Utjecaj proteina TRNP1 na proliferaciju primarne kulture stanica korteksa iz E14 miša

Foglar, Nika

University of Zagreb

Faculty of Science

Department of Biology

Nika Foglar

Effect of Trnp1 orthologs on proliferation of E14 mouse cortical primary culture

Graduation Thesis

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Graduation thesis

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Nika Foglar

Roosvelt square 6, 10000 Zagreb, Croatia

Through the evolution of mammals, the brain size has increased by folding the neocortex. The cause for differences in folding between species could be different regulation of some genes. Trnp1 gene overexpression causes self-renewal of neuronal stem cells *in vitro*, while *in vivo* knockdown leads to folding of the cortex in mice. Rate of evolutionary mutation was calculated for Trnp1 orthologs, and correlation between these rates and degree of gyrification was noticed. To determine if evolutionary mutations of Trnp1 have different effect on the self-renewal capacity of cortical primary culture cells, six Trnp1 orthologs were overexpressed in these cells. The six species were *Mus musculus, Otolemur garnettii, Macaca mulatta, Mustela putorius, Homo sapiens* and *Tursiops truncates*. Analysis showed that all Trnp1 orthologs increase proliferation, but the effect of each ortholog was similar. Therefore, it is unlikely that evolutionary mutations of Trnp1 caused the differences in folding between these species.

(34 pages, 18 figures, 3 tables, 25 references, original in: English)

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Supervisor: Dr sc. Miriam Esgleas

Cosupervisor: Dr. sc. Inga Marijanović, Asst. Prof.

Reviewers:

1) Dr. sc. Inga Marijanović, Asst. Prof.

2) Dr. sc. Martina Šeruga Musić, Asst. Prof.

3) Dr. sc. Jasna Lajtner, Asst. Prof.

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Utjecaj proteina TRNP1 na proliferaciju primarne kulture stanica korteksa iz E14 miša

Nika Foglar

Rooseveltov trg 6, 10000 Zagreb, Hrvatska

Kroz evoluciju sisavaca, veličina mozga se povećavala stvaranjem nabora u mozgu. Uzrok različite naboranosti mozga između sisavaca mogao bi biti različita regulacija nekih gena. Prekomjerna ekspresija gena Trnp1 uzrokuje samoobnavljanje živčanih matičnih stanica *in vitro*, dok *in vivo* smanjenje ekspresije uzrokuje stvaranje nabora u korteksu miša. Brzina evolucijskih mutacija je izračunata za Trnp1 ortologe te je primijećena korelacija između te brzine i stupnja naboranosti mozga. Kako bi se odredilo imaju li evolucijske mutacije gena Trnp1 različiti efekt na sposobnost samoobnavljanja stanica primarne kulture korteksa, šest ortologa Trnp1 je eksprimirano u stanicama. Odabrane vrste su bile *Mus musculus, Otolemur garnettii, Macaca mulatta, Mustela putorius, Homo sapiens* and *Tursiops truncates*. Analiza je pokazala da svi ortolozi Trnp1 povećavaju sposobnost proliferacije, ali su ortolozi imali podjednak učinak. Stoga je malo vjerojatno da su evolucijske mutacije gena Trnp1 uzrokovale razlike u stupnju naboranosti mozga između tih vrsta.

(34 stranica, 17 slika, 3 tablica, 25 literaturnih navoda, jezik izvornika: engleski)

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Voditelj: Dr. sc. Miriam Esgleas

Suvoditelj: Dr. sc. Inga Marijanović, izv. Prof.

Ocjenitelji:

1) Dr. sc. Inga Marijanović, izv. prof.

2) Dr. sc. Martina Šeruga Musić, izv. prof.

3) Dr. sc. Jasna Lajtner, izv. prof.

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Contents

1. Introduction

1.1. Evolution and development of mammalian brain and folding

Vast changes in size of amniote brain that occurred throughout evolution can be contributed to development of the dorsal telencephalon. It was the evolution of neocortex that defined all mammals and increased the brain size and complexity. The neocortex is a sheet of six horizontal layers, with each layer consisting of specific neuronal subtypes (Lui et al., 2011). Although this basic structure can be seen in all mammals, neocortical size, shape and organization continued to evolve, becoming very variable in mammals (Cárdenas and Borrell, 2019). As mammals evolved, the need for an increased number of neurons was satisfied by folding the neocortex. These folds, consisting of gyrus and sulcus, expanded the surface of the neocortex within the limits of the skull (Kelava et al., 2013). But within the same mammalian superorder, species with both smooth, or lissencephalic, and folded, or gyrencephalic brain can be found (Lui et al., 2011). If the classical view of neocortex evolution was correct, it would mean that the early mammals had smaller, smooth brains which gradually grew and evolved into folded brains. This would require independent evolution of gyrencephaly in all mammalian orders. Therefore, it is more probable that the first mammal was gyrencephalic, and some lineages became lissencephalic through secondary loss of gyrencephaly (Kelava et al., 2013).

To understand the formation of neocortical folding, we must look into the embryonic development and its mechanisms (Figure 1). First step that influences the size of the brain is proliferation of neuroepithelial cells (NEC), the first progenitor cells of the cerebral cortex. Their number directly influences the final amount of other progenitor cells and can be increased by delaying neurogenesis, which was noticed when comparing rodents and primates (Fernández et al., 2016). Neurogenesis begins with the loss of epithelial features in NEC and their transformation into apical radial glia cells (aRGC). Some acquired features of these cells are expression of Pax6, Vimetin and positioning their body in the ventricular zone (VZ) (Götz and Huttner, 2005). With their cell fate restricted, these cells can divide in four different ways. The proliferative division can be symmetric, generating two aRGC, or asymmetric, generating one aRGC and a different type of progenitor. Similarly, in neurogenic division, asymmetric division gives one aRGC and a neuron, and symmetric produces two neurons (Taverna et al., 2014). One

type of progenitor cells generated by asymmetric proliferative division of aRGC is intermediate progenitor cells (IPC). These multipolar cells are located basal of the VZ, forming a secondary germinal layer, the subventricular zone (SVZ) (Borrell and Götz, 2014). In lissencephalic animals, such as mouse, most IPCs undergo terminal division, producing two cortical neurons. In gyrencephalic animals, these cells can self-amplify, increasing their numbers as well as producing neurons (Pontious et al., 2007). Another type of progenitor cell that can be found in the SVZ is basal radial glia cell (bRGC). It is present in scarce amounts in the embryonic mouse cortex, while being abundant in gyrencephalic species. These cells are highly neurogenic in mouse, but cannot self-amplify (Fernández et al., 2016). Self-renewing potential is found in bRGCs and IPCs from gyrencephalic mammals. This enables their accumulation, which causes enlargement of the SVZ and its division into inner and outer SVZ (Cárdenas and Borrell, 2019). The accumulation also causes the increase in the final number of neurons and in the number of radial scaffolds composed of bRGC's processes attached to the basal surface (Cárdenas and Borrell, 2019).

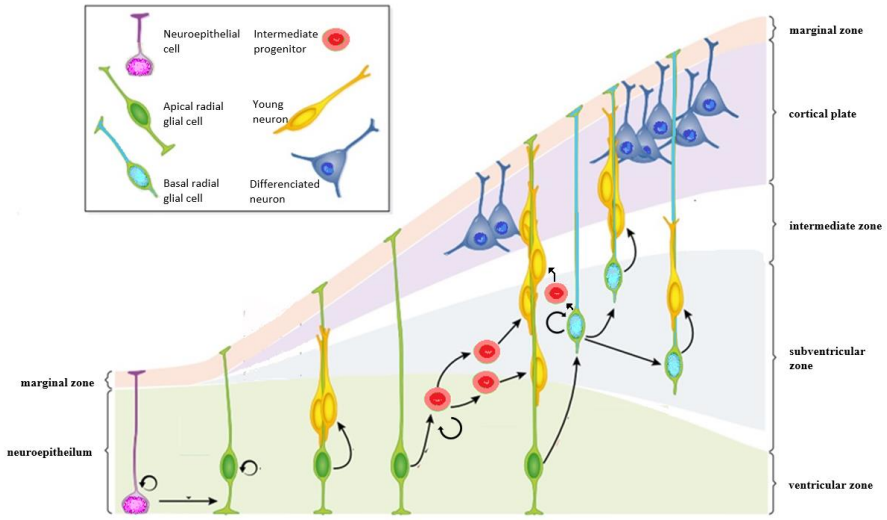


Figure 1. Development of the neocortex with depicted main germinal layers and cell types. All progenitors in the SVZ have self-renewing potential in gyrencephalic mammals, as well as the ability to produce any other type of bRGC or IPC. This enables the growth of SVZ not seen in lissencephalic mammals. Adapted from V. Fernández et al., (2016.)

There are several hypotheses trying to explain the evolution of gyrencephaly. According to the radial unit hypothesis, extending the proliferation time of NEC and delaying neurogenesis would exponentially increase the number of NEC and subsequently aRGCs (Rakic, 2009). This hypothesis explains the dramatic increase in the number of progenitor cells in gyrencephalic brains, but when tested on transgenic mice, the increased number of NEC led to folding in VZ, a feature that cannot be found in gyrencephalic brains (Borrell and Götz, 2014). Another hypothesis, the Intermediate Progenitor hypothesis, suggested that the number of neurons and location of folds are determined by number and non-homogenous distribution of IPCs in the developing brain. But experiments showed that increase in IPCs in mice does not lead to folding despite the increase in neuron numbers (Borrell and Götz, 2014). The major issue they found was insufficient number of scaffolds for the increased number of migrating neurons. These neurons were unable to separate laterally and increase the surface area, thus creating thicker cortical layers (Fernández et al., 2016). Therefore, increasing the number of bRGCs, whose processes can serve as radial scaffolds, could expand the cortical surface (Lewitus et al., 2013). This is proposed in the Tangential Divergence hypothesis, which states that the scaffold can be expanded at the basal surface by addition of radial fibers from proliferating bRGCs in the OSVZ (Figure 2.) (Borrell and Götz, 2014). This modifies the trajectory of migrating neurons, enabling the lateral expansion across the expanding basal surface (Lewitus et al., 2013). When comparing species with smooth and folded cortices, a difference in number of bRGCs can be noticed. As predicted by the hypothesis, species with more fissures and folds have a higher number of bRGCs (Borrell and Götz, 2014).

