The age-regulated zinc finger factor ZNF367 is a new modulator of neuroblast proliferation during embryonic neurogenesis

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Global population aging is one of the major social and economic challenges of contemporary society. During aging the progressive decline in physiological functions has serious consequences for all organs including brain. The age-related incidence of neurodegenerative diseases coincides with the sharp decline of the amount and functionality of adult neural stem cells. Recently, we identified a short list of brain age-regulated genes by means of next-generation sequencing. Among them znf367 codes for a transcription factor that represents a central node in gene co-regulation networks during aging, but whose function in the central nervous system (CNS), is completely unknown. As proof of concept, we analysed the role of znf367 during Xenopus laevis neurogenesis. By means of a gene loss of function approach limited to the CNS, we suggested that znf367 might act as a key controller of the neuroblast cell cycle, particularly in the progression of mitosis and spindle checkpoint. A candidate gene approach based on a weighted-gene co-expression network analysis, revealed fancd2 and ska3 as possible targets of znf367. The age-related decline of znf367 correlated well with its role during embryonic neurogenesis, opening new lines of investigation also in adult neurogenesis to improved maintenance and even repair of neuronal function.

The age-related incidence of many brain diseases coincides with a reduced adult neurogenic potential. The regenerative capability and the amount of adult neural stem cells (aNSCs) decline with age, contributing to the reduced functionality of the aged brain1. Despite the great interest in age related diseases, the molecular factors responsible for age-dependent decay of aNSCs function and the transition between stemness and differentiating properties of these precursors are almost unknown. Recently, we identified a set of evolutionarily-conserved genes expressed in aNSCs and age-regulated by RNA-seq analysis in the short-lived fish Nothobranchius furzeri, a well-established animal model in aging studies2. Among them, zinc finger protein 367 (znf367) was suggested to occupy a central position in a regulatory network controlling cell cycle progression and DNA replication. We found that znf367 is expressed in the adult brain of N. furzeri, where its RNA level decreases with age, and in neuroblast and retinoblast of developing Zebrafish embryos3. Znf367 belongs to the Zinc finger (ZNF) transcription factors family that represents a large class of proteins that are encoded by 2% of human genes4,5. Their functions include DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding6. Zinc finger proteins have an evolutionarily conserved structure and the ones containing the Cys2-His2 motif, constitute the largest family7. The function of the majority of ZNF genes is largely unknown, but some of them play a critical role in the development and differentiation of the nervous system. For instance, the Kruppel-like zinc finger transcription factor Zic has multiple roles in the regulation of proliferation and differentiation of neural progenitors in the medial forebrain and cerebellum8. The Ikaros family of transcription factors is characterized by two sets of highly conserved Cys2His2-type zinc finger motif and is involved in the maturation and differentiation of striatal medium spiny neurons9. Znf367 gene (also known as ZFF29 or ZFP367) has been initially isolated in human fetal liver erythroid cells10. In the human genome, this gene is on chr 9q11.21

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two alternative mRNA splicing products were identified and designated ZFF29a and ZFF29b. They both code for nuclear proteins, but only ZFF29b seems to act as an activator factor of erythroid gene promoters. In Human SW13 adrenocortical carcinoma cell line, znf367 is overexpressed and in this cell line Znf367 downregulation caused an increase of cellular proliferation, invasion and migration. Furthermore, znf367 was also identified as a potential tissue-specific biomarker correlated with breast cancer where its expression level is dysregulated influencing cell proliferation, differentiation and metastatic processes. To our knowledge, there are no data available regarding the putative role of znf367 in the Central Nervous System (CNS) during embryonic and adult neurogenesis. A recent research paper analyzed the transcriptome of different pools of nASC that comprise quiescent and activated neural stem cells in the mouse sub-ventricular zone. Interestingly in the Supplementary materials (Table S7), the authors compared the transcriptome of young (3–4 month-old) quiescent neural stem cells to the one obtained from old (19–22 month-old) quiescent neural stem cells and znf367 emerged among the genes significantly down regulated in the old mice. Despite this data confirmed that, even in mammals, znf367 is an age-regulated gene in the adult brain, its function in the CNS remained unknown. To shed light on the znf367 role in vertebrates CNS, we analyzed its function during neurogenesis in Xenopus laevis embryos. The clawed frog Xenopus is the favorite animal model to perform functional screening of genes. In Xenopus, it is possible to microinject mRNAs or morpholino oligos in just one side of the early cleaving embryo and compare, in each embryo, the manipulated side of the embryo with its wild-type counterpart that represents a perfect internal control. This unique vertebrate model also provide the possibility, to rapidly perform gene loss of function experiments in a tissue specific manner thanks to the well-defined fate map of each blastomere of the early cleaving embryo. This allowed us to target specific znf367 morpholinos to the central nervous system without interfering with the normal development of the other tissues. In this paper, we show that znf367 is expressed in the developing CNS in Xenopus and it could have a key role in primary neurogenesis and in the neuroblast progression of mitosis. These finding, together with the znf367 gene expression decline observed during CNS aging, lay the groundwork for future studies aimed to unveil znf367 role in adult neurogenesis and CNS aging.

