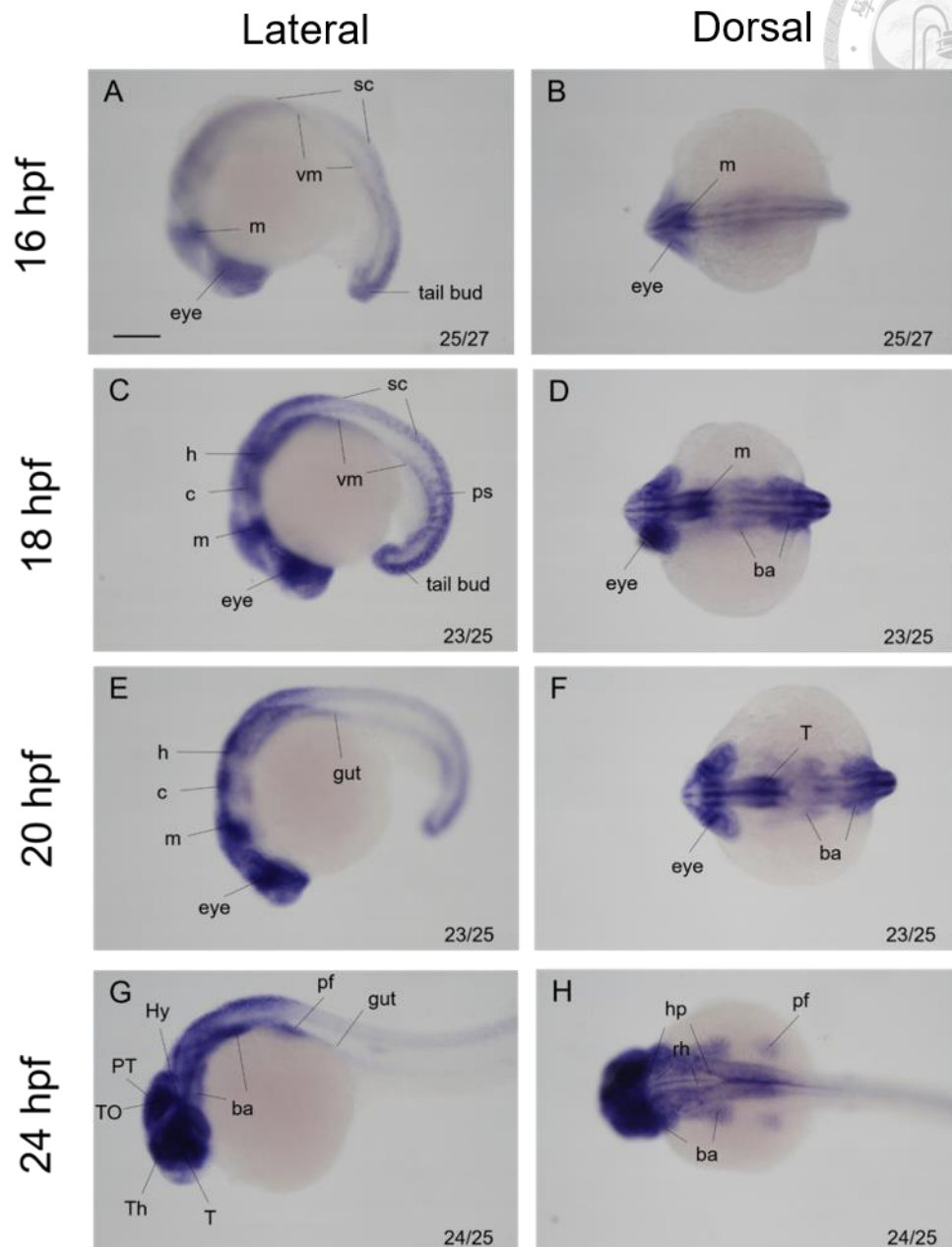


Fig. 5. Spatial expression pattern of stathmin1a during segmentation stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stam1a*. (A), (C), (E), (G) are lateral view of embryos. (B), (D), (F), (H) are dorsal view of embryos. ba, branchial arches; c, cerebellum; h, hindbrain; hp, hindbrain proliferative zone; Hy, hypothalamus; m, midbrain; PT, posterior tectum; pf, pectoral fin; ps, posterior somite; rh, rhombomeres; c, spinal cord; T, tegmentum; TO, tectum opticum; Th, thalamus; vm, ventral mesoderm. eye, gut and tail bud are labeled. Scale bars: 200 μ m