1.2. Molecular mechanisms regulating cerebral cortex expansion and folding

The proliferative capacity of neural stem cells depends on both intrinsic factors, such as certain transcription factors, and extrinsic factors from, for example, the extracellular matrix (Fernández et al., 2016). Before neurogenesis, self-amplification of NEC is promoted by ß-catenin pathway among others. Once NECs start to express FGF10, they undergo differentiation into aRGCs. When activated in aRGC, FGF pathway inhibits neurogenesis and shortens the G1 phase, promoting self-renewal and cortical growth (Rash et al., 2011). Another pathway with similar function is Notch pathway. It also promotes NEC to aRGC transition and later inhibits neurogenesis and production of IPC (Fernández et al., 2016). Some other well-known pathways

are Wnt, and BMP. Wnt is a time regulated pathway, in early stages it promotes aRGC self-renewal, and in later, production of IPCs together with Shh and FGF2 (Viti et al., 2003). BMP first induces neurogenesis while later it promotes astrocyte differentiation (Fernández et al., 2016). As all of these pathways are conserved and important in many functions, probably other proteins are responsible of the differences between lissencephalic and gyrencephalic brains. The discovery of Trnp1 protein expressed exclusively in mammals, paved a new path in which the brain could evolve.

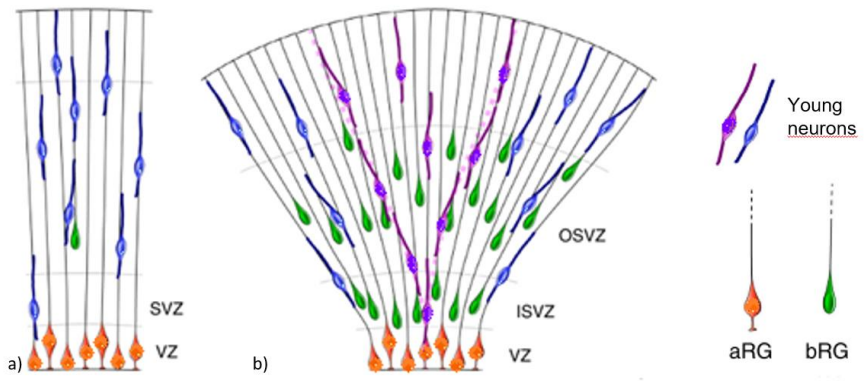


Figure 2. Role of radial fibers in cerebral cortex expansion. a) in lissencephalic brain radial fibers and neurons (blue) migrating along them follow parallel trajectories, coming from the aRGCs (orange) in ventricular zone, making the basal and apical surface the same size. b) in gyrencephalic brains, bRGC (green) increase the number of radial fibers attached to the basal surface, expanding the cortical surface area and enabling the lateral migration of neurons (purple). Adapted from Borrell and Götz (2014).

1.3. Trnp1 as a key factor in brain folding

TMF- regulated nuclear protein, or Trnp1, is a DNA-associated protein expressed during cerebral cortex development in apical progenitors of the VZ and newborn neurons in the cortical plate (Stahl et al., 2013). It was shown that Trnp1 overexpression causes increased self-renewal of neuronal stem cells *in vitro*, maintaining the cell fate and inhibiting symmetric neurogenic divisions (Stahl et al., 2013). The *in vivo* knockdown of Trnp1 causes an increase in number of basal progenitors like bRGC and IPC, which creates a thicker SVZ. This causes significant radial expansion and leads to increased frequency of cortical folding in postnatal stages of development (Figure 3.a) (Stahl et al., 2013).

In the experiments, local knockdown of Trnp1 caused increased generation of IPC which gave rise to more neurons in that area (Stahl et al., 2013). It also increased the generation of bRGCs, whose radial fibers provided a scaffold, establishing new paths for the neurons to migrate laterally through the cortex (Stahl et al., 2013). In mouse, a naturally lissencephalic species, almost all neuroepithelial cells express Trnp1, and therefore self-renew and do not generate IPCs. At later stages of development, expression of Trnp1 is lowered randomly in cells, which then divide to produce IPCs (Stahl et al., 2013). Trnp1 expression was also measured in gyrencephalic species like human and ferret. The method used to detect Trnp1 mRNA in sections of fetal human cerebral cortex was in situ hybridization because there is no antibody recognizing these species Trnp1. Hybridization showed different areas with low or high Trnp1. Lower Trnp1 expression was found in areas that further develop gyri, and higher expression was found in areas that form the future sulci (Figure 3.) (Stahl et al., 2013).

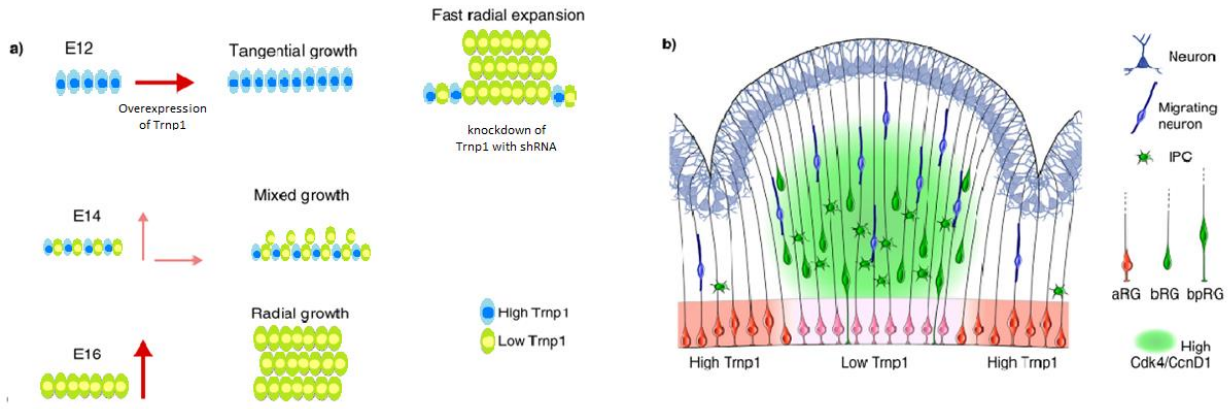


Figure 3. Differences in Trnp1 expression during mouse and human development. a) in mouse, during early neurogenesis (E12), Trnp1 concentration is high, maintaining the proliferation of RGC and causing tangential expansion. Local Trnp1 knockdown causes increased generation of bRGC and basal progenitors (BP) leading to radial expansion and folding of naturally smooth mouse cortex. At mid neurogenesis, around E14, Trnp1 expression lowers in salt and pepper fashion, causing equal tangential and radial growth. At the end (E16), low Trnp1 enables radial growth. b) in human development, alternating regions of low and high Trnp1 concentrations exist, enabling the formation of gyri and sulci. Adapted from Borrell and Götz (2014).

1.4. Evolution of Trnp1 sequence

The sequence of Trnp1 gene is highly conserved among mammals, with conservation of 86% between human and mouse orthologs (Stahl et al., 2013). As gyrencephaly occurs only in some mammalian species, either difference between Trnp1 orthologs, or different regulation of Trnp1

could cause the difference in folding. To measure the rate of evolution of Trnp1 sequence, protein substitution rates were calculated in collaboration with Wolfgang Enard and Zane Kliesmete (Anthropology and Human Genomics, Faculty of Biology, LMU). The Trnp1 protein evolution (dN/dS ratio) was calculated by dividing the rate of non-synonymous mutations altering the amino acid (dN) with the rate of synonymous mutations not affecting it (dS). When the dN/dS ratio is higher than one, it means there is positive selection acting on that amino acid site. If its equal to 1, the amino acid site is evolving naturally, and if its lower than one, there is constraint, or negative selection/conservation, acting on the amino acid site. To depict the brain phenotype, encephalization quotients (EQ) and gyrification indices (GI) were calculated (Figure 4). Gyrification index calculates the degree of cortical folding by dividing the contour following all gyri and sulci with the outer contour lining the outside. With GI, mammals can be divided into two groups. Low-GI group has less folding compared to high-GI group with highly gyrified brains (Lewitus et al., 2014). The encephalization quotient is a ratio between observed and expected brain mass. If EQ is less than one, it means the species of certain body mass has a brain smaller than expected. If it's equal or larger than one, the brain size is as expected or larger for the body mass, respectively. EQ is used to compare the relative brain size between species (Minervini et al., 2016).

$$EQ = \frac{brain\ mass\ (g)}{(0{,}12 * body\ mass(g)^{2/3})}$$

$$GI = \frac{inner\ cortical\ contour}{outer\ cortical\ contour}$$

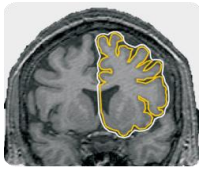


Figure 4. Formulas used to numerically express brain phenotypes. EQ was calculated with a formula developed for mammals. For GI the inner yellow line of the brain is measured and divided with the outer white line.

The evolution rates of Trnp1 were correlated with encephalization quotients, gyrification indices (Figure 5.) and body mass. There was no expected correlation with body mass which served as a negative control. The correlation was higher for GI than EQ. To research this further six different Trnp1 orthologs (whose Trnp1 sequence was known and their GI were different (Figure 5.b)) were chosen. The species were *Mus musculus, Otolemur garnettii, Macaca mulatta, Mustela putorius, Homo sapiens* and *Tursiops truncates*.

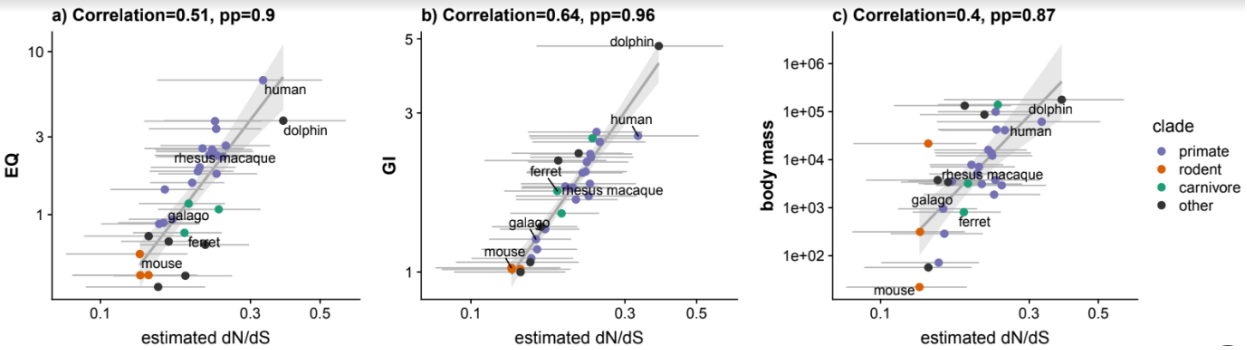


Figure 5. Correlations between Trnp1 protein evolution and three morphological traits. Estimated Trnp1 dN/dS range: 0,11-0,4. a) encephalization quotients (0,1-9) with estimated dN/dS show correlation of 0,51. b) gyrification index (1-5) shows high correlation with estimated protein evolution. c) correlation with body mass used as a negative control. Species chosen for further experiments are marked on the graph b). Personal communication from W. Enard lab.