Results

Embryonic expression analysis of znf367. To verify the evolutionary conservation of znf367 sequence in vertebrates we performed an in silico analysis of the amino acid sequences of ZNF367 in Homo sapiens (both splicing variants: ZFF29a and ZFF29b), Nothobranchius furzeri and Xenopus laevis (both splicing variants: znf367a and znf367b). This approach revealed a high conservation of znf367 with a 66% of identity between the human and Xenopus amino acid sequence that reached 98% at the level of the zinc finger domains (Fig. 1) suggesting a conserved putative znf367 function in vertebrates, from fish to tetrapod and primates. To analyze the spatio-temporal gene expression pattern of znf367, whole mount in situ hybridization (WISH) was performed on Xenopus embryos at different stages. Znf367 is expressed maternally in the animal pole in Xenopus embryos at blastula stage (Fig. 2A,B) when compared to sense control probe treated siblings (Fig. 2A). At neurula stage znf367 is expressed in the neural tube, in the eye fields, in the pre-placodal territory and in the neural crest cells (NCC) (Fig. 2C). At the tadpole stage, znf367 is widely expressed in the central nervous system, in the eye, in the otic vesicle and in the NCC migrated in the branchial pouches (Fig. 2D). At larval stages of development, znf367 is widely expressed in the CNS as shown in transverse sections (Fig. 2E,F).

Znf367 Knockdown inhibits neuronal differentiation in Xenopus laevis embryos. To investigate the znf367 function during neurogenesis in Xenopus, we performed knockdown experiments using a specific antisense oligonucleotide morpholino designed to block the translation of the endogenous mRNA (ZNF367-MO). For all the experiments described here, injections were performed unilaterally into one dorso-animal blastomere at the four-cell stage to target the neural tissue. The un-injected side served as an internal control and the co-injection with gfp (250 pg) RNA was used to screen for correctly injected embryos (Fig. 3A). The standard Gene Tools Control-morpholino (co-MO) was used to control for non-specific embryo responses. At neurula stage (st.18), znf367 morphants showed a strong reduction of cells expressing the markers of post-mitotic neurons
Although ZNF367-MO did not affect the expression of ngr1, a proneural marker necessary for the specification of primary neurons\(^\text{13}\) (N = 53) (Fig. 3H–H’), suggesting a role for znf367 during neuronal differentiation, but not in neuronal specification. The lack of differentiated primary neurons in znf367 morphants could be due to an increase in cell apoptosis during the differentiation process. To evaluate this aspect, we performed a TUNEL assay in znf367 morphants at the neurula stage and did not detect a significant increase in TUNEL positive cells between the znf367 injected side and the un-injected control side of each analyzed embryo (Fig. 3I).