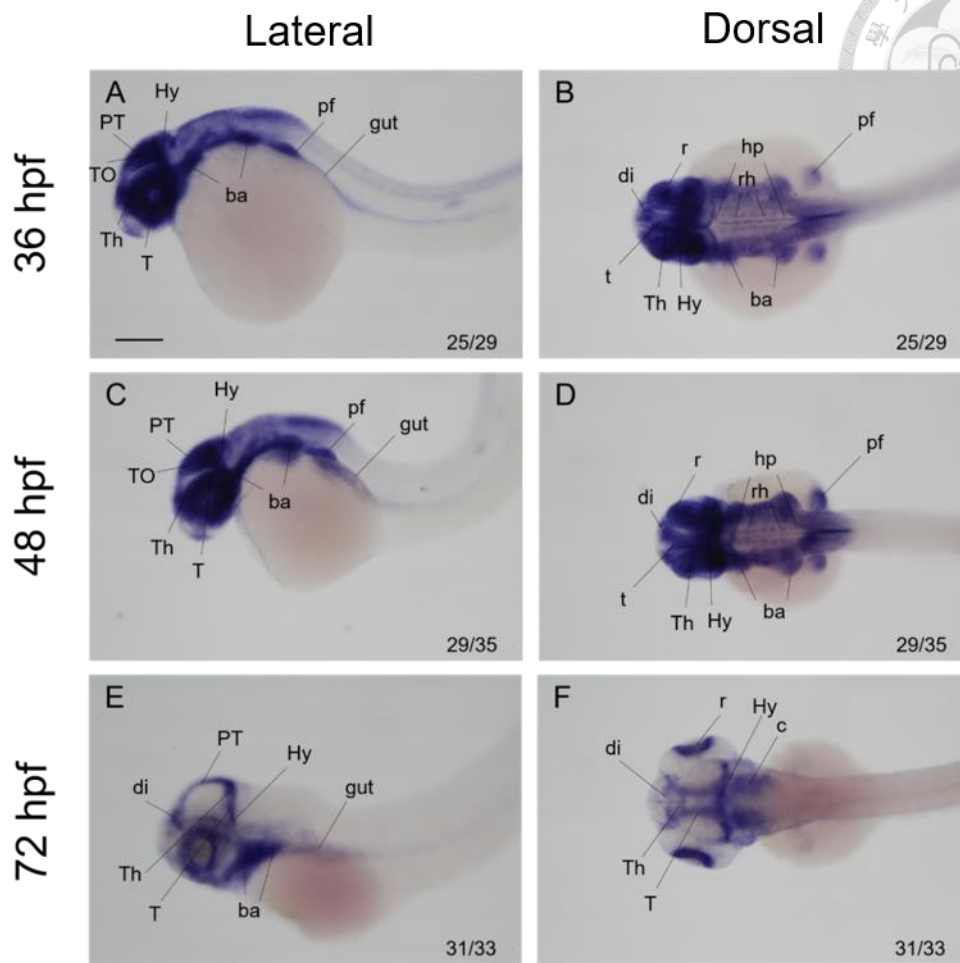


Fig. 6. Spatial expression pattern of stathmin1a early larval stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stathmin1a*. (A), (C), (E) are lateral view of embryos. (B), (D), (F) are dorsal view of embryos. ba, branchial arches; c, cerebellum; di, diencephalon; DT, dorsal thalamus; h, hindbrain; hp, hindbrain proliferative zone; Hy, hypothalamus; m, midbrain; PT, posterior tectum; pf, pectoral fin; ps, posterior somite; r, retina; th, rhombomeres; sc, spinal cord; T, tegmentum; TO, tectum opticum; Th, thalamus; t, telencephalon; rh, rhombomeres; vm, ventral mesoderm. eye and gut have been labeled. Scale bars: 200 μ m.