1.5. Aims of research

The aim of this study was to determine whether the effect of Trnp1 on cortical folding in lissencephalic and gyrencephalic species can be consequence of the different sequences in the different Trnp1 orthologs. For this, the effect of the different orthologs on proliferation of cortical primary culture cells was measured. If orthologs have different effect on proliferation, the Trnp1 from species with higher GI would be expected to cause a higher increase in proliferation when compared to Trnp1 from lissencephalic species with lower GI.

2. **Materials**:

2.1. Plasmid cloning

2.1.1. Plasmids and restriction enzymes

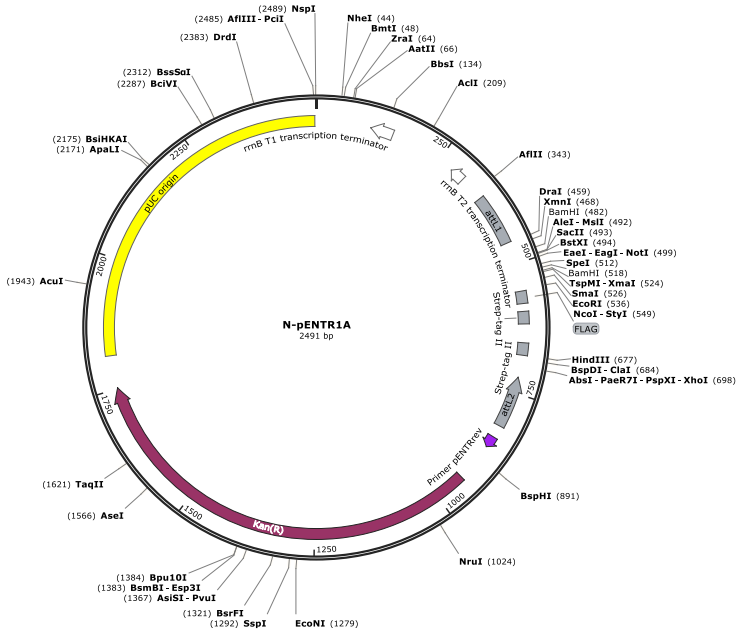


Figure 6. Gateway entry plasmid N-pENTR1A with kanamycin resistance, multiple cloning site between attL1 and attL2 sequences and bacterial pUC origin of replication

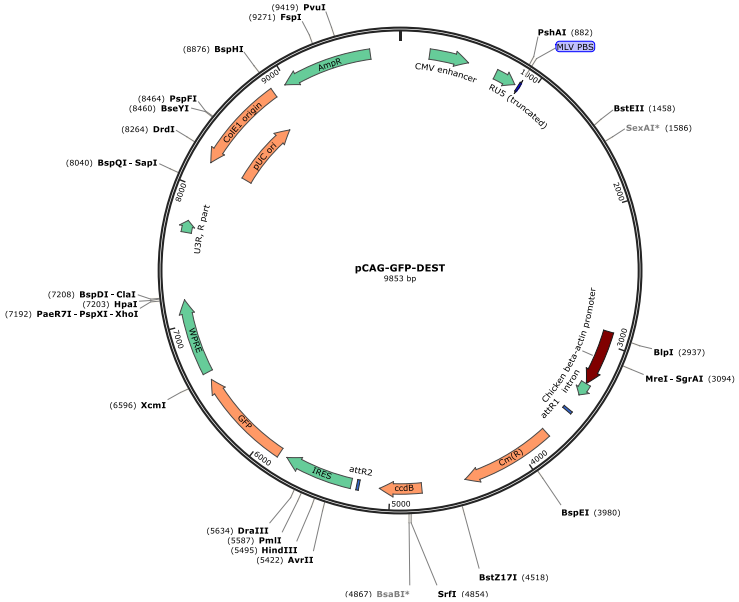


Figure 7. Gateway destination plasmid pCAG-GFP-DEST with Ampicillin resistance, GFP marker and toxic ccdB gene between attR1 and attR2 sequences

JetGene commercially ordered plasmids with: *Otolemur garnettii* (galago) Trnp1 gene

Macaca mulatta (macaca) Trnp1 gene

Tursiops truncatus (dolphin) Trnp1 gene

Restriction enzymes: BamHI High Fidelity (New England BioLabs)

XhoI (New England BioLabs),

NheI HF (New England BioLabs),

NotI (New England BioLabs)

2.1.2. Buffers and mediums

TAE buffer 50x and 1x

CutSmart Buffer (New England BioLabs)

TE Buffer (Invitrogen)

Blocking buffer (10 ml PBS with 2% BSA and 0,5% Triton X-100)

LB (Luria-Bertani) medium:

tryptone	10 g
NaCl	10 g
yeast extract	5 g
distilled water	up to 1 L
pH	7.0

Agar plates:

bacto-agar	7.5 g
LB-Medium	500 ml
Ampicillin or Knamycin	100 µg/ml or 50 µg/ml

liquid poured into 10 cm culture dishes and stored at 4°C

Bacterial strain Top 10 *E. coli* (genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G)

2.2. Cell culture

2.2.1. Animal line

Mus musculus line 657BL6

2.2.2. Buffers and mediums

PBS (8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4, 240 mg of KH_2PO_4, distilled water to 1 L, pH ≈ 7.4)

Primary culture proliferation medium:

GlutaMAX GIBCO 61965 (Thermo Fisher Scientific)	45 ml
Bovine Serum Albumin (Sigma)	5 ml
Penicillin/Streptomycin (50 µg/ml) (Life technology)	0,5 ml

Primary culture differentiation medium:

GlutaMAX GIBCO 61965 (Thermo Fisher Scientific)	50 ml
B27 (Life technology)	1 ml
Penicillin/Streptomycin (50 µg/ml) (Life technology)	0,5 ml

Opti-MEM Reduced Serum Medium GIBCO (Thermo Fisher Scientific)

2.3. Primary and secondary antibodies

Anti-GFP (chicken antibodies, IgY Fraction, Aves labs, inc.)

G3G4 anti-BrdU (Developmental Studies Hybridoma Bank)

Anti-Mouse/Rat Ki67 purified (eBioscience)

Goat anti-chicken IgY (H+L) secondary antibody, Alexa Fluor 488 (Invitrogen by Thermo Fischer Scientific)

Goat anti-mouse IgG1 Alexa Fluor 546 (Invitrogen)

Goat anti-rat Alexa Fluor 647 (Invitrogen)

3. Methods:

3.1. Cloning

3.1.1. Digestion of plasmids for extraction of Trnp1 gene

From the six orthologs used in this study, plasmids containing human, mouse and ferret Trnp1 were already available in the lab and ready for use, while the plasmids expressing macaca, dolphin and galago Trnp1 genes needed to be cloned. The sequence of Trnp1 in these species is known, so plasmids containing Trnp1 gene, synthetized by the provided sequence, were ordered from GeneScript (www.genscript.com). The sequences were flanked by the restriction enzyme sites for BamHI and XhoI, which were used to clone them into the gateway p-ENTR1A plasmid. The digestion mix for each sample was prepared in separate 1,5 ml Eppendorf tubes as described in Table 1. and the reaction was incubated in a Thermomixer Compact on 37°C for 2 hours. The expected fragment lengths were 2,26 kb and 0,85 kb for p-ENTR1A and around 2,5 kb and 700 bp for macaca, galago and dolphin Trnp1 gene. To separate the fragments a 1% agarose gel was prepared by dissolving 2 g of Agarose, Universal (VWR Life Sciences) in 200 ml of TAE buffer. Bands were visualized using Syber Safe DNA gel Stain (Invitrogen) in a concentration of 1:33000. After digestion, 5 µl of DNA loading dye 6x (Thermo Scientific) was added to the samples and they were loaded on the gel next to the GeneRuler, DNA Ladder mix 0.5µg/µl (Thermo Scientific) to be able to distinguish the band sizes. Electrophoresis was run for 1 h at 120 V. The bands were visualized on a Dark Reader transilluminator (Clare Chemical Research). The expected sizes needed for further purification were a band at 2,26 kb for the p-ENTR1A backbone and around 700 bp for the different Trnp1 inserts. These bands were cut out of the gel, and each placed in a different, pre-weighted Eppendorf tube and weighted. The Gene JET Gel Extraction Kit (Molecular Biology, Thermo Scientific) protocol was followed to purify the DNA from the gel. First the Binding buffer was added to gel slice in 1:1 volume. This mixture was incubated on 60°C for 10 min, then transferred to GeneJET purification column and centrifuged to discard the flow through. Column was washed with 700 µL of Wash Buffer with added ethanol. Residual wash buffer was removed by centrifuging the empty column for another minute, then the column was placed in a clean 1.5 mL microcentrifuge tube, and the DNA was

eluted with 50 µL of Elution Buffer. The concentration of purified DNA was measured by NanoDrop ND-1000 Spectrophotometer (PeqLab).

Table 1. Digestion mix for gateway entry plasmid and plasmids with Trnp1 orthologs.

	p-ENTR1A	Trnp1 macaca	Trnp1 galago	Trnp1 dolphin
DNA	6 µl	20 µl	20 µl	20 µl
H_2O	14,5 µl	0,5 µl	0,5 µl	0,5 µl
CutSmart buffer	2,5 µl	2,5 µl	2,5 µl	2,5 µl
BamHI HF	1 µl	1 µl	1 µl	1 µl
XhoI	1 µl	1 µl	1 µl	1 µl

3.1.2. Ligation

The restriction enzymes cut DNA asymmetrically at specific sites called palindromes and create single stranded DNA overhangs, called sticky ends. In the presence of the DNA ligase enzyme, the compatible overhangs create new phosphodiester bonds and join together. In order to ligate the plasmid with the insert in the correct orientation, as well as to avoid the empty plasmid ligating itself, two restriction enzymes that create different overhangs are used. For the ligation mix, 100 ng of plasmid was mixed with 100 ng of each insert. The total volume of the mix was 10 µl containing 1µl of T4 DNA Ligase buffer (BioLabs), 1µl of T4 DNA ligase 10X (BioLabs), the needed quantity of digested and purified insert and plasmid and the rest was sterile B. Brown water. The mix was incubated at room temperature for 3 h and then frozen for 2 h.

