Characterization of the Role of Macoilin in *Dugesia japonica*

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Dedication

This thesis is dedicated to my family for their support for my study and life in University of Minnesota, especially my mother, who believes in me and encourages me to pursue the master degree, and to my boyfriend, Jinming Zhang, for his understanding and help throughout these years.

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Materials and Methods

Introduction

Planarian Biology

Stem cell research is a topical issue for medical science. Cells that are highly differentiated (so called 'unipotent' cells) have lost the ability to become other kinds of cells. When unipotent cells area damaged they undergo programmed cell death or necrosis. In contrast, stem cells have maintained an ability to differentiate into different cell types, potentially restoration of damaged tissues. This restorative process, which occurs naturally during physiological turnover, or on ectopic damage is called "regeneration" [1]. The precise mechanisms of regeneration are varied and often undefined, but one way to understand the molecular mechanisms involved is to study spontaneously regenerating animals. In nature, many types of animals have regenerative abilities, unlike humans. Regenerative capabilities are broadly but unevenly distributed across species[1, 2]. One type of animal that shows remarkable regenerative capacity is the planarian flatworm. Planarians are non-parasitic flatworms that have the ability to restore any missing body parts after damage/cutting[1, 22, 23]. This regenerative function is caused by the presence of neoblasts, totipotent stem cells maintained throughout the planarian body which respond to injury and repair/replace the damaged cells/tissues through proliferation and differentiation[2, 22]. Previous data has shown that the process of regeneration in planarians involves neoblast migration to the wound followed by a rapid proliferation phase that forms an undifferentiated tissue called the blastema. New tissues with appropriate polarity and form will differentiate from the blastema. However, a key question remains: what signals direct this regenerative process[3]. Various research data has led researchers to

believe that the integration of neurons and other signaling pathways guide the process of differentiation[2, 22, 23]. Thus the resolution of regeneration-induced signaling pathways is important for helping scientists identify the mysteries behind regeneration.

Among the studies of regeneration process in planarian, the most common methods are gene manipulation (loss of function by *in vivo* RNAi) and/or drug exposure. These methods can cause abnormal phenotypes during regeneration of worms[6, 24]. One example is the discovery of bipolar phenotypes occurring after regeneration rather than the normal head-to-tail polarity[5]. Usually, after amputation of a planarian's head and tail, the remaining fragment ('trunk fragment', boxed in **Figure 1.1A**), will regenerate into an intact normal worm. However disturbing the process of regeneration by gene manipulation[4] (RNAi of β Catenin) or drugs[6] (Praziquantel, PZQ) can lead to a head instead of tail phenotype (**Figure 1.1B**), producing two-headed worms, which is called bipolarity.

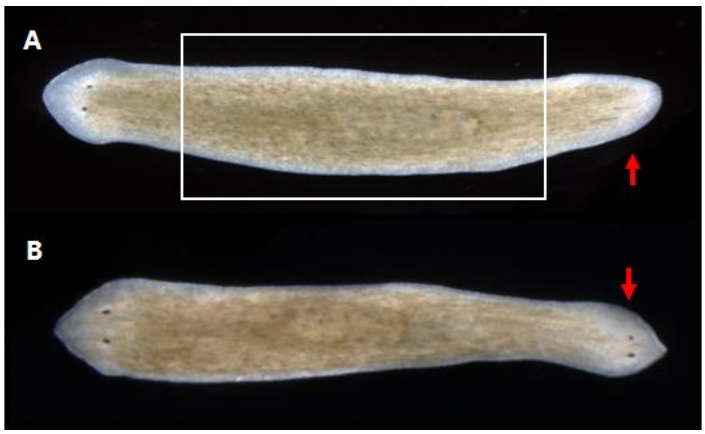


Figure 1.1 polarity regeneration of planarian
A, amputation of planarian, the white rectangular shows the position of "trunk fragment". ***B***, regenerative PZQ-induced head-instead-of-tail phenotype, the red arrows indicates the alternative head instead of a normal tail.

Effects of Drugs on Regeneration.

Praziquantel (PZQ), a pyrazinoisoquinoline anthelmintic drug, was discovered in 1970s and soon became a prevalent treatment for schistosomiasis, but the exact mechanism of action of this drug is still unknown. In parasitic schistosome worms, PZQ is believed to disrupt Ca^{2+} homeostasis to cause paralysis[7, 8]. Studies have shown that PZQ requires the presence of a specific schistosome voltage-gated calcium channel (VGCC, Ca_v) β subunit to activate Ca^{2+} entry [4, 8, 9]. Thus the results bring VGCC to the attention of scientists who are studying the mechanism of PZQ action. The researchers of our lab also observed the correlation between PZQ and calcium cation homeostasis. Our lab accidentally found that when PZQ was applied to regenerating planarian trunk fragments, PZQ exposure yielded a high penetrance of bipolar (two-headed) worms rather than the normal head-to-tail anterior-posterior polarity in naïve worms. Further: 1) higher calcium accumulation appeared in the PZQ-treated regenerative worms compared to control regenerative worms, 2) higher doses of calcium potentiated the penetrance of bipolarity caused by PZQ, and 3) the activity of PZQ on planarian was modulated by knockdown of specific voltage-gated calcium channel subunits. All these observations broadly agree with the calcium-related discovery on schistosomes, and thus we believe PZQ acts as an activator of calcium influx in planarians[5, 6, 11].

Voltage-gated Calcium Channel

To figure out which subunit of VGCC is the key regulator of PZQ activity, our lab studied the effects of different VGCC isoforms on planarian regeneration. Voltage-gated calcium channels (VGCC) are membrane protein complexes that contribute to impulse

propagation and regulation of intracellular Ca^{2+}, which provide Ca^{2+} influx for various Ca^{2+} dependent responses such as neurotransmitter release and secretion[11, 28]. Intracellular calcium signaling is critical for organisms to support various basic cellular activities such as cell division, meiosis, apoptosis motility and synaptic transmissions[11, 25, 26, 27]. VGCC have five components: α1, α2, β, δ and γ subunits, which play different roles in calcium channel function. $Ca_v\alpha1$ is the transmembrane, channel pore-forming subunit while $Ca_v\beta$, a cytoplasmic subunit, regulates channel inactivation activity and plasma membrane expression[29]. Then this family was distinguished by the duration of subunit-induced Ca^{2+} currents: Ca_v1 calcium channel, also called L-type VGCC, and Ca_v2 calcium channel, induce long lasting currents; while T-type channels induce transient currents[27]. For most invertebrates, the organism encodes only one subtype for either of these VGCC families. However, schistosomes and planarians, instead of having a single subtype for each type of channel, contain more than one subtype of Ca_v1 and Ca_v2 calcium channels[11, 30, 31]. However the exact role of each subunits and isoforms of VGCC in processes such as movement and neuronal activities is unclear[12]. Though the VGCC subunits are expansive, and the function of most of these subtypes remains elusive, our lab has identified five VGCC subunits and found only one of subunits, Ca_v1 has effects on PZQ-evoked bipolarity of planarian during regeneration and the Ca^{2+} influx activated by PZQ[10]. Ca_v1 family has two subtypes called Ca_v1A and Ca_v1B in planarian, which show an opposite effect on worm's polarity to each other and **Figure 1.2** illustrates the simplified working model for these two proteins. *In vivo* RNAi of Ca_v1B in planarian will potentiate PZQ's function of evoking bipolarity in the regeneration of planarian, while Ca_v1A deteriorate this function by PZQ[10]. The previous data suggests a potential role of VGCC subunits in

the function of PZQ in planarian, and, more specifically, the potential inhibition effect of Ca_v1B on PZQ-evoked bipolarity. But how VGCC is activated in PZQ-induced signaling pathway and how VGCC regulates the further downstream signaling pathway is still less clear. Thus I believe voltage-gated calcium channel is not the only protein or protein complex that modulate PZQ efficacy in planarians and have potential roles in planarian nervous system.

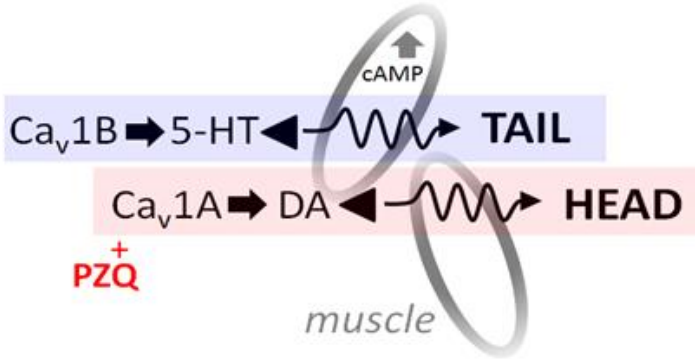


Figure 1.2 Schematic model for Ca_v1 family in modulating neurons and polarity of worms. 5-HT, serotonergic neurons; DA, dopaminergic neurons

Macoilin

Here I attempted to bring new idea into the VGCC-PZQ pathways and focus my interests on macoilin protein after a series of targets screening. Macoilins are conserved proteins from human to protozoa which are specifically expressed in neurons[13]. In the mouse, macoilin is active mostly in post-mitotic and post-migratory neurons which are downregulated at the end of maturation[14]. It's predicted to have five transmembrane domains at N-terminus and four coiled coil domains in the C-terminus of the protein in *C. elegans.* (And at least three transmembrane domain and four coiled coil domains in the C-terminus of the protein in planarians, see **Figure 1.3**)[16, 17]. Previous co-localization

experiments showed that macoilin located in the endoplasmic reticulum (ER) of neurons, indicating that it may be involved in the folding, assembly, or traffic of secreted or transmembrane proteins, and probably takes part in regulating calcium storage and release by modulating membrane-bounded calcium channel on through modulating the maturation of calcium channel protein families.