**Znf367 knockdown increases proliferation markers in Xenopus laevis embryos.** To determine whether the observed loss of post-mitotic neurons in znf367 morphants was the consequence of impairment in the maintenance of the neuronal progenitors pool, we visualized cells expressing the stemness genes sox2 and rx1, in the injected embryos. Sox2 and rx1 are involved in maintaining neuroblast and retinoblast as cycling precursors in the neural plate\(^\text{14,15}\). The expression domains of sox2 and rx1 were expanded on the ZNF367-MO injected side of the embryo as compared to either the un-injected and Co-MO injected sides (Fig. 4A,B) indicating a larger population of progenitors. These data were confirmed by qRT-PCR analysis that showed a significant increase of both mRNAs in znf367 morphants (Fig. 4C). On the base of these results, we can suggest that the znf367 knockdown enhances self-renewal of progenitors at the expense of differentiation. For testing the specificity of the ZNF367-MO to induce this phenotype, we performed functional rescue experiments by co-injecting 9 ng ZNF367 MO together with 500 pg full-length Xenopus znf367 mRNA. We observed a restoration of the phenotype by 25% of rescue for znf367 (N = 114; 25% of rescue for elrC N = 100) (Fig. 4D). To further verify whether znf367 downregulation could alter the regulation of neuroblast proliferation, we also examined the mRNA expression of pcna (proliferating cell nuclear antigen) and we directly counted mitotically active cells marked by the anti-phosphorylated H3 (p-H3) antibody. Znf367 morphants showed an increased pcna mRNA expression both in WISH (Fig. 5A,B) and qRT-PCR experiments (Fig. 5C). The p-H3 staining showed a significant increase in mitotic cells number upon ZNF367-MO injection as compared to the control side (Fig. 5D,E). Given that a larger pool of neuroblasts did not correspond to an increased number of differentiated cells in the absence of apoptotic cell death, it is tempting to speculate that znf367 could be required to exit the M phase or control the mitotic checkpoint that precedes the anaphase. To test this hypothesis, we first evaluated the relative expression of cyclin B1 that is expressed predominantly during M phase of the cell cycle\(^\text{16}\), by qRT-PCR analysis of znf367 morphants. This experiment revealed a significant increase of cyclin B1 expression in znf367 morphants (Fig. 5F) indicating again that znf367 deficient neuroblasts could enter M phase, but they could not correctly exit mitosis and differentiate. The differentiation of neuronal progenitors requires the withdrawal from the cell cycle, driven by cell cycle inhibitors, such as pak3 (p21) and p27\(^\text{17,18}\). Concomitantly with the increase in mitotically active cells, a mild loss of p27 expression (phenotype 55%, N = 93) was observed in neurula morphants indicating that znf367 depleted neuroblasts are unable to exit the cell cycle (Fig. 5G–H’). These data led us to hypothesize that znf367 could be involved in the cell cycle exit and/or for the initiation of maintenance of a differentiated state. Finally, we examined morphants at the tailbud stage

Figure 2. Znf367 gene expression pattern during Xenopus laevis development. (A,B) Znf367 expression at blastula stage (stage 3) using sense control probe (A) and antisense probe (B). (C,D) At neurula (C) and at tadpole stages (D) znf367 is expressed in the neural tube (black arrow), the developing eye (black arrowhead), the neural crest cells (white arrowhead) in the otic vesicle (white arrow) and in the most anterior regions of the nervous system. (E,F) Stage 40 embryo is shown in lateral view (E) and in a transversal section at the level of the hindbrain (F). MB, midbrain; NCC, skeletogenic neural crest cells; RE, retina.
by performing WISH using *rx1* and *elrD* (also known as *HuD*). *ElrD* labels post-mitotic neurons in the neural tube and the developing cranial ganglia19. As stated above, *znf367* knockdown, but not control MO, caused an increase in *rx1* gene expression (52%, *N* = 72) (Fig. 5I) while inhibiting neuronal differentiation, thus affecting the
expression of *elrD* (54% N = 64) (Fig. S1L). These data showed that the effects of depletion *znf367* are not recovered even in the late phases of primary neurogenesis.