3.1.3. Transformation

For generation of a large number of plasmids, special competent bacterial strains are used. In these bacteria, plasmids are generated in large number and can be extracted for further usage. Top 10 is a *E. coli* strain that provides a high efficiency transformation. One microliter of the ligation mix was added to 50 µl of competent bacteria and heat shock transformation (42°C for 20 seconds) was done. After that, bacteria were put on ice for 2 min and 200 µl of LB medium was added to the bacteria and incubated for 1 h at 37°C and 180 rpm. 150 µl of the mix was plated on kanamycin plates and placed in the incubator overnight.

3.1.4. Isolation of plasmid DNA

A single bacterial colony on the plate is a clone of single bacteria and contains one type of plasmid. To get smaller amounts of plasmid for next steps in cloning, plasmid DNA was isolated with miniprep. A single colony was picked from the plate with a plastic pipette tip and placed in 5 ml of liquid LB medium with 5 µl of kanamycin (1:1000). For each plasmid, 7 colonies were picked and placed in separate tubes with LB media. Bacteria were left to grow over night in the incubator at 37°C and 180 rpm. Next day the tubes were centrifuged at 4000 rpm for 10 min in Heraeus Multifuge X3R Centrifuge. The supernatant was discarded, and the bacterial pellet was used to purify the plasmids following the protocol provided with GeneJet Plasmid Miniprep Kit (Thermo Scientific Molecular). To lyse the cells, pallets were resuspended in 250 µL of Resuspension Solution, then 250 µL of Lysis Solution was added, tube was inverted 5 times and incubated for exactly 5 min to avoid denaturation of supercoiled plasmid DNA. To stop the lysis 350 µL of Neutralization Solution was added and the tube was inverted 4-6 times. After centrifugation the supernatant was transferred to a Thermo Scientific GeneJET Spin Column centrifuged again. The column was washed twice with 500 µL of Wash Solution and an empty column was centrifuged for 1 min. The purified DNA was eluted with 50 µl of B. Brown H_2O. The concentrations were measured with NanoDrop ND-1000 Spectrophotometer (PeqLab).

Another plasmid isolation technique used at the end of the cloning process was maxiprep. This technique is used when larger amounts of plasmid DNA need to be isolated. In maxiprep, the bacteria are cultured in more liquid medium, leading to more bacterial growth and higher plasmid concentrations after isolation. To grow the bacteria for Maxi prep, 200 µl of ampicillin was added to 200 ml of LB medium. 50 µl of bacteria kept from miniprep after recombination step was added, one for each plasmid, and the flasks were incubated overnight on a shaker at 37°C and 180 rpm. For plasmid extraction the flasks were centrifuged for 15 min at 4000 rpm and the protocol of the PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen) was followed. For this protocol, Filtration Cartridge was equilibrated with 30 mL Equilibration Buffer. The pallet was resuspended with 10 mL Resuspension Buffer with RNase A, lysed with 10 mL Lysis Buffer by gently inverting four times and incubating the mixture for 5 min. Then 10 mL of Precipitation Buffer was added, and the mixture was filtered through filter papers placed in funnels over the Filtration Cartridge. The filter papers were discarded, the column was washed

with 10 mL Wash Buffer. To elute the plasmid, the column was placed over a sterile 50-mL centrifuge tube and 15 mL of Elution Buffer was added. The plasmids were precipitated with 10.5 mL isopropanol, centrifuged and the supernatant was discarded. The pellet was washed with 5 mL 70% ethanol, centrifuged, and left to air dry. The plasmids were resuspended in water and concentrations were measured.

3.1.5. Selection of clones by digestion

To be able to identify the right clone it is necessary to digest the plasmids isolated with Miniprep. This is done with specifically chosen restriction enzymes that produce fragments of different size when cutting the plasmid with or without the insert. The p-ENTR1A containing dolphin, macaca or galago Trnp1 gene were cut with NheI (NEB) generating fragments of 2309 + 670 bp, 2285 + 673 bp and 2297 + 675 bp respectfully. If the plasmid was empty fragment size after digestion would be 3121 bp. Around 1 µg of DNA was digested to be visible on the gel as a band. To cut 1 µg of DNA, 0,5µl of NheI HF were used with the CutSmart buffer diluted from 10x to 1x with water. Digestion was incubated in a Thermomixer Compact on 37°C for 1,5 hours. After digestion, DNA ladder mix and samples were loaded on a 1% agarose gel and electrophoresis was run for 1 h at 120 V. The picture of the gel was taken with BIO-RAD ChemiDoc XRS+. Correctly ligated clones were located on the picture (Figure 11), and one for each gene was sent for sequencing for additional confirmation of the gene sequence.

These methods were repeated after recombination to select the bacterial clones containing properly inserted Trnp1 gene in the Gateway expression plasmid. The transformation and mini prep were done as previously described, with ampicillin for antibiotic resistance selection. Four colonies were picked from each plate and 50 µl of bacteria from each mini prep was transferred to Eppendorf and kept for maxi prep. For digestion, NotI restriction enzyme was used. The expected fragment size for the desired clone of dolphin was 7422 bp and 1520 bp, for macaca it was 7033 bp and 1520 bp and for galago 7416 bp and 1520 bp. The size of 6938 bp and 2900 bp would indicate the plasmid was not recombined correctly. The protocol used was the same as previously described. The correct clones were also sent for sequencing.

3.1.6. Recombination

p-ENTR1A is a high-copy replication and maintenance plasmid that does not enable expression of inserted genes, which are flanked by attL1 and attL2 sites. To produce the Expression Clone, the gene was subcloned into a destination vector (gateway) containing the gene for ampicillin resistance, and two recombination sites, attR1 and attR2, that flank a gene for negative selection, *ccdB*, whose protein product is toxic to bacteria. The destination vector used was a retroviral vector with pCAG promoter that also expresses the GFP gene. The Gateway system uses *in vitro* versions of site-specific recombination properties of bacteriophage lambda to move the gene of interest into a destination vector. This recombination is called LR Reaction and it is directional and specific, so that *att*L1 only reacts with *att*R1 and *att*L2 with *att*R2. The recombination yields two constructs: the intended expression clone and a by-product, composed of entry vector and *ccdB* gene (Figure 8). For this step in cloning, Gateway LR clonase II kit (Invitrogen) was used. 100 ng of entry clone and 100 ng of destination vector were mixed, TE buffer was added to 8 µl and the enzyme was added. Reaction was left for 3 h at room temperature and then the1 µl of proteinase K was added and incubated on 37°C for 10 min to stop the reaction.

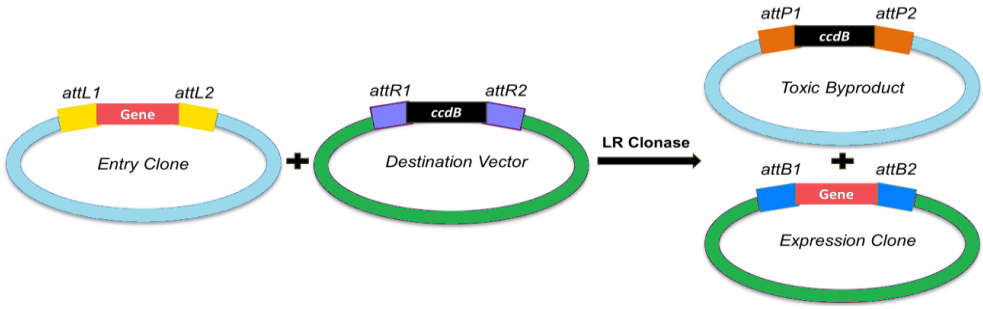


Figure 8. LR reaction in Gateway cloning. Specific att sites recombine enabling the transfer of gene into another plasmid without restriction enzymes.

3.2. Primary culture

To test the effect of Trnp1 orthologs, primary cultures needed to be prepared. Cells of cortical primary culture only attach if the surface is coated with poly-D-Lysine (PDL). Therefore, PDL coated coverslips were used. Sterile coverslips were placed in the P24 well plates in sterile conditions. PDL was diluted in ddH_2O to a final concentration of 10 µg/ml and the coverslips were covered with the PDL solution and left in the incubator overnight. Next day, the PDL was aspirated, the wells were washed with ddH_2O three times and left to dry for minimum 3 h. More

plates were prepared and stored in the fridge at +4°C for maximum 2 weeks. The starting material for primary culture was E14 mouse embryos. To prepare the culture, first the brain was removed from the embryos head. Then the olfactory bulbs were cut away. Using scissors and forceps the cortex was cut as shown in Figure 9. The cortex cells were dissociated mechanically, with fire polished glass pipette, centrifuged for 10 min at 1200 rpm and resuspended in proliferation medium. Cell suspension was placed as a drop in the middle of the PDL coated coverslip in a P24 well plate.

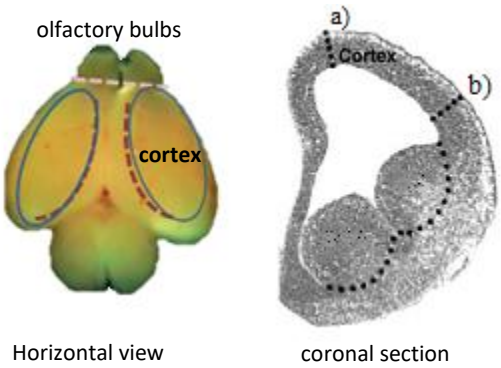


Figure 9. Cortex isolation for primary culture. With small scissors, brain is cut on the line a), the cortex is opened, and a second incision is made from the inside, before ganglionic eminence, below line b). The cortex is separated from the meninges. Adapted from H. Toresson, et al (1999.)

The cells were left to attach for 30 min, then the drop of medium was aspirated and 500 µl of proliferation medium was added. The cells were placed in the incubator at 37°C and 5% CO_2 to recover for 2 h.

3.3. Transfection

Seven plasmids were used for transfection: RV-pCAG-ires-GFP, RV-pCAG-mouse-ires-GFP, RV-pCAG-human-ires-GFP, RV-pCAG-ferret-ires-GFP, RV-pCAG-galago-ires-GFP, RV-pCAG-macaca-ires-GFP and RV-pCAG-dolphin-ires-GFP. After incubation for at least two hours, the cell medium was changed to Opti-MEM Reduced Serum Medium. To each well, 200 µl was added. The 24 well plate was divided into 7 rows. Three wells were used for transfection with each plasmid, making three technical replicates. Lipofectamine 2000 (Invitrogen) was used to transfect the cells. For one well, 1 µg of plasmid was mixed with 25 µl of Opti-MEM in a clean Eppendorf tube. In another tube, 1 µl of Lipofectamine 2000 was mixed with 25 µl of Opti-MEM per well. Mixtures were left for 5 min and then both reactions were mixed at RT. After 30

min the mix was used to transfect the cells by drop transfection. Next day, the medium was changed to half proliferation, half differentiation medium. The experiment was repeated five times, making five biological replicates.