It is also been supported by research that macoilin might be involved in the regulation of

Figure 1.3 Predicted schematic domains of macoilin subtype MACO-1
Predicted structure of MACO-1 gene family of *C. elegans* and *D. japonica*. Solid and open bars show the predicted positions of transmembrane domains and coiled coil domains, respectively. TM, transmembrane domains; CC, coiled coil domain.

the interaction of actin cytoskeleton and membrane components[17]. Research has shown that in macoilin deficient worms, the nervous system, although largely intact, exhibited behavioral defects, and more specifically, signaling defects. Also, what is interesting is that the behavioral defects of macoilin mutants is similar to those caused by defects in particular calcium channel components[13]**.** Research on *C. elegans* showed that although baseline Ca^{2+} level in macoilin mutant worms remains normal, loss of macoilin leads to failure of Ca^{2+} transients in the O_2-sensing neuron PQR, which gave an idea that the activation of macoilin may be lead to recruitment of Ca_v channel[13]**.**

Furthermore, based on the fact that TMEM57 protein is an alias of macoilin protein, I carried out a literature search with the keyword “macoilin/TMEM57” in PubMed database for acquiring more information on this protein but got only three references

back (see reference 13, 17, 21) to date. These few reported studies of macoilin is only the tip of the iceberg and thus it's worth further researching. Therefore the purpose of this thesis is to investigate the role of another nervous system protein, macoilin, which may have the similar functions to that of VGCC subunit isoforms, and thus potentially providing a new pharmaceutical target for future anthelminthic medications.

Here, my research firstly proposed that planarian macoilin protein may play an important role in the regeneration process of planarian and this these is intended to identify the regenerative performance of planarian under PZQ treatment after knockdown of its macoilin protein. I used a species of dugesiid triclad called *D. japonica* to conduct my experiments. I carried out:

(i) I presented the *in vivo* imaging of human macoilin protein to show the localization of macoilin protein in primary neuronal cells. I co-transfected human macoilin-GFP plasmid and a red endoplasmic reticulum marker into neuronal cells to see the distribution of fluorescence protein by confocal microscope.

(ii) bioinformatic analysis of the macoilin family.

(iii) plasmids construction, aiming at constructing different plasmids contains different macoilin isoforms, MACO-1, MACO-2 and MACO-3. So far, I totally got 5 constructs in T7 promotor system.

(iv) a series of RNAi experiments, aiming at exploring if the knockdown of macoilin will have any effect on worm regeneration and motility. I scored the 2-head penetrance at different PZQ concentration and then recorded the movement of worms coming from different cohorts to see if there were any defects of movement in each cohort.

Results

In vitro expression of human macoilin protein

Macoilin is a highly conserved protein found in diverse species[17, 21]. An N-terminus GFP-tagged human macoilin protein plasmid was commercially acquired (GenScript USA Inc.) based on the sequence of human macoilin (GenBank ID: 59897093). To identify the localization of macoilin, the plasmid was transfected into a primary culture of natal mouse neuron cells, to do fluorescence imaging. The imaging assay (**Figure 2.1**) suggests that macoilin is a non-nuclear protein, possibly showing co-localization with an endoplasmic reticulum marker (DsRed-ER).

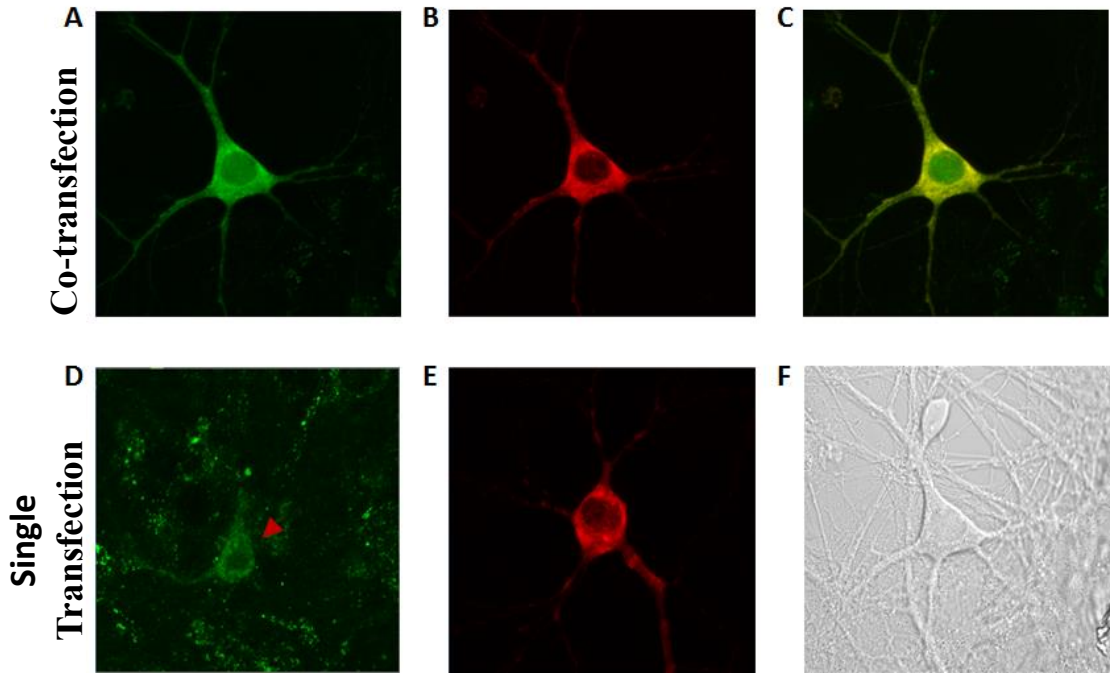


Figure 2.1 Neuron imaging of human macoilin protein. Green: GFP-tagged human macoilin protein; Red: dsRed-tagged endoplasmic reticulum (ER) marker
A-B. Co-localization assay. Co-transfection of ER marker and GFP-macoilin. Both of the proteins are mostly detected in neuron cytoplasm compared to nucleus. C. the merge of A and B. D. Single transfection of GFP-macoilin. The red arrow shows the position of cell body. E. Single transfection of Red-ER marker. F. Bright field of co-localization assay utilized in A, B, C.

***In silico* identification of planarian macoilins.**

A blast of planarian macoilin proteins based on the mammalian macoilin protein was launched on Galaxy tBLASTn program, aiming at finding homologs in planarian. One Homo sapiens macoilin protein sequence (GenBank ID: 59897093) was submitted as the template in the search of the transcriptome of *Dugesia japonica* genome. When analyzing the results of Galaxy tBLASTn, I used a filter of E value < 1.00E-10 to filter the results. The output showed a total of 7 sequences whose E value is smaller than the cutoff (**Table 1**).

Specific name	Planarian translated nucleotide	Ident	Hs_macoilin		Nucleotide		E value	Hit score	FPKM value
			From	To	From	To			
MACO-1	comp143153_c0_seq4	49.23	1	192	2002	1418	1.00E-67	238	40.91
MACO-2	comp140642_c1_seq5	41.62	1	193	2177	1587	7.00E-56	199	12.025
MACO-2	comp140642_c1_seq2	41.62	1	193	2192	1602	8.00E-56	199	4.20167
MACO-2	comp140642_c1_seq7	41.62	1	193	2308	1718	1.00E-54	195	0.55167
MACO-2	comp140642_c1_seq8	41.62	1	193	2323	1733	1.00E-54	195	0.10333
MACO-3	comp143153_c0_seq7	30.63	500	610	598	266	9.00E-14	59.7	2.385
MACO-2	comp140642_c1_seq4	23.84	385	657	1133	309	7.00E-12	53.1	1.59167

Table 1 tBLASTn results of human macoilin (gi59897093) protein sequence in planarian translated nucleotide genome. All of the listed genes are sorted by E value from the smallest to the biggest.

These 7 sequences represented 3 different products, called MACO-1, MACO-2 and MACO-3. These predicted macoilin protein sequences are 678, 770, 301 amino acids for MACO-1, MACO-2, and MACO-3. MACO-1 is highly homologous with Homo sapiens macoilin protein at its N-terminus which is the transmembrane domains, and at its C-terminus which is the coiled-coil domain as shown in **Figure 2.2**. The sequence

comp140642_c1_seq8 was chosen as MACO-2, due to the result of gene alignment which shows that comp140642_c1_seq8 is the longest sequences and it contains comp140642_c1_(seq2, seq4, seq5, seq7). MACO-2, through the result of gene alignment, had five transmembrane domains to its N terminus. But rather than having coiled coil domains at its C-terminus, it contained four transmembrane domains at the C-terminus. Comp143153_c0_seq7, which was called MACO-3, was highly similar to the C-terminus MACO-1, which, the result of gene alignment showed that it was homologous at C-terminus at which it had the coiled-coil domains, while it was not homologous at the N-terminus with MACO-1.