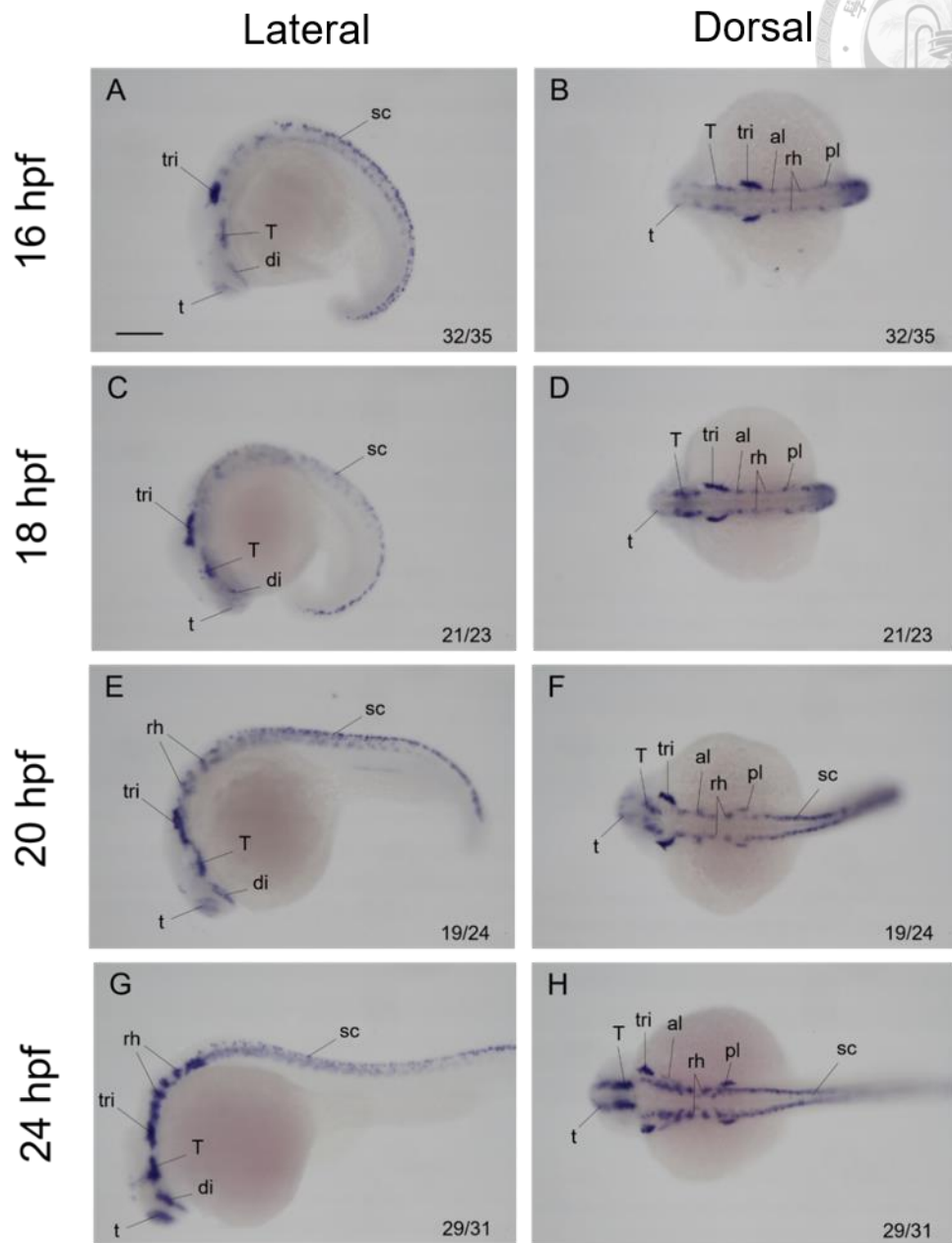


Fig. 7. Spatial expression pattern of stathmin2b during segmentation stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stam2b*. (A), (C), (E), (G) are lateral view of embryos. (B), (D), (F), (H) are dorsal view of embryos. al, anterior lateral line ganglion; c, cerebellum; di, diencephalon; pl, posterior lateral line ganglia; rh, rhombomeres; sc, spinal cord; T, tegmentum; t, telencephalon; tri, trigeminal ganglia. Scale bars: 200 μ m.