3.4. BrdU treatment and fixation

5-Bromo-2′-deoxyuridine (Sigma-Aldrich) was given to the cells 36 h after transfection. BrdU replaced thymidine base in the DNA of cells that were in the S phase of the replication cycle, marking them. 40 min after administration, cells were fixed with 4% PFA for 10 min, then washed three times with PBS for 5 min and placed in the fridge.

3.5. Immunostaining of proliferating cells with Ki67 and BrdU antibodies

For staining, fixed cells were washed three times for 5 min with PBS and then 400 µl of blocking buffer was added and incubated for 1 h. Anti-rat Ki67 and anti-mouse BrdU antibodies were used to mark the proliferating cells and to distinguish transfected cells anti-chicken GFP antibody was used. Antibody for BrdU requires an acid treatment to open up the chromatin and enable the antibody the access to integrated BrdU. This acid treatment can extinguish the fluorescence of secondary antibodies, alter the epitopes for other antibodies and disrupt their binding. Therefore, a longer, two-step staining protocol was tested and used. Primary antibodies against Ki67 (1:250), and GFP (1:500) were added for 2 h at RT, then the cells were washed three times for 5 min with PBS, fixed again with PFA and again washed as before. Then 3M HCl was added to the cells for 30 min, then washed with sodium borate (pH 8.3), two times for 15 min. Again, cells were washed with PBS as before, and primary antibody against BrdU (1:25) was added and incubated overnight at +4°C. Next day, cells were washed with PBS as before and secondary antibodies were added in dilution 1:500. Anti-chicken 488, anti-mouse IgG1 546, anti-rat 647 and DAPI (4′,6-diamidino-2-phenylindole) were used. After 1h in the dark, the cells were washed again three times for 10 min at RT and fluorescence was checked. To ensure the staining worked, the BrdU+ cells had to be positive for Ki67 proliferation marker, which is present in all phases of the cell cycle, but not in the G0 phase (Scholzen and Gerdes, 2000). Since BrdU marks only cells in the S phase, not all Ki67+ should be BrdU+, otherwise that would indicate the cross reaction of the antibodies. Coverslips with cells were mounted with Aqua Poly/Mount (Poly Sciences) and left to dry overnight.

3.6. Imaging on epifluorescent microscope and quantification

Pictures of the whole coverslips were taken with Zeiss Imager.M2 epifluorescent microscope. The channels used were DAPI, GFP 488 nm, DsRed 546 nm and Cy5 647 nm for far red. A big picture, composed of approx. 100 tiles for each coverslip, was taken. From the pictures, using the ImageJ program, cells overexpressing Trnp1 were distinguished from the non-transfected cells by GFP fluorescence. Only these green fluorescent cells were counted and checked for the signal from Ki67 and BrdU. Usually, 100-250 GFP+ cells were counted per coverslip. The percentage of double positive cells, e.g. GFP+ Ki67+, was calculated. These percentages were normalized to the GFP control, which does not contain Trnp1, enabling the comparison between the biological replicates. For statistical analysis, non-parametric Mann-Whitney test was used.

4. Results

4.1. Cloning of Trnp1 via Gateway system

The aim of this research was to determine if accumulated evolutionary mutations in Trnp1 orthologs have different effects on proliferation of primary culture cells and in that way contribute to differences in cortical folding between species. Therefore, all orthologs needed to be cloned in expression plasmids which would overexpress Trnp1 protein in transfected cells alongside the GFP protein. The starting material were plasmids containing macaca, galago and dolphin Trnp1 genes, which needed to be moved into p-ENTR1A plasmid for gateway cloning. The first step, digestion of p-ENTR1A plasmid and plasmids containing the Trnp1 genes was successful. The digestion gave two clear bands of expected sizes. The backbone of p-ENTR1A at 2,26 kb and inserts at 700 bp were cut one by one out of the gel as seen in Figure 10, purified and ligated.

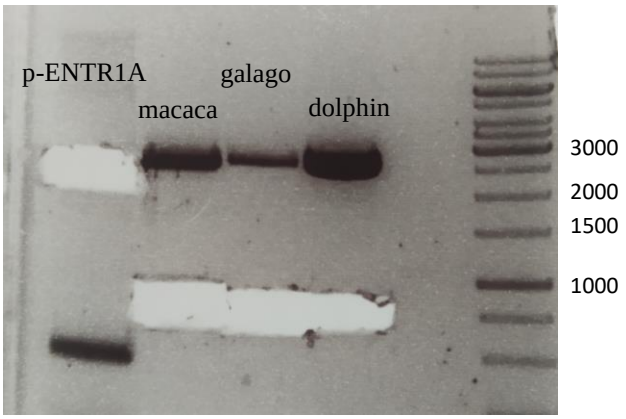


Figure 10. Digestion of p-ENTR1A and gene containing plasmids into two linear fragments. p-ENTR1A fragment sizes are 2,26 kb backbone and 0,85 kb fragment, gene plasmid's fragment sizes are 2,5 kb backbone band and ~0,7 kb gene band. White spots show the excision of p-ENTR1A backbone band and genes TRNP1 macaca, galago and dolphin for purification.

The bacteria transformed with the ligation mix grew into colonies that were picked for mini prep. After digestion and electrophoresis of purified plasmids, the picture showed all clones were ligated correctly (Figure 11).

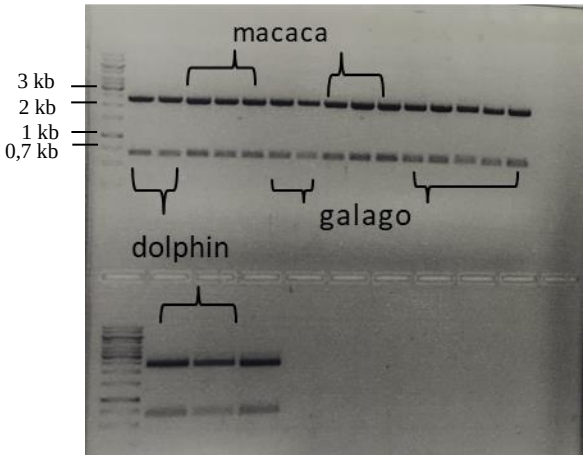


Figure 11. Digestion of ligated plasmids for clone selection. All bands are around 2300 bp + 670 bp, the expected size for good clones.

One sample of plasmid with Trnp1 from each species was sent for sequencing. The sequence of genes matched the original, meaning no error in gene sequence occurred during the cloning process (Appendix: Figure 1, 2 and 3).

The last step in cloning, the LR reaction, was successful in all but one dolphin clone. The rest of the bands were of expected sizes (Figure 12).

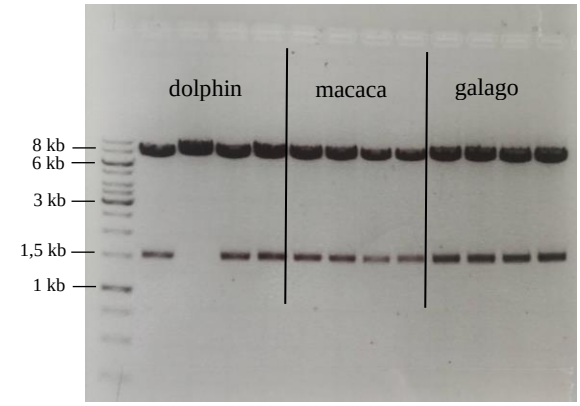
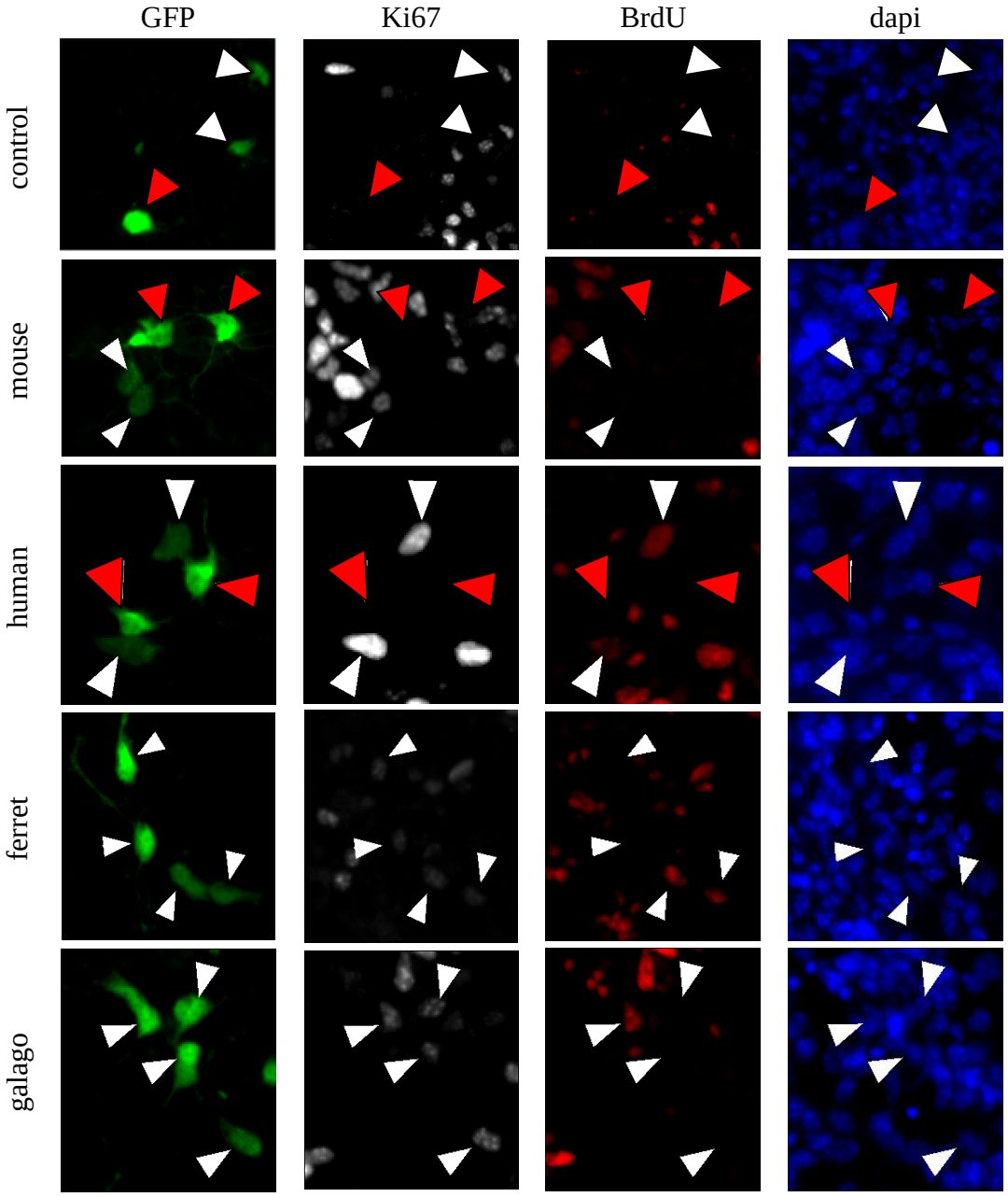


Figure 12. Verifying the success of LR reaction via digestion. Only the clone of dolphin in second well wasn't correct size and was discarded.