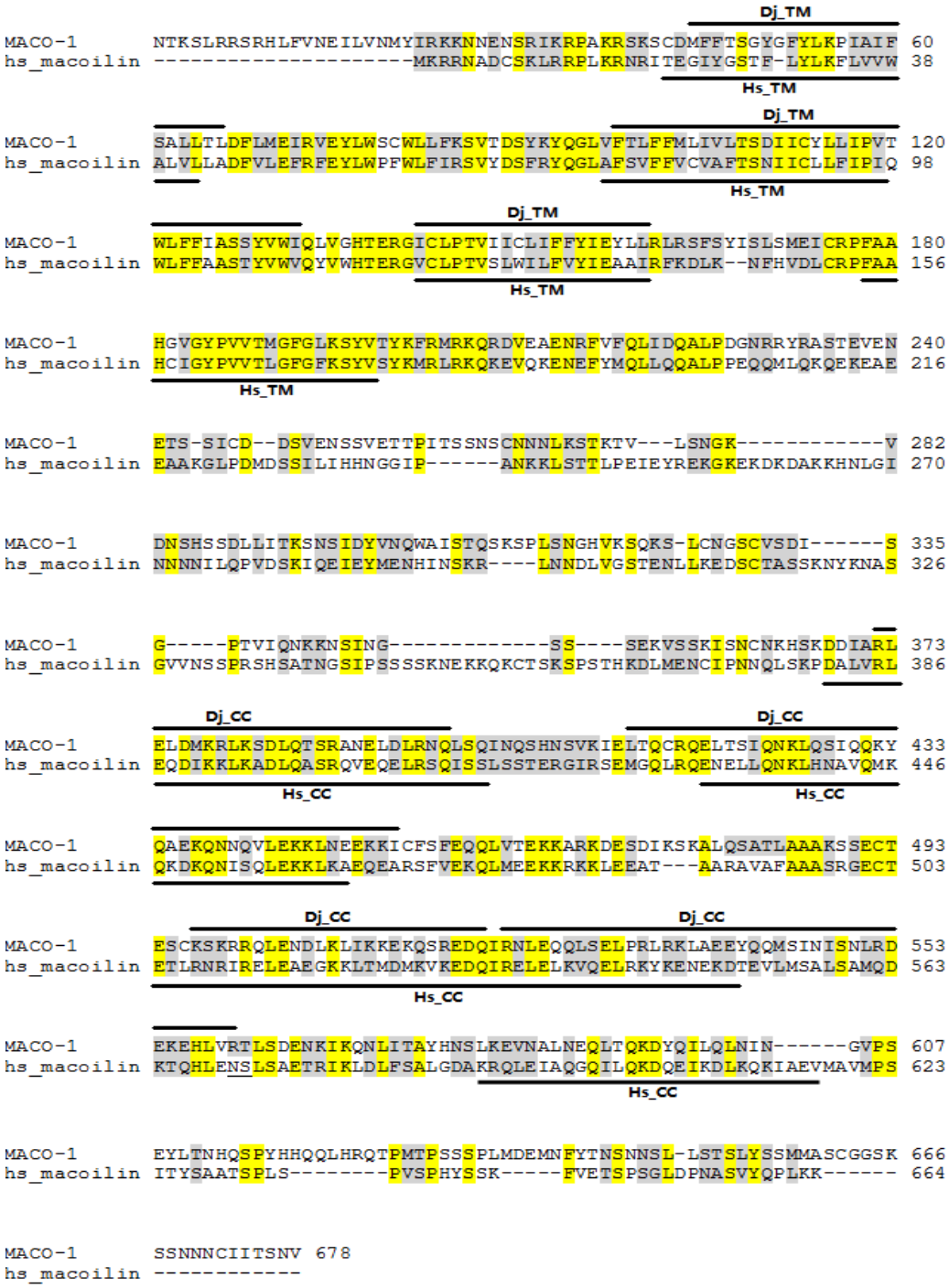


Figure 2.2 alignment of MACO-1 with human macoilin

The predicted macoilin protein sequence MACO-1 comes from EST assembly. The lengths of these two aligned proteins are 678, 664 amino acids for MACO-1(dj_macoilin), and human macoilin (hs_macoilin). Yellow highlights show the identical amino acid residues and grey highlights indicate the similar residues. Functionalized domains are marked by black line. TM stands for transmembrane domain and CC stands for coiled coil domain. Dj: *D. japonica*; Hs: *H. sapiens*

The alignment of multiple macoilin protein sequences was done by SEAVIEW (**Figure 2.3**) and these inspections of the predicted planarian *D. japonica* macoilin sequence MACO-1 confirms the highly conservation of macoilin protein between mammalian animals like human and mouse and worms like *C. elegans* and planarians.

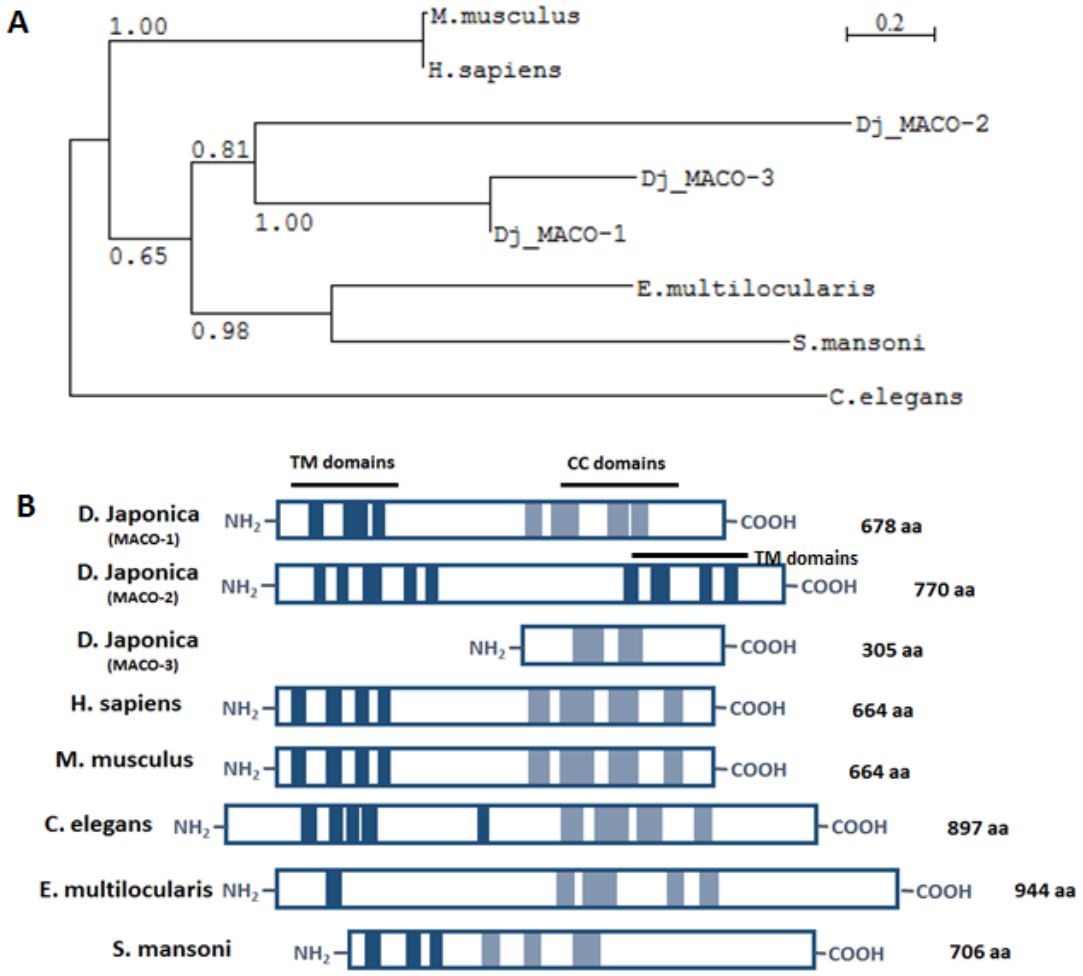


Figure 2.3 Alignment of macoilin protein family in diverse species.
A. Alignment used SEAVIEW and displayed as a rooted tree analyzed by LG model with an aLRT (SH-like) branch. Numbers around nodes are bootstrap values. Accession numbers for macoilin protein family are 59897093 (*Homo sapiens*), 40254350 (*Mus musculus*) and 351060762 (*Caenorhabditis elegans*). *S. mansoni* and *E. multilocularis* macoilin protein sequences were predicted and got from blast through their genome using H. sapiens macoilin protein as template. B. Schematic illustration of the predicted domains of macoilin protein family. The dark bars represents transmembrane domains (TM domains) and the light bars show the position of coiled coil domains (CC domains). Numbers at the left of structures indicate the lengths of protein, aa: amino acid.

Generation of RNAi constructs

Based on the bioinformatics analysis of the D. *japonica* macoilin isoforms, I designed several pairs of PCR primers to amplify the three *in silico* predicted macoilin sequences. Different primer pairs targeted different domains (transmembrane domain or coiled coil domain, shortened as TM and CC, respectively) on those sequences. Primers design was based on the NCBI Primer-BLAST system. Detailed individual primers used in this PCR experiments are discussed in "Materials and Methods" based on the strategy illustrated in **Figure 2.4**.

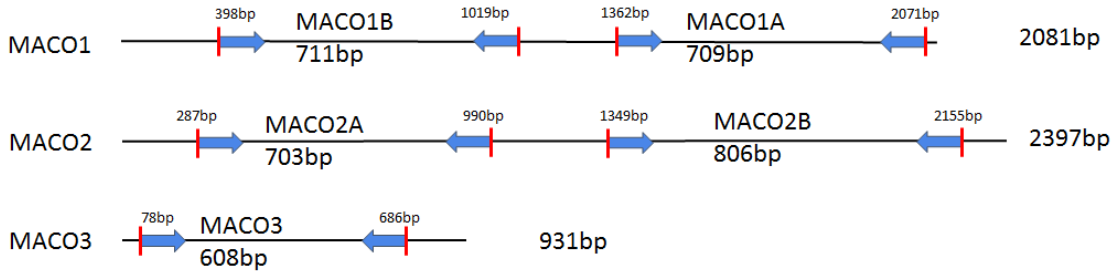


Figure 2.4 Schematic illustration of primers' locations.
Two blue arrows indicate the position of one construct. Numbers under the constructs indicate their lengths. Numbers beside isoforms show the length of each isoform, bp: base pair.