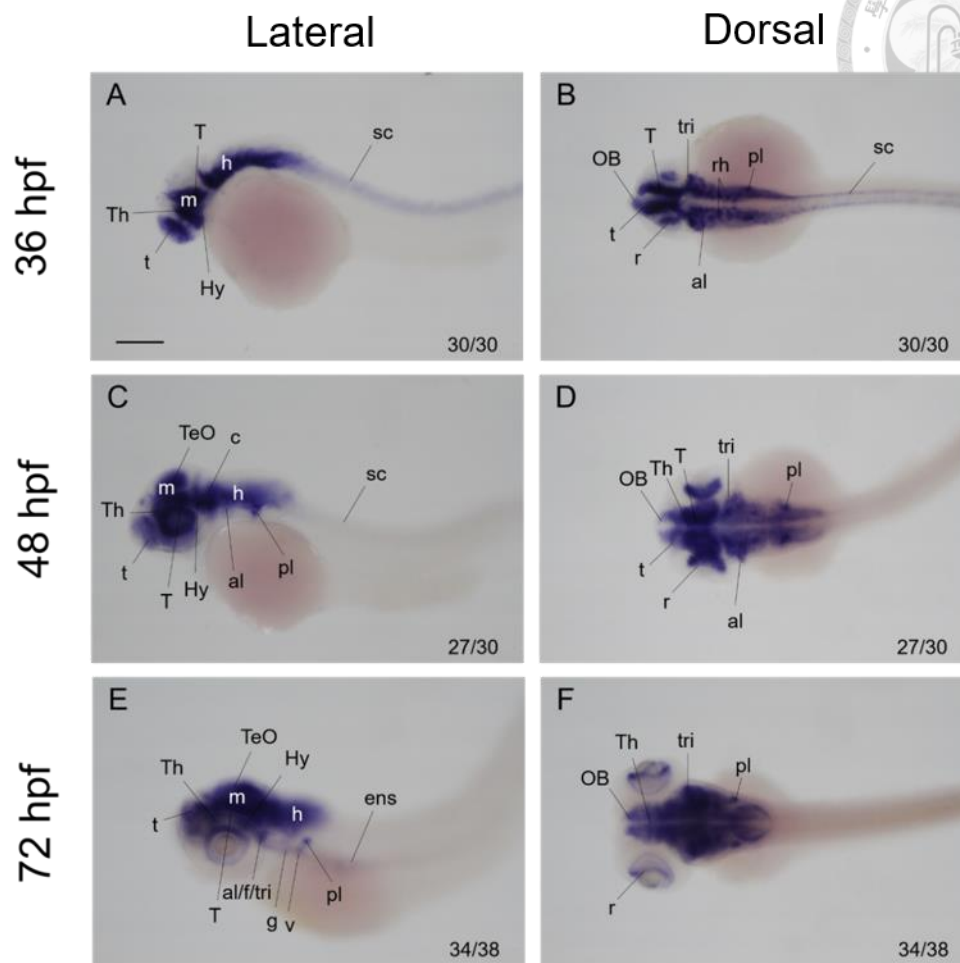


Fig. 8. Spatial expression pattern of stathmin2b early larval stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stam2b*. (A), (C), (E) are lateral view of embryos. (B), (D), (F) are dorsal view of embryos. Stage of embryos are showed on the left panel. al, anterior lateral line ganglion; c, cerebellum; di, diencephalon; ens, enteric nervous system neuron; f, facial ganglia; g, glosso-pharyngeal ganglia; Hy, hypothalamus; h, hindbrain; m, midbrain; OB, olfactory bulb; pl, posterior lateral line ganglia; r, retina; rh, rhombomeres; sc, spinal cord; T, tegmentum; Th, thalamus; t, telencephalon; tri, trigeminal ganglia; TeO, tectum opticum; v, vagal ganglia. Scale bars: 200 μ m.