4.2. GFP signal amplification and testing for cross reactions of proliferation markers

As plasmids expressing Trnp1 also express GFP protein, transfected cells emit green fluorescence. Some transfected cells do not divide many times and their signal is bright. But the plasmid in the cells cannot replicate itself, and therefore, it dilutes in rapidly proliferating cells. To be able to visualize even small amounts of GFP in these dividing cells, the signal was

amplified by immunostaining for GFP protein. This led to the differences in the intensity of GFP signal, with brighter cells more often being negative for proliferation markers than cells with dimmer GFP signal. Antibodies for proliferation markers rat Ki67 and mouse BrdU. As these species' antibodies are relatively similar, it was necessary to check if secondary antibodies recognized only one or both species. There was no such cross reaction between Ki67 and BrdU antibodies, meaning all BrdU+ cells were also Ki67+, but not the other way around (Figure 13).



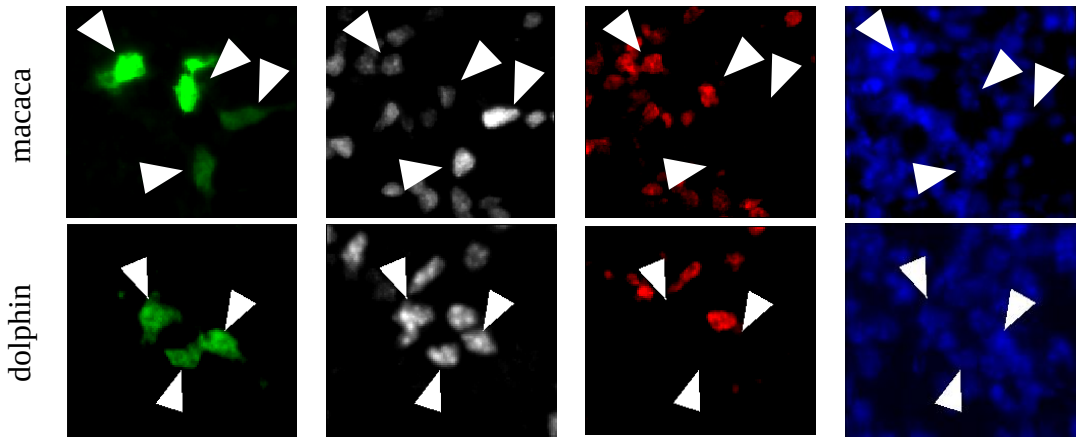
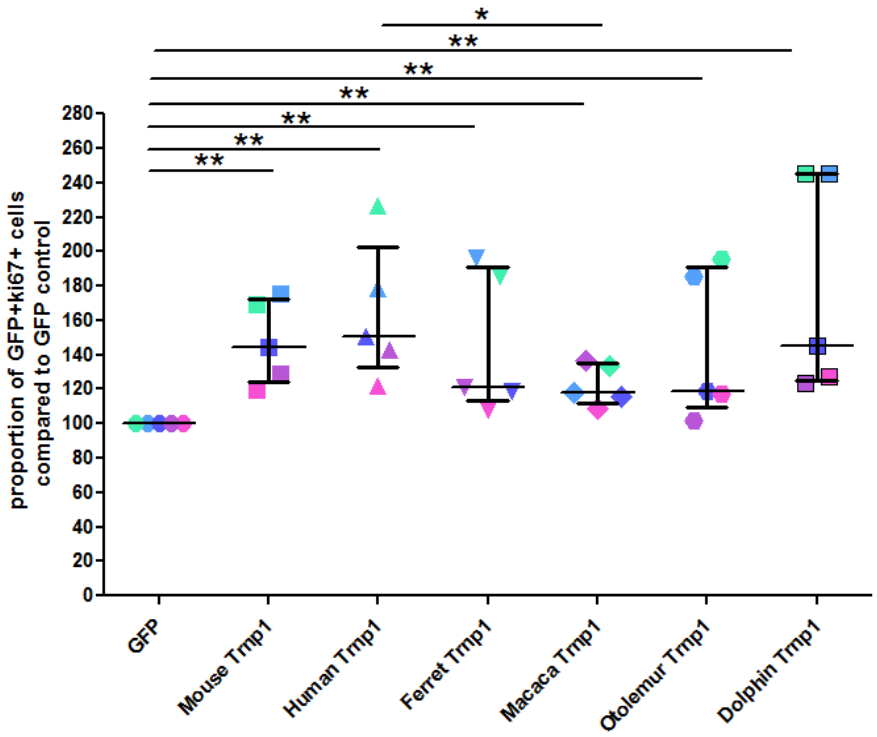


Figure 13. Immunostaining of control and all orthologs. All GFP+ cells were counted, as marked, and checked for signals of Ki67 and BrdU. Red arrows indicate cells negative for Ki67 and white arrows cells positive for Ki67

4.3. Quantification of proliferating cells

There was a significant increase in proliferation when comparing GFP control and any ortholog. The addition of mouse Trnp1 plasmid increased the proliferation for approx. 150% compared to control considered as 100%. The Trnp1 from human showed even bigger increase in proliferation, while the ferret Trnp1 showed an increase between mouse and human. These results were expected and served as a positive control, as this increase has been experimentally shown before in the lab. Proliferation increased slightly more with dolphin Trnp1 than with human Trnp1. As dolphin brains are highly gyrencephalic, this Trnp1 ortholog should cause increased proliferation. Surprisingly, macaca ortholog resulted in lowest increase in proliferation, increasing the proliferation less than mouse Trnp1. This was surprising because macaca is a primate with gyrified brain. Galago Trnp1 increased the proliferation similarly to mouse Trnp1. Galago also has a relatively high GI, plotted between mouse and macaca (Figure 5.b). Some differences between biological replicates were noticed. The transfection rates, which are generally around 5-10% in primary cultures, were even lower, possibly due to older reagents such as lipofectamine2000. This affected the number of counted cells, which varied between 100 and 250 transfected cells. N5 and N6 had a relatively low increase in proliferation in all orthologs when compared to other replicates. Their transfection rate was worse than in other replicates. Plasmid dilution also caused problems in detecting transfected cells.

With all the data combined, statistical analysis showed that there was a significant increase in proliferation between GFP control and all orthologs, with p-value lower than 0,01 and a significant difference between human and macaca Trnp1, p>0,05 (Figure 14). No significant difference was found between other orthologs using this test.



N=5; Mann-Whitney test (non-parametric test); * p<0,05; **p<0,01

Figure 14. Quantification results for proliferating cells marked with Ki67 marker, statistically analyzed in Prism program using non-parametric Mann-Whitney test. The percentage of proliferating cells was calculated by dividing the number of transfected cells (GFP+) with the number of proliferating cells (Ki67+). Each ortholog's percentage of proliferating cells was divided by the percentage in control GFP, enabling the comparison between species. Each color represents one biological replicate (N1-N5). There is a statistically significant (**) increase in proliferative capability caused by overexpression of each Trnp1 ortholog, compared to GFP control. When comparing the orthologs, only statistically significant difference (*) was between human Trnp1 and macaca Trnp1 gene.

Table 2. Supplementary table for Figure 14. Shows color coded percentage of proliferating (GFP+Ki67+) cells/GFP control for each Trnp1 ortholog (mouse-dolphin). These percentages are plotted on the y axis of Figure 14. Each color is one biological replicate (N1-N5).

	GFP control	mouse	human	ferret	macaca	galago	dolphin
N1	100	168,9655	226,7275	185,5144	133,7054	195,5839	245,0000
N2	100	175,7525	178,6471	196,4316	118,1507	185,6654	245,4603
N3	100	144,3864	150,8814	118,7241	115,6580	118,8267	145,1460
N4	100	128,5320	142,8285	120,8513	136,7073	102,0554	123,3219
N5	100	119,6323	122,0376	108,2111	108,3296	117,3313	127,3505

4.4. Determining the number of cells in S-phase

The second proliferation marker, BrdU, marked the cells in S-phase at the time of BrdU administration. During the 40 min after BrdU was added to the cells, those that were in or entering S-phase were able to incorporate it into their DNA. The goal was to be able to estimate the length of cell cycle. If the ortholog increased the self-renewing potential by shortening the cell cycle, more cells would be in S phase, and would be marked with BrdU. But the BrdU counting results were highly variable and inconclusive. When normalized to the control, the percentage of GFP+BrdU+ cells varied between biological replicates from 63% to 176% for mouse, 89% to 260% for human etc. There was no consistency between the replicates.

5. Discussion

Trnp1 is a gene found only in mammals, whose expression is found in self-renewing apical progenitor cells of the VZ (Stahl et al., 2013). As some species have lissencephalic cortex and others gyrencephalic, it is possible that evolutionary accumulated mutations in the Trnp1 gene contributed to these differences by controlling proliferation of cells. In that case, Trnp1 gene from species with higher gyrencephalic index (GI) would increase cell proliferation capacity more than Trnp1 from species with lower GI. Every Trnp1 ortholog caused a significant increase in the self-renewal capacity of embryonic cortical primary cultures, compared to the GFP alone negative control. This was expected, since Trnp1 is a master regulator whose expression stimulates symmetric cell division, causing tangential growth, while lowered concentration enables generation of bRGCs and radial growth *in vivo* (Stahl et al., 2013). As species within the GI range from 1 to 5 were taken (Figure 5), those with higher GI were expected to show an increased proliferation potential necessary for the creation of folds (Lewitus et al., 2014). Orthologs ordered by GI from lowest to highest were mouse, galago, ferret, macaca, human, dolphin. In the same order, Trnp1 orthologs were expected to have increased effect on proliferation. But the slight differences between orthologs did not match these expectations. The ortholog to show the highest increase in proliferation after dolphin and human Trnp1 was mouse Trnp1. This was followed by ferret, galago and lastly macaca. None of these differences were statistically significant, except for the difference between human and macaca orthologs, which was unexpected. As macaca has high GI, the increase in proliferation was lower than anticipated. The precursors in developing macaca brain go through consecutive proliferative divisions and shorten their cell cycle (Betizeau et al., 2013). But proliferative divisions have to be tightly regulated through both intrinsic factors, for example by controlling symmetrical or asymmetrical cell divisions (Sun and Hevner, 2014) and external factors (Fietz et al., 2012). Perhaps the low proliferation observed was due to the slight differences of macaca Trnp1 from the mouse endogenous protein, sufficient to impair the function in mice cells. Another reason could be that the cofactors for Trnp1 function may be different or have different sequences in other species. These experiments were performed in mouse cells, which were previously used for similar experiments in the lab. But also, for ethical reasons, the brains of each species could not be used to produce primary cultures. Therefore, Trnp1 orthologs had only mouse endogenous cofactors

to work with, which could influence their function. Other pathways may interact with Trnp1 pathway in regulating cell proliferation, such as FGF or Notch (Sun and Hevner, 2014). Since mouse is a naturally lissencephalic species, these potentially interacting pathways may be differently regulated than they would be in macaca cells, thereby hindering the effect of this Trnp1 ortholog.