PCR products from planarian cDNA were examined by gel electrophoresis. The results showed in **Figure 2.5A** indicates the corresponding sizes of different macoilin products which are matched with my *in silico* prediction. The sequences were validated by gene sequencing after cloning into pGEMT-EZ vector system. RNAi vectors (pDONRdT7) were then prepared as described below.

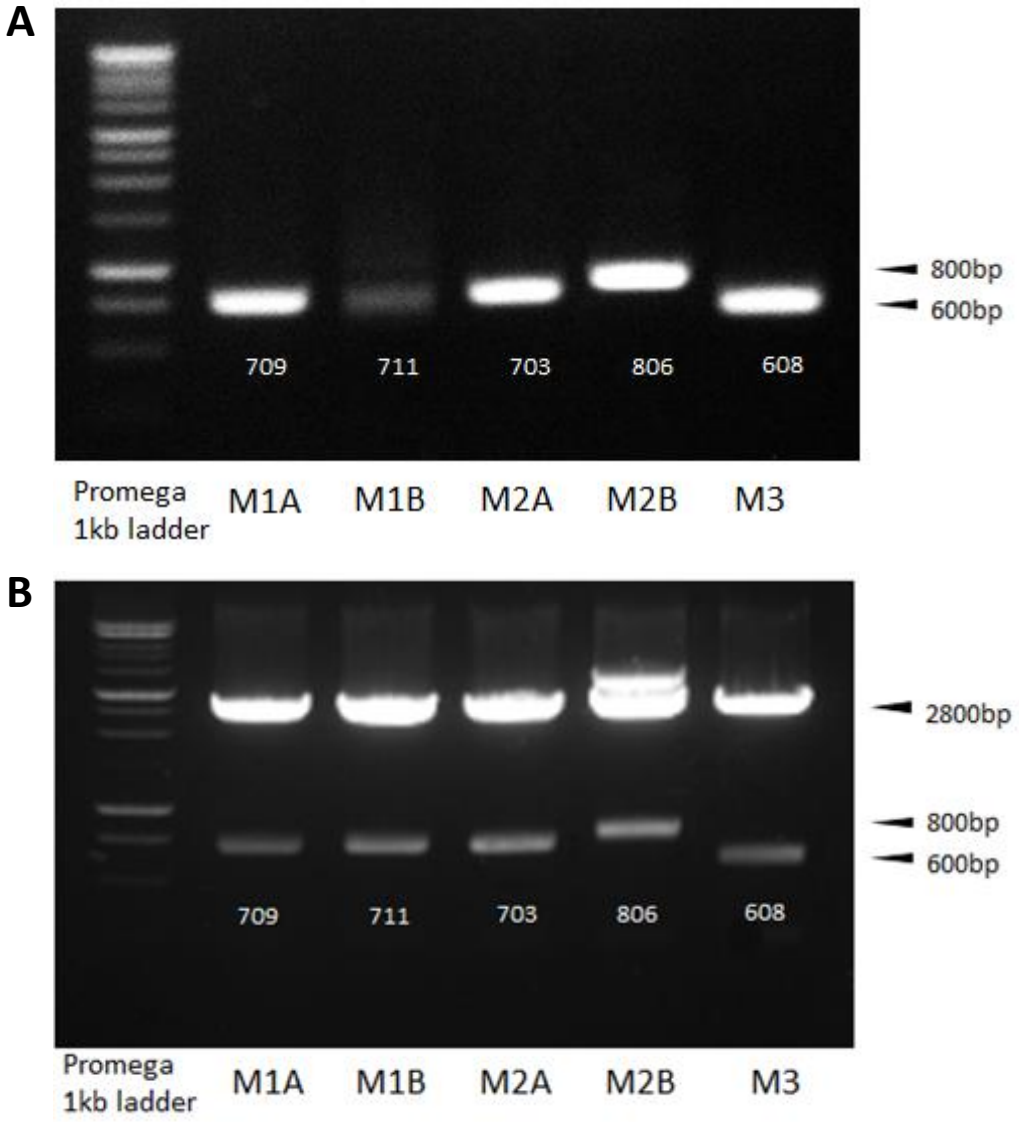


Figure 2.5 Gel results of gene cloning experiments. A. Gel results of PCR products from planarian cDNA library. B. Gel results of SfiI digested plasmids (pDONRdT7 system)
Each lane represents one product. M1A: MACO-1A; M1B: MACO-1B; M2A: MACO-2A; M2B: MACO-2B; M3: MACO-3; Ladder: Promega 1kb DNA ladder; Gene of interests: range from 600bp to 800bp, number under each of lane indicates the length of each gene, unit: bp. Vector: pDONRdT7 2.8kb (only in B). Double bands happened in M2B lane could be due to the over-digestion by SfiI.

To prepare RNAi experiments, I constructed a series of plasmids to generate double-stranded RNA (dsRNA) in the worms. I amplified the 5 macoilin constructs from pGEMT-EZ vector system and recombined them into the modified pDONRdT7 system as illustrated in **Figure 2.6**, with two T7 promoter flanked besides the insert[18]. The T7 promoters can be induced to express T7 polymerase after adding isopropyl β-D-thiogalactoside (IPTG)[18], and leads to the formation of dsRNA products. Sfil digestion and gel electrophoresis was done to examine the size of insert. The results are showed in **Figure 2.5B**. Sequences were verified by gene sequencing service.

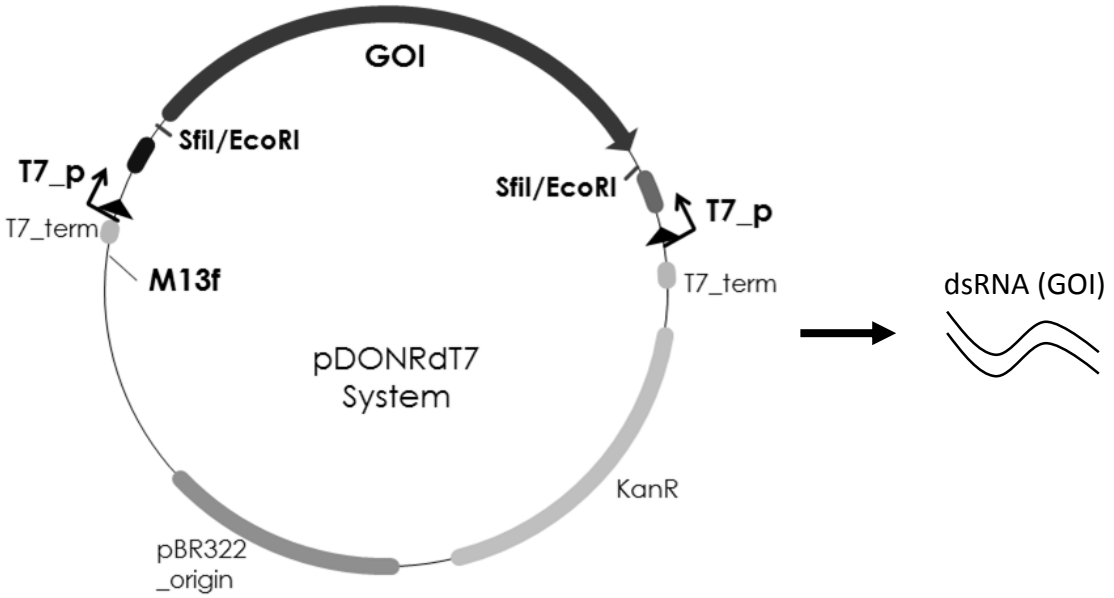
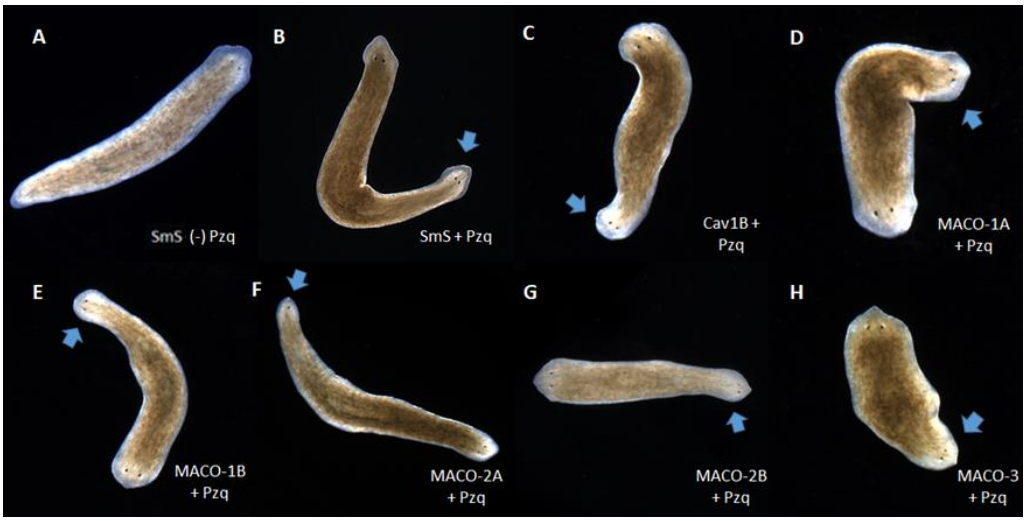


Figure 2.6 illustration of the structure of pDONRdT7 vector system
T7_p: T7 promoter; T7_term: T7 terminator; M13f: M13 forward primer; GOI: Gene of interest; KanR: Kanamycin resistance; SfiI/EcoRI: restriction digestion sites, including SfiI and EcoRI digestion site. dsRNA: double-stranded RNA carries GOI sequences.