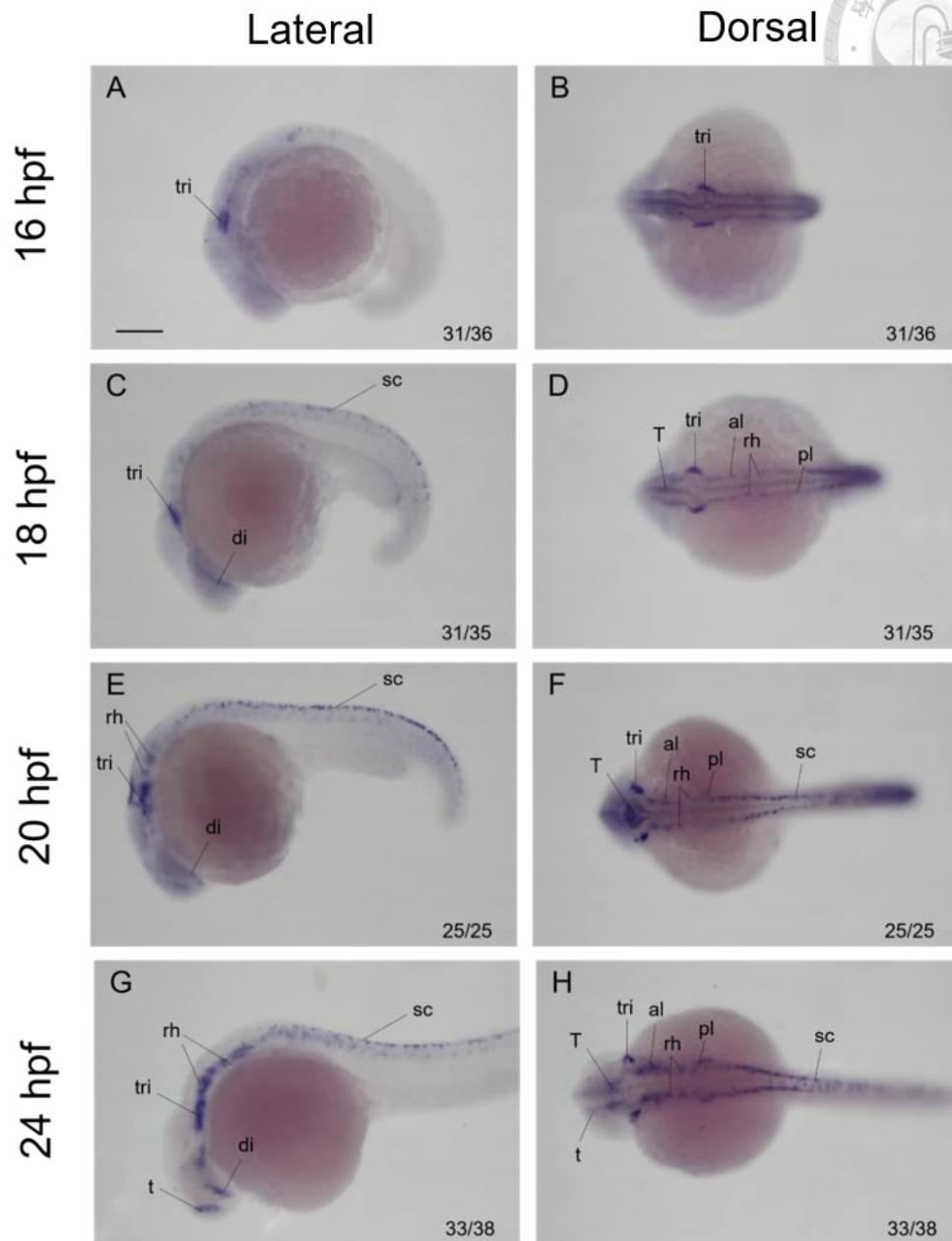


Fig. 9. Spatial expression pattern of stathmin41 during segmentation stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stam41*. (A), (C), (E), (G) are lateral view of embryos. (B), (D), (F), (H) are dorsal view of embryos. al, anterior lateral line ganglion; di, diencephalon; pl, posterior lateral line ganglia; rh, rhombomeres; sc, spinal cord; T, tegmentum; t, telencephalon; tri, trigeminal ganglia. Scale bars: 200 μ m.