The BrdU staining results were inconsistent and varied between the biological replicates. Due to these large differences within replicates of each ortholog, it was impossible to get a proper mean value to compare between the orthologs. A possible explanation for BrdU staining results was the presence of damaged or dying cells in the cultures. Perhaps, the DNA of damaged cells could have incorporated BrdU in the repair process (Limsirichaikul et al., 2009) and interfered with signaling, creating a higher background and false positive signals by overlapping with live cells.

When comparing the ortholog protein sequences, evolutionary mutations between these orthologs weren't located in the first 15 amino acids (aa) and the 16^{th} was only different in galago. Based on the previous research in the lab (Esgleas et al, under revision), the first 16 aa are highly conserved part responsible for increased proliferation after Trnp1 overexpression (Figure 15). This offers an explanation as to why no differences in ortholog's effect on proliferation were observed. Next region of Trnp1 is intrinsically disordered region (IDR). In proteins, IDR is a flexible part of the protein without stable structure, able to form interactions with many other proteins, nucleic acids and other interacting partners (Oldfield and Dunker, 2014). This region is most variable when comparing Trnp1 orthologs, where most evolutionary accumulated mutations are located. These differences could strengthen or weaken interactions with other proteins, causing changes in Trnp1 regulation. It is not only the strength of Trnp1 effect on proliferation that is important in development, but also the timing of its expression and distribution (Borrell and Götz, 2014). These things could be affected by other proteins interacting with Trnp1, changing its expression or ability to perform its function. The next domain in Trnp1 creates a secondary structure, α-helix capable of forming a coiled coil, and the C-terminus of Trnp1 important in nuclear localization. Both of these regions are relatively well conserved parts of the protein. The ferret ortholog is the only of the six used in this experiment whose protein sequence is shorter and very different from the rest in those two domains. If the

other five are aligned and compared, around 5 evolutionary mutations can be found in the region for forming an α-helix, and five in the C-terminus. Two of those substitutions are for an aa with very similar properties, while the others usually involve substitution with the simplest aa, glycine. As proteins are carried through the nuclear pores by cytoplasmatic carriers (Adam, et al., 1990), evolutionary mutations at the C-terminus could affect this interaction between mouse carriers and Trnp1 from other species (Lange et al., 2007).

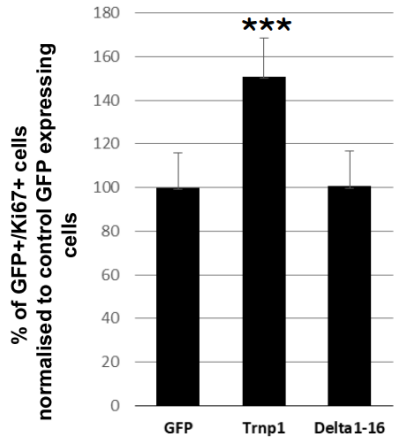


Figure 15. Deletion of first 16 aa impairs Trnp1 ability to increase proliferation. Cells were transfected with control GFP, full length Trnp1 and Δ1-16 Trnp1. While full length Trnp1 caused a significant increase in proliferation, no increase was observed in truncated Trnp1 when compared to control. Esgleas et al. under revision

Another gene with the capacity to change self-renewing potential of NSC is ARHGAP11B. This gene evolved from ARHGAP11A by partial duplication and became specific for humans after single nucleotide substitution. This C→G base change introduced a new splice site in the mRNA, causing the truncation of GAP domain. This caused a frame shift in the mRNA, creating a unique 47-residue long C-terminal sequence (Florio et al., 2016). ARHGAP11B mRNA was first found in human aRGCs and bRGCs but not in cortical neurons. This gene did not have a mouse ortholog. To understand its function, the gene was expressed in E13.5 mouse neocortex by in utero electroporation. It caused an increase in bRGCs by promoting their self-amplification. This caused thickening the SVZ and induced cortical folding at E18.5 in half of the cases. The ARHGAP11B mRNA was also microinjected into single aRGC of organotypic slice culture of E14.5 mouse neocortex. The expression of ARHGAP11B in aRGC caused the switch from

producing two daughter aRGCs to producing two bRGCs daughter cells, identified with Tbr2 (Florio et al., 2015).

Trnp1 knockdown and ARHGAP11B expression both cause the bRGC proliferation. bRGCs played an important role in cortex expansion and formation of a folded brain. Their number is always low in lissencephalic mammals, and increasing it causes the formation of gyri (Borrell and Götz, 2014). If the first mammal was gyrencephalic, evolution of Trnp1 and its regulation could have caused changes in its expression and lowered or increased the number of bRGCs, making the brain smoother or more folded, respectively (Kelava et al, 2013). On the other hand, ARHGAP11B gene evolution in human further increased the bRGCs proliferation, making human cortex among the largest.

6. Conclusion

Overexpression of all Trnp1 orthologs increases the proliferation of primary culture cells. When comparing the orthologs, no statistically significant differences were found in the capacity to promote proliferation, possibly due to the fact that we used mouse cells. It is unlikely that differences between Trnp1 orthologs are solely responsible for the differences in brain size and folding capacity. Most evolutionary mutations are located on the IDR of Trnp1 protein, important in oligomerization and other interactions. Therefore, the differences in GI could be caused by different regulation of Trnp1 protein by other interacting partners.

7. References

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8. Appendix

```
pENTR1A-Dolphin      GTAGACATTCGTCGACTGGATCCATGCCGGGCTGCCGCATCAGCGCCTGCGGCCCGGGGG     60
dolphin              -----------------GGATCCATGCCGGGCTGCCGCATCAGCGCCTGCGGCCCGGGGG     43
                                      *****************************************

pENTR1A-Dolphin      CCCAGGAAGGGTCGGCGGAACCGGGGTCCCCATCGCCGCCGCCCGGGGAGCGCCTATTCT     120
dolphin              CCCAGGAAGGGTCGGCGGAACCGGGGTCCCCATCGCCGCCGCCCGGGGAGCGCCTATTCT     103
                     ************************************************************

pENTR1A-Dolphin      CCCCTCAGCCCCCGTCCCCAACTCCGACCTTGACCCCGACCCCGGCTCAGGCCTCACCGC     180
dolphin              CCCCTCAGCCCCCGTCCCCAACTCCGACCTTGACCCCGACCCCGGCTCAGGCCTCACCGC     163
                     ************************************************************

pENTR1A-Dolphin      AGCCCGAAGTGGCCCAGGAGTCGGCGGGCTCGGCCGAGGGGCAGGAGCTGCAGCGCTGGC     240
dolphin              AGCCCGAAGTGGCCCAGGAGTCGGCGGGCTCGGCCGAGGGGCAGGAGCTGCAGCGCTGGC     223
                     ************************************************************

pENTR1A-Dolphin      GCCAGGGCGCTAGCGGGGGCGCGGGGCGCACCGGGCCGGCAGGGGGCGCGGGCGGCGGCC     300
dolphin              GCCAGGGCGCTAGCGGGGGCGCGGGGCGCACCGGGCCGGCAGGGGGCGCGGGCGGCGGCC     283
                     ************************************************************

pENTR1A-Dolphin      CGGTCGCGGCGGCGGCGGCGGCGGCGGCAGGGGGCCGTGCGCTTGAGCTGGCCGAAG     360
dolphin              CGGTCGCGGCGGCGGCGGCGGCGGCGGCAGGGGGCCGTGCGCTTGAGCTGGCCGAAG     343
                     ************************************************************

pENTR1A-Dolphin      CGCGGCGGCGACTGCTGGAGGTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCC     420
dolphin              CGCGGCGGCGACTGCTGGAGGTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCC     403
                     ************************************************************

pENTR1A-Dolphin      GCGTGCTGCAGCTGCACTGCGTCTTCCTGGCGGCCGAGCTGCGCCTGGCGCACCGCGCCG     480
dolphin              GCGTGCTGCAGCTGCACTGCGTCTTCCTGGCGGCCGAGCTGCGCCTGGCGCACCGCGCCG     463
                     ************************************************************

pENTR1A-Dolphin      AGAGCCTGGGCCGCCTGGGCGGCGGCGTGGCGCAGGCCGAGCTCTATCTGGCGGCGCACG     540
dolphin              AGAGCCTGGGCCGCCTGGGCGGCGGCGTGGCGCAGGCCGAGCTCTATCTGGCGGCGCACG     523
                     ************************************************************

pENTR1A-Dolphin      GGTCACGCCTCAAGAAGGGCTCGCGCCGCGGCCGTCGCGGCCGCCCGCCCGCGCTGCTTG     600
dolphin              GGTCACGCCTCAAGAAGGGCTCGCGCCGCGGCCGTCGCGGCCGCCCGCCCGCGCTGCTTG     583
                     ************************************************************

pENTR1A-Dolphin      CCTCTGCGCTCGGTCTGGGCGGCTGCGTGCCCTGGGGCGCCGGGCGCCTGCGGCGGGGCC     660
dolphin              CCTCTGCGCTCGGTCTGGGCGGCTGCGTGCCCTGGGGCGCCGGGCGCCTGCGGCGGGGCC     643
                     ************************************************************

pENTR1A-Dolphin      ACTGCCCCGAGCCCGATTCGCCCTTCCGCCGGAGCCCGCCCCGCGGCCCCGCCTCCCCCC     720
dolphin              ACTGCCCCGAGCCCGATTCGCCCTTCCGCCGGAGCCCGCCCCGCGGCCCCGCCTCCCCCC     703
                     ************************************************************

pENTR1A-Dolphin      AGCGCTGACTCGAGATATCTAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAG     780
dolphin              AGCGCTGACTCGAG----------------------------------------------     717
                     **************

pENTR1A-Dolphin      CATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATT     840
dolphin              ------------------------------------------------------------     717
```