RNAi experiments

Worms were fed with individual constructs to let them express dsRNA of interest as described in Methods. As previously described in Introduction, treatment of regenerating worms with PZQ yielded a significant fraction of bipolar ('two-headed') worms (**Figure 2.7A-B**)[5]. The SmS originated from *S. mansoni* Six-1 genes is used as negative control due to its lack of target mRNA in planarian. The percentage of bipolar worms caused by PZQ was enhanced in the Ca_v1B RNAi cohort (**Figure 2.7I**) [10], which is used as a positive enhancer in these assays.

The RNAi experiments revealed different activities of macoilin isoforms. RNAi of three macoilin genes, MACO-1A, MACO-1B, MACO-3, all potentiated the penetrance of 2-head compared to the negative control, SmS group, and the phenotypes of these three cohorts was really similar to that of Ca_v1B RNAi worms, while the RNAi of the other 2 genes, MACO-2A and MACO-2B had less of a change on penetrance compared to SmS worms. (Phenotypes shown in **Figure 2.7D-H**, quantification data shown in **Figure 2.7I**.). A similar effect was observed under a lower dose of PZQ (**Figure 2.8**).



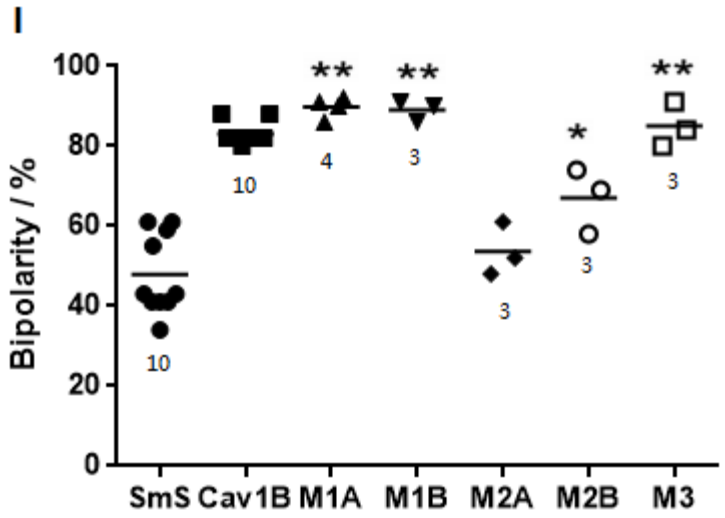


Figure 2.7 RNAi scoring results.
A-H. B-H Images show bipolar phenotypes after regeneration and PZQ exposure in SmS, Cav1B, MACO-1A, MACO-1B, MACO-2A, MACO-2B and MACO-3 RNAi worms compared to the polarity worms without PZQ showed in A. Blue arrows indicate the additional heads instead of normal tails in bipolar phenotypes. B. quantification of bipolarity penetrance of regenerated worms in each cohort under exposure of 75uM PZQ. Number under each cohort indicates the independent experimental trials, black short line shows the average percentage of each cohort. The asterisks above data indicate significant changes of experimental cohort compared to SmS, two stars stand for P< 0.01 and one star stands for P < 0.05.

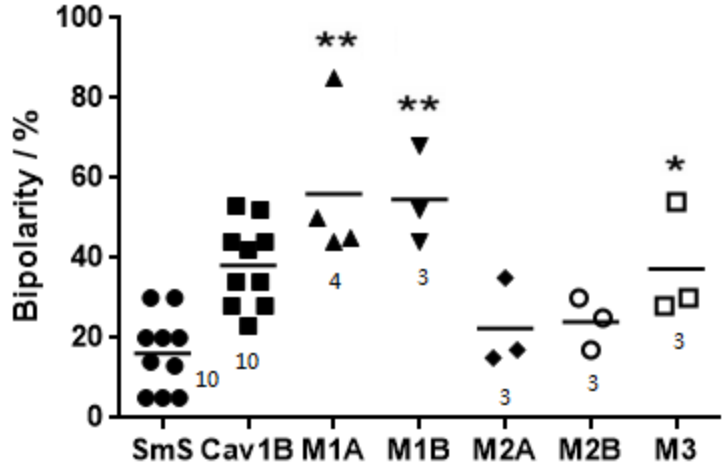


Figure 2.8 Bipolarity under lower dose (50uM) treatment of PZQ
A. Regeneration happened in 75uM PZQ; B. Regeneration happened in 50uM PZQ. Number under each cohort indicates the independent experimental trials, black short line shows the average percentage of each cohort. The asterisks above data indicate significant changes of experimental cohort compared to SmS, two stars stand for P< 0.01 and one star stands for P < 0.05.

In addition to changes in PZQ-evoked bipolarity, some of the RNAi worms also showed movement defects, just as seen in worms caused by Ca_v1B RNAi. To quantify this effect, I took videos to record the tracks of worms for each RNAi cohort. The z-stack images by maximum intensity projection of ImageJ are shown in **Figure 2.9A** to present the overlay stacks of worm tracks. The quantification of average moving distance of worms for each cohort is shown in **Figure 2.9B**. It shows that among experimental worms, MACO-1A. MACO-1B and MACO-3 RNAi worms exhibited a movement defect by the inhibition of *D. japonica* gene, MACO-1 and MACO-3, respectively. The average moving distance of MACO-1A and MACO-3 RNAi group is lowered by 50% compared to that of SmS RNAi group, while the rate of MACO-1B is 60% slower than negative control. The inhibition of MACO-2 gene in *D. japonica* had no influence on the movement of worms, based on the fact that both of the MACO-2A and MACO-2B RNAi worms didn't show defected motility compared to SmS. The results showed that the proteins which potentiate the effect of PZQ also could cause movement defects on the RNAi worms.

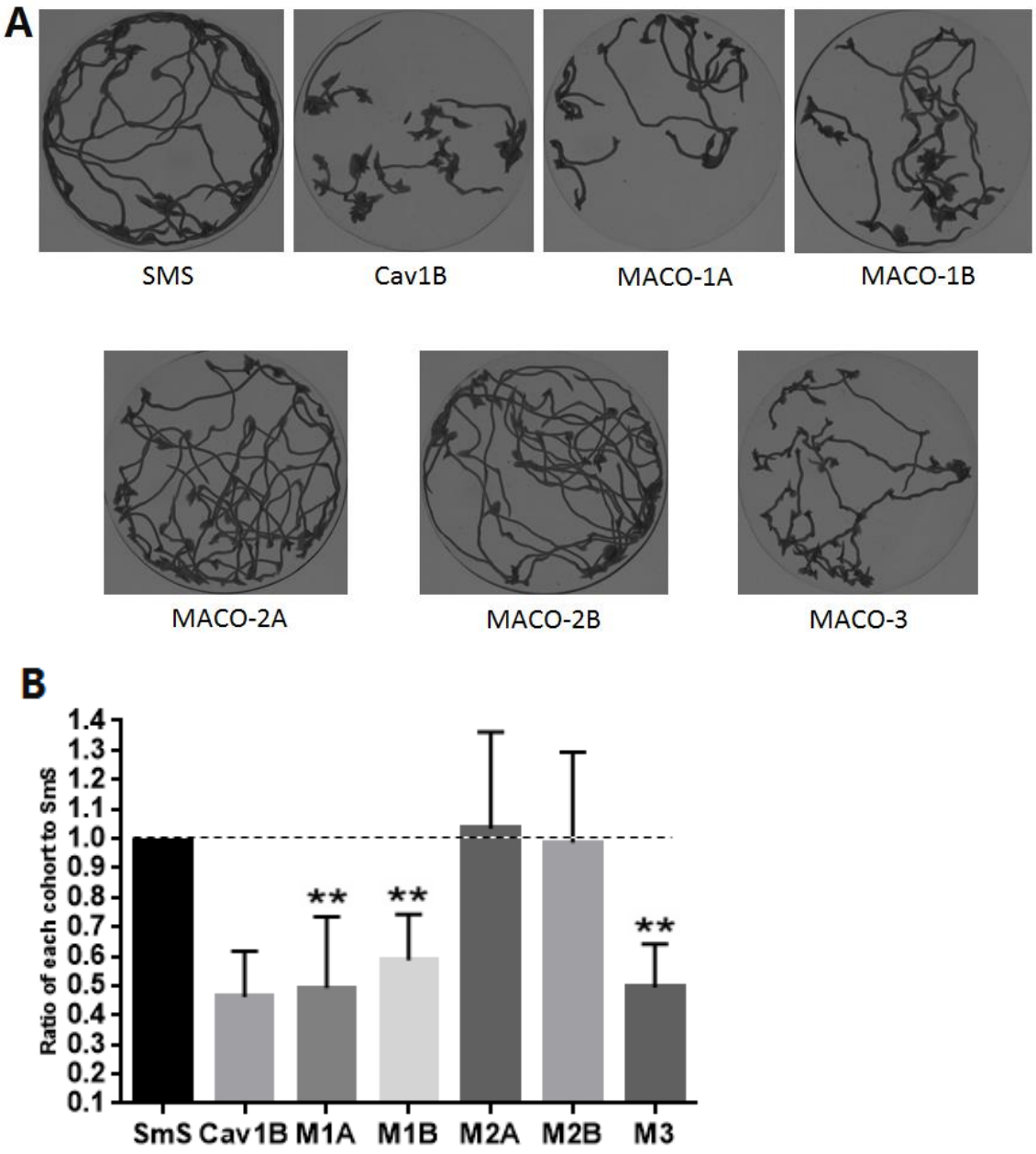


Figure 2.9 Movement tracking of SmS (negative control), Cav1B (positive control) and experimental groups (MACO-1A, MACO-1B, MACO-2A, MACO-2B, MACO-3) RNAi worms
A. Z-projection of RNAi worm moving tracks. Each cohort contains 10 RNAi worms and each experiment was repeated three times (n=3). Each video is taken over 2 minutes B. Quantification of average worm-moving distance, y-axis shows the percentage moving-distance compared to SmS RNAi worms, the horizontal dash line shows the level of SmS RNAi worms; the asterisks above data indicate significant changes of experimental cohort compared to SmS RNAi worms, two stars stand for $P < 0.01$.