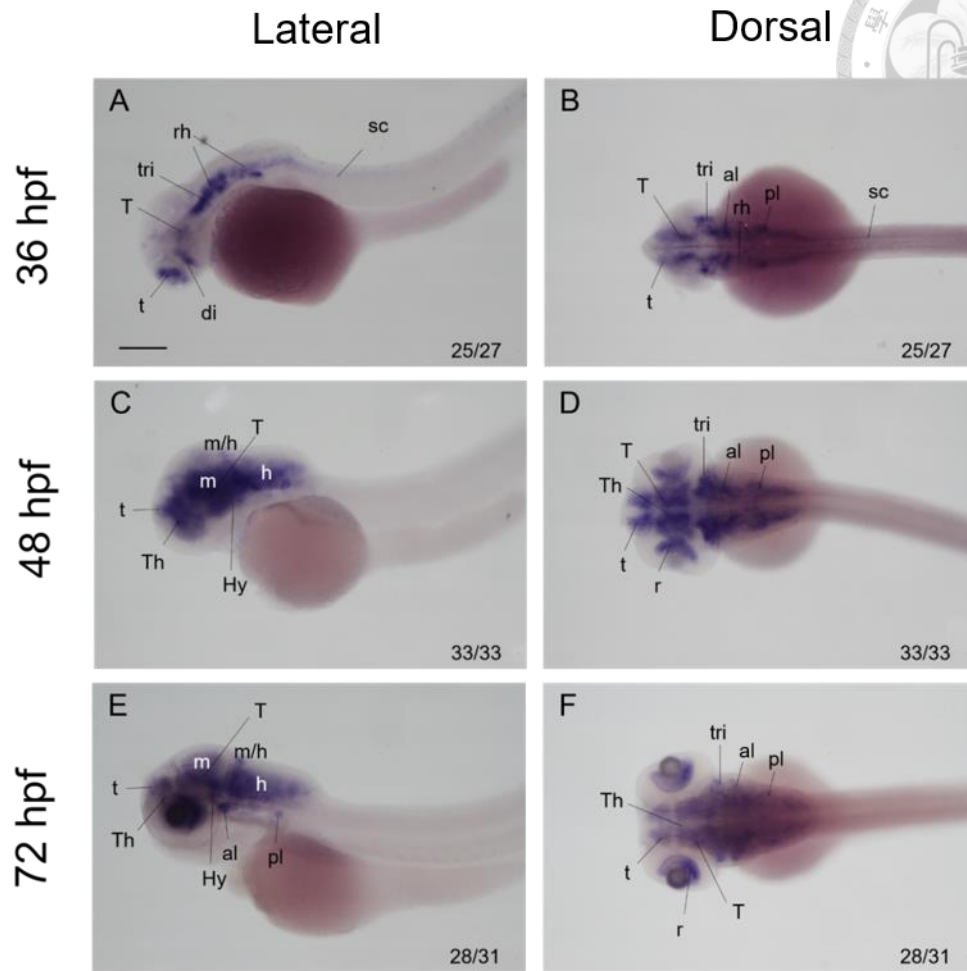


Fig. 10. Spatial expression pattern of stathmin4l during early larval stages. Embryos were fixed at designated stages (shown on the left) and subjected to whole-mount *in situ* hybridization against *stam4l*. (A), (C), (E) are lateral view of embryos. (B), (D), (F) are indicated dorsal view of embryos. al, anterior lateral line ganglion; di, diencephalon; Hy, hypothalamus; h, hindbrain; m, midbrain; m/h, midbrain and hindbrain boundary; pl, posterior lateral line ganglia; r, retina; rh, rhombomeres; sc, spinal cord; T, tegmentum; Th, thalamus; t, telencephalon; tri, trigeminal ganglia. Scale bars: 200 μ m.