Figure 1. Sequencing of dolphin Trnp1 gene inserted into pENTR1A plasmid and alignment with the dolphin Trnp1 sequence.

```
pENTR1A-macaca      GTAGACATTCAGTCGACTGGATCCATGCCGGGCTGCCGCATCAGCGCCTGCGGCCCGGGG      60
macaca              ------------------GGATCCATGCCGGGCTGCCGCATCAGCGCCTGCGGCCCGGGG      42
                                      ****************************************

pENTR1A-macaca      GCCCAGGAAGGGACGGCAGAGCCGGGGTCGCCGCCGCCGCCCCGGGAGCCCATGCCGTTC      120
macaca              GCCCAGGAAGGGACGGCAGAGCCGGGGTCGCCGCCGCCGCCCCGGGAGCCCATGCCGTTC      102
                    ************************************************************

pENTR1A-macaca      TCTCAGCCCCCGCCCCCAACTCCGACCTTGACCCCTACCCCGACCCCGGGTCAGTCCCCG      180
macaca              TCTCAGCCCCCGCCCCCAACTCCGACCTTGACCCCTACCCCGACCCCGGGTCAGTCCCCG      162
                    ************************************************************

pENTR1A-macaca      CCGCTGCCGGACGCAGCCGGGGGCTTCGGCAGGCGCGGCCGAAGGCCAGGAGCTGCAGCGC      240
macaca              CCGCTGCCGGACGCAGCCGGGGGCTTCGGCAGGCGCGGCCGAAGGCCAGGAGCTGCAGCGC      222
                    ************************************************************

pENTR1A-macaca      TGGCGCCAGGGCGCTAGCGGGGTCGCGGGGCTCGCCGGCCCCGGAGGGGGCTCTGGCGCG      300
macaca              TGGCGCCAGGGCGCTAGCGGGGTCGCGGGGCTCGCCGGCCCCGGAGGGGGCTCTGGCGCG      282
                    ************************************************************

pENTR1A-macaca      GCGGCGGGGACAGGGGGCCGCGCGCTGGAGCTGGCCGAAGCACGGCGGCGGCTGCTGGAG      360
macaca              GCGGCGGGGACAGGGGGCCGCGCGCTGGAGCTGGCCGAAGCACGGCGGCGGCTGCTGGAG      342
                    ************************************************************

pENTR1A-macaca      GTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCCGCGTGCTGCAGCTGCACCGC      420
macaca              GTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCCGCGTGCTGCAGCTGCACCGC      402
                    ************************************************************

pENTR1A-macaca      GTTTTTTTGGCGGCTGAGCTGCGCCTGGCGCACCGCGCGGAGAGCCTGAGCCGCCTGAGC      480
macaca              GTTTTTTTGGCGGCTGAGCTGCGCCTGGCGCACCGCGCGGAGAGCCTGAGCCGCCTGAGC      462
                    ************************************************************

pENTR1A-macaca      GGCGGCGTGGCGCAGGCCGAGCTCTACCTGGCGGCTCACGGGTCGCGCCTCAAGAAGGGC      540
macaca              GGCGGCGTGGCGCAGGCCGAGCTCTACCTGGCGGCTCACGGGTCGCGCCTCAAGAAGGGC      522
                    ************************************************************

pENTR1A-macaca      CAGCGCCGGGGCCGTCGCGGCCGCCCCCCAGCGCTGCTGGCCTCGGCGCTGGGCCTGGGC      600
macaca              CAGCGCCGGGGCCGTCGCGGCCGCCCCCCAGCGCTGCTGGCCTCGGCGCTGGGCCTGGGC      582
                    ************************************************************

pENTR1A-macaca      GGCTGCGTGCCCTGGGGCGCCGGGCGACTGCGGCGCGGCCACGGCCCCGAGCCCGACTCG      660
macaca              GGCTGCGTGCCCTGGGGCGCCGGGCGACTGCGGCGCGGCCACGGCCCCGAGCCCGACTCG      642
                    ************************************************************

pENTR1A-macaca      CCCTTCCGCCGCAGCCCGCCCCGCGGCCCCGCCTCCCCCCAGCGCTGACTCGAGATATCT      720
macaca              CCCTTCCGCCGCAGCCCGCCCCGCGGCCCCGCCTCCCCCCAGCGCTGACTCGAG------      696
                    ****************************************************

pENTR1A-macaca      AGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTG      780
macaca              ------------------------------------------------------------      696
```

Figure 2. Sequencing of macaca Trnp1 gene inserted into pENTR1A plasmid and alignment with the macaca Trnp1 sequence.

```
pENTR1a-otolemur    GTAGACATTCAGTCGACTGGATCCATGCCGGGCTGCCGCATCAGCGCTTGCGGCCCCGGG    60
otolemur            ------------------GGATCCATGCCGGGCTGCCGCATCAGCGCTTGCGGCCCCGGG    42
                                      ****************************************

pENTR1a-otolemur    GCCCAGGAAGCGACGGCAGAACCGGGGTCCCCGCCGCCGCTGCCCCGGGAGCCCCTGCCA    120
otolemur            GCCCAGGAAGCGACGGCAGAACCGGGGTCCCCGCCGCCGCTGCCCCGGGAGCCCCTGCCA    102
                     ************************************************************

pENTR1a-otolemur    TCCCCTCAGCCCCCTCCCCCAACCCCGACCTTGACCCCGACCCCGACCCAGGTTCAGGCC    180
```

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otolemur              TCCCCTCAGCCCCCTCCCCCAACCCCGACCTTGACCCCGACCCCGACCCAGGTTCAGGCC    162
                       ************************************************************

pENTR1a-otolemur      CCGCAGCTGTCGGAGACGGCCCGGGAGTCAGCCGGTGTGGCCGAAGGGCAGGAGCTGCAG    240
otolemur              CCGCAGCTGTCGGAGACGGCCCGGGAGTCAGCCGGTGTGGCCGAAGGGCAGGAGCTGCAG    222
                       ************************************************************

pENTR1a-otolemur      CGCTGGCGCCAGAGCGCTAGCGGGGGCGCGGGGGTCACGGGGCCCGGTGGGGGCGCGGGC    300
otolemur              CGCTGGCGCCAGAGCGCTAGCGGGGGCGCGGGGGTCACGGGGCCCGGTGGGGGCGCGGGC    282
                       ************************************************************

pENTR1a-otolemur      TGCGGCTCGGGAGCGGCGGCTGGAGCTGGAGGCCGCGCGCTGGAGCTGGCCGAAGCGCGG    360
otolemur              TGCGGCTCGGGAGCGGCGGCTGGAGCTGGAGGCCGCGCGCTGGAGCTGGCCGAAGCGCGG    342
                       ************************************************************

pENTR1a-otolemur      CGGCGCCTGCTGGAGGTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCCGCGTG    420
otolemur              CGGCGCCTGCTGGAGGTGGAGGGCCGCCGGCGCCTGGTGTCGGAGCTGGAGAGCCGCGTG    402
                       ************************************************************

pENTR1a-otolemur      TTGCAGCTGCACCGAGTTTTCCTGGCGGCTGAGCTGCGCCTGGCTCATCGCGCTGAGAGC    480
otolemur              TTGCAGCTGCACCGAGTTTTCCTGGCGGCTGAGCTGCGCCTGGCTCATCGCGCTGAGAGC    462
                       ************************************************************

pENTR1a-otolemur      CTGGGCCGCCTGAGCGGCGGCGTGGCGCAGGCCGAGCTCTACCTGGCGGCGCACGGGTCG    540
otolemur              CTGGGCCGCCTGAGCGGCGGCGTGGCGCAGGCCGAGCTCTACCTGGCGGCGCACGGGTCG    522
                       ************************************************************

pENTR1a-otolemur      CGCCTCAAGAAGGGCCAGCGCCGTGGCCGCCGCGGCCGCCCACCCGCGCTGCTGGCTTCA    600
otolemur              CGCCTCAAGAAGGGCCAGCGCCGTGGCCGCCGCGGCCGCCCACCCGCGCTGCTGGCTTCA    582
                       ************************************************************

pENTR1a-otolemur      GCTCTCGGCCTGGGTAGCTGCGTGCCCTGGGGGTGGGGGGCGCCTGC-------------    647
otolemur              GCTCTCGGCCTGGGTAGCTGCGTGCCCTGGGGTGGGGGGCGCCTGCGGCGAGGCCACGGT    642
                       *******************************  *****   *

pENTR1a-otolemur      ------------------------------------------------------------    647
otolemur              CCCGAGCCCGATTCGCCCTTCCGCCGCAGCCCGCCCCGCGGCCCCGCCTCCCCCCAGCGC    702


pENTR1a-otolemur      ---------       647
otolemur              TGACTCGAG       711
```

Figure 3. sequencing of otolemur (galago) Trnp1 gene inserted into pENTR1A plasmid and alignment with the otolemur Trnp1 sequence.

9. Curriculum vitae

NAME AND SURANME: Nika Foglar

EDUCATION:
2017 – 2020: Master studies in molecular biology; Faculty of Science, University of Zagreb, Croatia; Graduation thesis: Effect of Trnp1 orthologs on proliferation of E14 mouse cortical primary culture
2014 – 2017: Bachelor studies in molecular biology; Faculty of Science, University of Zagreb, Croatia
2010 – 2014: High school education; II. Gymnasium, Zagreb, Croatia

RESEARCH EXPERIENCE:
03/2019 – 08/2019: Internship at the Department of Physiological Genomics, Ludwig-Maximilians University, Munich, Germany, Dr.sc. Miriam Esgleas
03/2017 – 09/2017: Biochemistry Laboratory Skill Training, University of Zagreb, Faculty of Science, Department of Chemistry - Division of Biochemistry, doc. dr. sc. Jasmina Rokov Plavec
04/2018 – 11/2018: Molecular Biology Laboratory Skill Training, University of Zagreb, Faculty of Science, Department of Biology - Division of Molecular Biology, izv. prof. dr. sc. Petra Korać

LANGUAGES:
Croatian (mother tongue), English (independent user; Intellecta certificate; B2)

GRANTS:
2019: ERASMUS + Student Mobility of Placement (SMP); Department of Molecular Biology, Faculty of Science, Ludwig-Maximilians University, Munich, Germany

PROJECTS:
2017: 9th Biology Night
2018: 10th Biology Night