RT-PCR results

To make sure that the aforesaid phenotypes caused by macoilin-related genes RNAi was actually due to the knockdown of macoilin-related genes, I carried out RT-PCR experiments to assess whether macoilin RNA levels changed following macoilin RNAi. The results showed a decrease of mRNA of MACO-1 compared to the negative control (SmS), happened in the MACO-1 RNAi worms fed with either MACO-1A or MACO-1B constructs (see **Figure 2.10**).

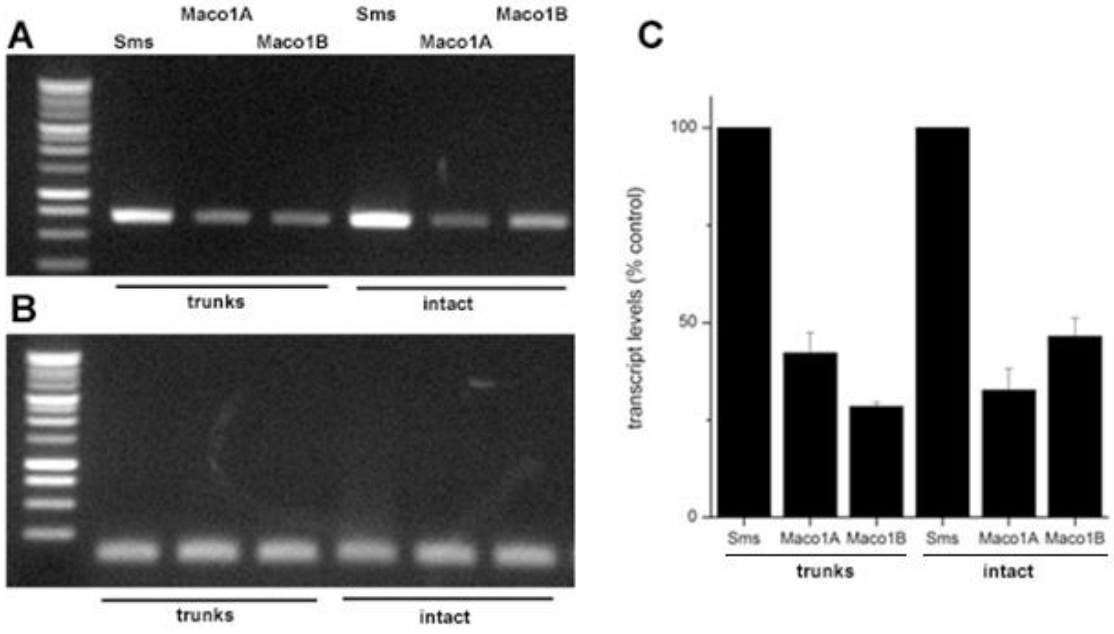


Figure 2.10 RT-PCR results of macoilin RNAi experiments
A-B. RT-PCR gel results. A. Amplification by MACO-1 primers. B. Amplification by β actin primers, control group. Different lanes represent different RNA extractions from different RNAi worms. Trunks: regenerative worms without head and tail. Intact: Intact worms without cutting. C. Quantification results of panel A.

Materials and Methods

Neuron imaging:

Primary rat embryonic cultures of hippocampal neurons were grown in Neurobasal medium at 37°C in an atmosphere of CO2 to maintain a density of 6000 cells/35mm Petri dish. Then the cell line was modified by transfecting a human macoilin protein encoded plasmid, an ER marker encoded plasmid or both (1:1) following Calcium Phosphate Transfection protocol[19]. Human macoilin protein encoded plasmid, obtained from Genscript, was cloned in pEGFP N1 vector. After a 48-hour incubation in 37°C incubator the cells was picked up for imaging by inverted confocal microscope. The objective lens needed to be moistened by 60x oil in advance. The laser power is as follows: co-transfection: 1.0W for green light and 4.0W for blue light; single-transfection: 1.0W for green light and 10.0W for blue light.

Genetics:

A blast of search based on the human macoilin protein was launched on Galaxy tBLASTn program, aiming at finding homologs in planarian. One Homo sapiens macoilin protein sequence (GenBank ID: 59897093) is submitted as the template in the search of the transcriptome of *Dugesia japonica* genome. This work identified three isoforms on three different scaffolds: MACO1 on comp143153_c0_seq4, MACO2 on comp140642_c1_seq8 and MACO3 on comp143153_c0_seq7 with the MACO-1 sequence predicted to be the most homologous with the tested Homo sapiens macoilin protein sequence.

PCR methods

PCR protocol of amplification from cDNA library was based on the Takara LA Taq HS protocol by Takara Bio Inc. Amplification of the insert for pDONRdT7 vector was carried out following Advantage HD polymerase protocol. Primers designed for these two PCR experiments are illustrated in **Table 2A and 2B**. The PCR condition is listed in **Table 2C**. To identify the sizes of PCR products, 1% agarose gel electrophoresis was carried out. Gel powder was purchased from Sigma. The uniform formula of 1% gel is 0.5g agarose gel/50ml 1xTAE buffer, while 1xTAE buffer formula is the common one by internet. 1kb DNA ladder from Promega was used as control, and Gel Purple dye from Biolab was used as the DNA loading dye. The condition of 50 voltage 40 minutes (sometimes an additional 10-minute running time under 100 voltage is needed) was used during electrophoresis. An alternative condition of 100 voltage 30 minutes was used in a quick protocol. DNA sequencing techniques were used to verify the sequences with the M13 forward primer (-20) as the sequencing primer.

A

Name	Primer Sequence	Tm (°C)	Gene target	Domain target	Predicted size (bp)
1A_F	TTGCTCTGTATCTCCGATTGCC	60.48	MACO-1A	Transmembrane domain	709
1A_R	AATACAAAATCTTTACGAAGGTCGC	58.46			
1B_F	AGCGCATTAACTTCCTTCAGTG	59.26	MACO-1B	Coiled coil domain	711
1B_R	AAGCAGTTCAGAAAAAGTCAGCA	59.3			
2A_F	GGTTGAATGACGACCACTACCT	60.03	MACO-2A	Coiled coil domain	703
2A_R	GCCACCAACCCAAACGAAAT	59.61			
2B_F	TTAGCACCGCCATTGGTAAGA	59.72	MACO-2B	Transmembrane domain	806
2B_R	TGGTCATGTTGGTTAATGTGTAGAT	58.52	MACO-2B		
3_F	CACAGGAGGCCATCATCGAA	59.82	MACO-3	Coiled coil domain	608
3_R	TGGTAAGCGTTACATAAATAACCAA	57.08	MACO-3		

B

Primers name	Tm (°C)	sequences
4F	60.90	GGCCATTACGGCCGGCCGCGGGAATTCGATT
4R	58.43	GGCCGAGGCGGCCGCCGCGAATTCACTAGTGAT
5F	58.28	AAACAGTTCGGTAGCATCCG
5R	59.53	AGGACAGAAACCTGGTGTGG

C

Protocol	LA Taq	Adv HD
Cycles	98°C for 1min 98°C for 10s 56°C for 15s 72°C for 1min (30 cycles) 72°C for 10 min	98°C for 1min 98°C for 10s 56.5°C for 5s 72°C for 1min (35 cycles) 72°C for 10 min

Table 2 Primer sequences and conditions used for PCR
A. Primer sequence used for amplifying genes from planarian macoilin cDNA library B. Primers for amplifying gene of interest from pGEMT-EZ and vector from pDONRdT7-containing plasmid. 4F/4R is the primer pair of macoilin-containing genes, 5F/5R is the primer pair for vector prep, and red highlighted parts indicate the position of SfiI digestion sites. C. PCR conditions used in the amplification of target genes from plasmids.

Gene cloning

PCR products from cDNA library was firstly ligated into pGEMT-Easy vector system by Promega and then transformed into Stellar Competent Cells from Clontech accordingly to its commercial protocol. After the 16 hours incubation for colonies growing on LB agar plate under 37°C, Miniprep (PureLink® Quick Plasmid Miniprep Kit) was done for extraction of plasmid from each single colony. Each plate contained at least 100 colonies and 6 colonies were chosen from them to screen the target plasmid. Restriction endonuclease EcoRI digestion (NEB) was performed for one hour in 1xCutsmart buffer

after miniprep. And then each colony extraction was loaded onto each well of 1% agarose gel to examine the size of fragments. The materials used in gel electrophoresis was the same as the method used in "PCR methods". Gel confirmed the general size of insert and DNA sequencing system by genomic center of UMN confirmed the sequence of insert.

The reason to use pGEMT-EZ vector firstly but not pDONRdT7 vector is due to the low efficiency of cDNA PCR experiments. The rather low amount of copies of interested genes from cDNA PCR need to be ligated into a highly efficient vector system firstly to create a pure and stable cloning system and then re-ligated to another functionalized vector (pDONRdT7) following a sticky ends ligation protocol, or T4 ligase reaction, by NEB. The cloning strategy was based on SfiI digestion enzyme system (NEB). The primers contains Sfil digestion sites to the double ends of PCR products. Then the insert and vector, the pDONRdT7 which contains two SfiI digestion sites, were digested by SfiI prior to ligation to create two easily sticky ends flanking the multiple function regions, respectively. The ligation reaction was then transformed into HT115 Competent Cells which are lacking of RNAse and plated onto LB agar plates for overnight. Miniprep by cloned tech was done for plasmids screening. One more SfiI digestion followed by agarose gel electrophoresis was carried out to examine the size of inserts. Gene sequencing confirmed the sequence of each plasmids.

RNAi methods:

In this part, I used a modified RNAi feeding protocol to do gene interference experiments (RNAi) on the worms[18, 20]. Time line for this RNAi feeding protocol is illustrated in **Figure 3.1**. The regenerated worms from the second cutting (X) was split into two groups,

one without PZQ exposure was recorded of their movement patterns, while the other with PZQ exposure was scored to identify the phenotypes by the inhibition of macoilin protein. Sample of scored worms were used to evaluate the changes of mRNA level caused by the inhibition of protein by RNAi (qPCR).

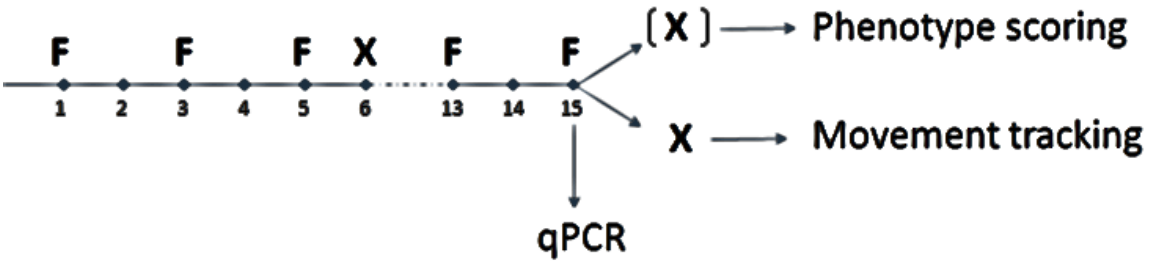


Figure 3.1 Time line of feeding protocol for planarian RNAi experiments
F stands for one single feeding, X stands for one single cutting which showed in Introduction Part, (X) stands for one single cutting and then exposure to Pzq (Praziquental, 75uM/50uM))

The RNAi feeding strategy used food (chicken liver) as an intermedia to deliver interested genes into worms. The target plasmid-carrying bacteria was inoculated one night prior to the experimenting day to create large copies of cells. The dsRNA expression was then induced by IPTG with 2-hour incubation and shaking under 37°C. Next the OD value of each cohort was checked for quantification, the extra IPTG was eluted by repeatedly centrifuging down the pellets of cells and resuspension by 2xYT medium solution. In order to reduce the difference between each cohort, I used the following quantification methods in all my RNAi experiments: Centrifuge the cell culture, decant most of the supernatant and leave 5ml of medium. Add 5/3 ml 80% glycerol (for cryogenic reservation) to reach a final concentration of 30% and a final volume of 6.67ml (5ml + 5/3ml) and re-suspended the pellet. The OD of this cell culture was assumed as

1.00, and the cell culture was dispensed into 10 EP tubes with 667μl/tube. If the OD value of a new cell culture equals to A, then the final volume of this new cell culture per EP tube is 667*1.00/A, unit: μl.

The quantified dsRNA-expressed HT115 cells then were mixed with 150 μl homogenized chicken liver (worm food) and a few microliters red food coloring (to visualize ingestion of food), and then were fed to worms. For feeding, the tubs of experimental worms were briefly rotated for keeping worms in the center of each tub. And the mixed RNAi smoothie then was pipetted around the cluster of worms. A single feeding step took around one hour before changing the medium of worms to spring water. An intact feeding mode involved 5 single feeding steps and 2 cutting steps, which is FFFxFFx**.** The secondary cut worms were exposure to varied concentration of PZQ (75uM/50uM/0uM) for 48 hours and then washed out by pure spring water for regeneration. The regeneration often took 6-8 days after cutting.

Movement tracking

For each cohort, 10 worms were picked from the RNAi worms tub. Three videos were taken for each group of 10 worms with a duration of 2 minutes per film. Image J were used to generate the Z-stack graphs for each group of 10 worms by Z project. Next a homemade matlab code was used to track the motion trails of worms to get an average moving distance in unit time and compared this number of experimental group to that of SmS worms.

RT-PCR

Prior to the exposure of PZQ, I took 25 worms from each experimental cohort to make up TRIzol reagent (Thermo Fish Scientific) and got the RNA extraction from TRIzol. The RNA then were digested by DNase I (room temperature, no more than 15 minutes to keep avoid of digesting RNA) to remove the remaining DNA. The mixture was converted to cDNA at the presence of reverse transcriptase (RT) following the First-strand Superscript Synthesis Kit by Invitrogen. 0.7μg of each cDNA sample was used in PCR to quantify the changes of mRNA in each experiment cohort and to visualize the influence of RNAi on worms' mRNA.

Conclusions

Up to date, little information about macoilin protein is known. Here I characterized the function of macoilin proteins in planarian *Dugesia japonica* based on the previous characterized human macoilin protein sequence and studied their effects on regeneration of planarian and worm's motility, and thus found that the similar influence on praziquantel-induced bipolarity and motility of worms compared to voltage-gated calcium channel (Ca_v). This indicate a synergy between macoilin protein and Ca_v1B function in the planarian system.

Human macoilin protein was said to localize to the nuclear membrane in non-neuronal cells (PTK19), and was identified and characterized as a non-nuclear protein in mice primary neurons. Besides a co-localization assay with ER marker suggested a possible co-localization of macoilin protein and the endoplasmic reticulum. Experiments with *C. elegans* macoilin protein corroborated this relationship between macoilin and endoplasmic reticulum[13].

Next, I tried to figure out the exact functions of macoilin in planarian by inhibiting several mRNAs encoding macoilin in planarian using modified RNAi plasmids that I constructed. The results of knockdown of three macoilin genes which I identified in planarian implies that macoilin may be involved in Ca_v signaling pathway in these worms. MACO-1 has the most similar structures with human macoilin protein since it has transmembrane domains and coiled coil domains at each terminus and a relative conserved sequence compared with other macoilin protein sequences from multiple species, thus it is believed to be a homologue of human macoilin in *D. japonica*.

Experimental data illustrated the activity of MACO-1 which not only potentiates the bipolar activity of PZQ on regeneration of worms but also regulates planarian motility.

MACO-3, though functionally alike to MACO-1 in RNAi assays, is possibly not an isoform but just part of MACO-1, which means the inhibition of MACO-3 by MACO-3 plasmids not only block MACO-3 mRNA but also MACO-1 mRNA in RNAi worms. MACO-2, due to the fact that the RNAi of MACO-2 having no effect on PZQ-induced polarity and motility of worms, is not likely to be a functionally relevant isoform of macoilin protein in the planarian *D. japonica*. Further experiments would be required to understand the relationship between these sequences.

Part of the function of macoilin protein has been studied now and results provide a strong indication that macoilin plays a leading role in the regulation of regeneration of planarian. However, the exact mechanism involved in this regulation, like where in the worm it is localized, through which other effectors the functions are mediated and the relationship between Ca_v1B, calcium concentration and macoilin, is still a puzzle. I postulate that macoilin regulates the function of planarian neurons. The aforesaid hypothesis results from RNAi experiments that show that knockdown of macoilin affects the motility of worms, resulting in movement-defective worms after regeneration. Also, the imaging results of macoilin has shown that human macoilin protein possibly resides on the endoplasmic reticulum in primary mice neurons cells. So a postulation that the planarian macoilin is located on planarian neurons is reasonable. However, the research did not take steps to prove whether macoilin resides on the network structure of endoplasmic reticulum in neurons of planarian and if it is indeed a membrane-spanning protein. An *in*

situ hybridization, which can clearly provide information on the localization of macoilins in planarian, and a protein extraction experiment combined with western blotting could further investigate this issue.

PZQ induces cell death in schistosome and thus can treat schistosomiasis. The mechanism, though not explored clearly, is probably mediated by disrupting the balance of Ca2+ level in the worm's cells and more specifically, by regulating voltage-gated calcium channel (VGCC)[10]. And, in planarian, PZQ exposure can result in bipolarity phenotype after worm regeneration. However, whether the RNAi of macoilin can increase cellular calcium concentration is still unknown. This research can draw the attention to PZQ influences on planarian through the similar mechanism as that in schistosomes, by changing the Ca^{2+} level in planarian. As RNAi of macoilin with exposure to PZQ can potentiate the effect of PZQ by raising the penetrance of bipolar phenotype, this suggests a similar mode of action. However further experiments need to be done, such as by carrying out Ca^{2+} imaging experiments, to bring fresh data to this question.

Further, based on the fact that PZQ may possibly regulate VGCC in schistosomes and the experimental results that macoilin plays a synergic role with Ca_v1B (one subtype of VGCC in planarian) on PZQ-induced bipolarity effects in planarian, there is probably an interaction between VGCC and macoilin protein in planarian regarding their pharmacological mechanisms, and more likely, these two proteins can function in the same signaling pathway involved in the regeneration of worms. A proposed model is illustrated in **Figure 4.1**. In order to prove the hypothesis and determine the upstream and

downstream proteins functioning with Ca_v1B and macoilin, separate quantitative analysis of macoilin mRNA and Ca_v1B mRNA by qPCR can be carried out. Due to VGCC located on cell membrane and macoilin probably showed in ER, thus it is appropriate to propose the supposal that macoilin modulates the activity of Ca_v1B by facilitating the maturation of Ca_v1B in ER.

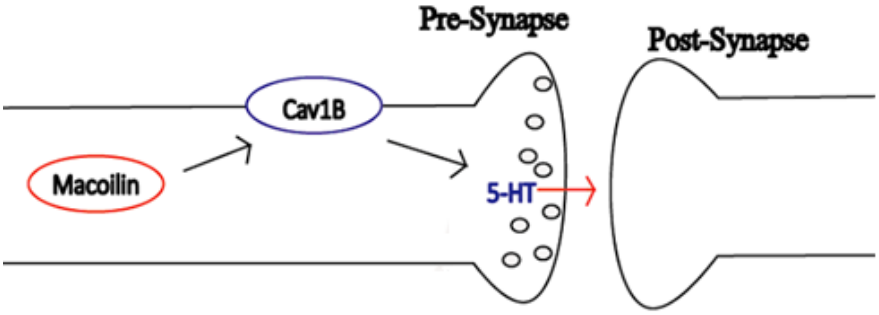


Figure 4.1 a proposed model for the mechanism of macoilin in regulating planarian activities

Furthermore, the role of 5-HT in macoilin-RNAi-induced movement defects is still required to be thoroughly studied. The macoilin protein is believed functioning in pre-synapse neurons in *C. elegans*[13, 21], so it has highly possibility to regulate the activities of neurons by modulating neurotransmitters. Previous study showed a closed relationship between praziquantel and serotonin: specifically that the effect of praziquantel could be potentiated or deteriorated by 5-HT regulator[4]. Therefore based on the regulation of macoilin on praziquantel, further experiments needed to be done to test if an inhibition of macoilin by RNAi can lead to a down-regulation of 5-HT and if a 5-HT shot can rescue the motion defects caused by macoilin RNAi.

Finally, the macoilin protein is believed functioning in pre-synaptic neurons in *C. elegans*[13, 21], but the location of macoilin in planarian nervous system is still unknown. To

completely unmask the mysteries of macoilin, this thesis is just a start and large amounts of studies are still needed to be done, but there is no doubt that we are on the right way, to unlock the mysterious function of invertebrate macoilins.

References

1. Alvarado AS. Q&A: What Is Regeneration, And Why Look to Planarians for Answers? BMC Biology 2012; 10: 88. doi:10.1186/1741-7007-10-88.
2. Eisenhoffer GT, Kang H, Alvarado AS. Molecular Analysis of Stem Cells And Their Descendants during Cell Turnover And Regeneration in the Planarian Schmidtea mediterranea. Cell stem cell 2008; 3(3):327-339.
3. Lobo D, Beane WS, Levin M. Modeling Planarian Regeneration: A Primer for Reverse-Engineering the Worm. Gojobori T, ed. PLoS Computational Biology 2012; 8(4):e1002481. doi:10.1371/journal.pcbi.1002481.
4. Reuter H, März M, Vogg MC, Eccles D, Grífol-Boldú L, Wehner D, Owlarn S, Adell T, Weidinger G, Bartscherer K. B-catenin-dependent Control of Position Information along the AP Body Axis in Planarians Involves A Teashirt Family Member. Cell Rep. 2015 Jan 13; 10(2):253-65.
5. Chan JD, Agbedanu PN et. al. 'Death and Axes': Unexpected Ca^{2+} Entry Phenologs Predict New Anti-schistosomal Agents. PLoS Pathog 2014 10(2): e1003942
6. Nogi T, Zhang D, Chan JD, Marchant JS. A Novel Biological Activity of Praziquantel Requiring Voltage-Operated Ca2+ Channel β Subunits: Subversion of Flatworm Regenerative Polarity. PLoS Negl Trop Dis 3(6): e464.
7. Greenberg RM. Are Ca^{2+} Channels Targets of Praziquantel Action? International Journal for Parasitology 2005 35 1–9
8. Wolde Mussie E, Vande Waa J, Pax RA, Fetterer R, Bennett JL. *Schistosoma mansoni*: Calcium Efflux And Effects of Calcium-free Media on Responses of The Adult Male Musculature to Praziquantel And Other Agents Inducing Contraction. Experimental Parasitology 1982 53: 270-278
9. Kohn AB, Anderson PAV et. al. Specific Sites in The Beta Interaction Domain of A Schistosome Ca^{2+} channel β Subunit Are Key to Its Role in Sensitivity to The Anti-schistosomal Drug Praziquantel. Parasitology 2013 127:349-356
10. Kohn AB, Roberts-Misterly JM, et. al. Schistosome Calcium Channel β Subunits. Unusual Modulatory Effects And Potential Role in The Action of The

Antischistosomal Drug Praziquantel. Journal of Biological Chemistry 2001 40: 36873-36876

11. Zhang D, Chan JD et. al. Opposing Roles of Voltage-gated Ca2+ Channels in Neuronal Control of Regenerative Patterning. J Neurosci. 2011 Nov 2; 31(44): 15983–15995.
12. Robert M. Greenberg. Ca2+ Signaling, Voltage-gated Ca2+ Channels, and Praziquantel in Flatworm Neuromusculature. Parasitology. 2005 131 Suppl: S97-108.
13. Colins JJ III, Newmark PA. It's No Fluke: The Planarian As A Model for Understanding Schistosomes. PLoS Pathog 2013 9(7): e1003396.
14. Arellano-Carbajal F, Briseño-Roa L, Couto A, Cheung BHH, Labouesse M, de Bono M. Macoilin, a Conserved Nervous System–Specific ER Membrane Protein That Regulates Neuronal Excitability. PLoS Genet 2011 7(3): e1001341.
15. Kuvbachieva A, et. al. Identification of A Novel Brain-specific and Reelin-regulated Gene That Encodes A Protein Colocalized with Synapsin. European Journal of Neuroscience 2004 Vol. 20, pp. 603–610, 2004.
16. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting Transmembrane Protein Topology with A Hidden Markov Model: Application to Complete Genomes. Journal of Molecular Biology 2001 305: 567–580.
17. Lupas A, Van Dyke M, Stock J. Predicting Coiled Coils from Protein Sequences. Science 1991 252: 1162-1164.
18. Newmark PA, Reddien PW, Cebrià F, Sánchez Alvarado A. Ingestion of Bacterially Expressed Double-stranded RNA Inhibits Gene Expression in Planarians. Proc Natl Acad Sci U S A. 2003 100 Suppl 1:11861-5.
19. Giordano G, Costa LG. Primary Neurons in Culture and Neuronal Cell Lines for *in vitro* Neurotoxicological Studies. Methods Mol Biol. 2001 758: 13-27.
20. Chan JD, Marchant JS. Pharmacological and Functional genetic assays to manipulate regeneration of the planarian *Dugesia japonica*. Journal of Visualized Experiments 2011 54; e3058: 1-4
21. Miyara A, et al. Novel and conserved protein macoilin is required for diverse neuronal functions in *Caenorhabditis elegans*. PLoS Genet. 2001 7(5): e1001384.

22. Baguñà J, Saló E, Auladell C. Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. Development 1989 107: 77-86.
23. King RS, Newmark PA. The cell biology of regeneration. The Journal of Cell Biology 2012 196(5):553-562. doi:10.1083/jcb.201105099.
24. Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Alvarado AS. Identification of Genes Needed for Regeneration, Stem Cell Function, and Tissue Homeostasis by Systematic Gene Perturbation in Planarian. Developmental Cell 2005 Vol.8, 635-649
25. Machaka K. Ca^{2+} Signaling, Genes and Cell Cycle. Cell Calcium. 2011 49(5):323-30.
26. Kondratskyi A, Kondratska K, Skryma R, Prevarskaya N. Ion Channels in The Regulation of Apoptosis. Biochim Biophys Acta. 2015 1848(10 Pt B):2532-46.
27. Sutherland DJ, Pujic Z, Goodhill GJ. Calcium Signaling in Axon Guidance. Trends Neurosci. 2014 37 (8):424-32.
28. Zamponi GW, Striessnig J, Koschak A, Dolphin AC. The Physiology, Pathology, and Pharmacology of Voltage-Gated Calcium Channels and Their Future Therapeutic Potential. Pharmacol Rev. 2015 67 (4):821-70.
29. Minor DL Jr, Findeisen F. Progress in the structural understanding of voltage-gated calcium channel (Ca_V) function and modulation. Channels (Austin). 2010 4(6): 459-74.
30. Eberl DF, Ren D, Feng G, Lorenz LJ, Van Vactor D, Hall LM. Genetic and Developmental Characterization of Dmca1D, A Calcium Channel α1 Subunit Gene in Drosophila melanogaster. Genetics 1998 148:1159 -1169.
31. Jeziorski MC, Greenberg RM, Clark KS, Anderson PA. Cloning And Functional Expression of A Voltage-gated Calcium Channel Alpha1 Subunit from Jellyfish. J. Biol. Chem. 1998 273: 22792-22799.