**Identification of putative Znf367 targets: a candidate gene approach.** Our previous results suggested that *znf367* represents a hub in the control of gene expression in the *N. furzeri* brain [5]. In order to test the conservation of this co-regulation across species, we analyzed CORTECON [20] a public dataset of RNA-seq during cortical differentiation of human embryonic stem cells (hESCs) using weighted-gene co-expression network analysis (WGCNA) [21]. WGCNA constructs co-expression networks based on topological criteria. It was shown to be more robust than simple correlation and it has become the method of choice for gene expression studies in the nervous system [22,23]. This method identifies gene modules that are sets of tightly co-expressed genes, within the modules hub genes are identified as the genes with highest number of connections (highest connectivity) and are the putative drivers of the coherent expression of the genes within the module [21]. We therefore tested the conservation of gene co-expression networks between *N. furzeri* and human neuronal differentiation *in vitro* (Fig. 6A). We identified a conserved module that contains *znf367* as a hub gene and then tested whether *znf367* can be considered a hub in both species by computing its connectivity. *Znf367* was among the top connected genes in the gene module in both species (98% percentile in *N. furzeri* and 92% percentile in human cells). Gene Ontology overrepresentation analysis revealed that cell cycle-related terms are highly overrepresented in this module. It should be also noted that all these genes have high expression in the hESCs, they are down-regulated during early differentiation, and show a peak of expression around 12 days of differentiation *in vitro* that correspond to the period of cortical specification [20]. Among the genes that showed the highest topological overlap, we noted enrichment in genes known to be involved in the progression of mitosis and in the mitotic spindle checkpoint (Fig. 6B). This corroborates the idea that *znf367* has a role in the control of cell cycle and it could be preeminent in mitosis, when the dividing cell has the fundamental task of correctly arranging the genetic content of the two daughter cells. To verify our hypothesis, we decided to test by qRT-PCR and by whole mount *in situ* hybridization the expression of three of the genes closest to *znf367* in the network (Fig. 6B). We analyzed the expression level of *smc2, ska3* and *fancd2* in *znf367* morphants. The *smc2* gene codes for one of the condensin components of the Structural Maintenance of Chromosomes (SMC) protein complexes, which play key roles in the regulation of higher-order chromosome organization and its role is crucial in the chromatin compaction in prophase [24,25]. *Ska3* is one of the spindle and kinetochore-associated (Ska) proteins required for accurate chromosome segregation during mitosis [52]. During mitosis the cyclin-dependent kinase Cdk1 phosphorylates Ska3 to promote its direct binding to the Ndc80 complex, (also present in the Znf367 network). This event is required for the overcoming of spindle checkpoint and the beginning of anaphase [56,57]. *FANCD2* encodes for a nuclear effector protein that is monoubiquitinated in response to DNA damage, targeting it to nuclear foci where it preserves chromosomal integrity [58]. Mutations in the FANC family are causative of the Fanconi Anemia in humans [59]. Greater than 60% of Fanconi anemia patients have developmental defects, such as growth retardation, short stature, microcephaly, and microphthalmia at birth, in addition to a highly elevated risk of bone marrow failure in the first decade of life [60]. This gene draws our attention as its knockdown in zebrafish embryos induced microcephaly, microphthalmia and pericardial edema [61]. It has been demonstrated that this factor is crucial for the S-phase rescue of damaged DNA, but also for the safeguarding of chromosome stability during mitosis [31,32]. The results obtained in three
independent experiments, showed a significant increase in fancd2 and ska3 gene expression in znf367 morphants. The smc2 gene expression level followed the same trend without statistical significance (Fig. 6C). These results were also confirmed by WISH experiments: the expression domains of fancd2 (50%, N = 50), ska3 (53%, N = 50) and smc2 (48%, N = 50) were expanded on the ZNF367-MO injected side of the embryo (Fig. 6D–F’).

These suggest that znf367 could have a major role in the control of chromosome stability and the functionality of the spindle check point.

**Discussion**

We chose Xenopus laevis as a model system to directly modulate the znf367 expression in the CNS without affecting other tissues and to unveil its role in tetrapods. Znf367 is expressed in the neural tissue of the early Xenopus laevis embryo including the eye field and in the neural crest cells. The spatial expression pattern suggested a role in the context of primary neurogenesis. This was further supported by the marked loss of post mitotic neurons upon knockdown of znf367, suggesting that znf367 could be essential for neuronal differentiation. In Xenopus znf367 morphants, we did not observe an increase in apoptosis rate suggesting that the loss of post-mitotic neurons was not due to unspecific morpholino toxicity or to a specific triggering of apoptotic pathways. Indeed, we found that the loss of znf367 function led to an increased expression of genes involved in the maintenance of
neuroblast and retinoblast as proliferating precursors. Accordingly, we observed a significant increase in the number mitotic cells in znf367 morphants. The co-ordinate regulation of cell proliferation and differentiation is of fundamental importance in the development of the central nervous system. At early developmental stages, a period of extensive proliferation is needed to generate the required number of progenitor cells for correct tissue and organ formation, accompanied or closely followed by differentiation. After the closure of the neural tube, the epithelial lining of the ventricles becomes specialized, consisting of a single sheet of progenitor cells (neuroepithelial cells). These cells undergo symmetrical cell divisions during the proliferative period to self-renew and expand the pool of progenitors. The subsequent transition from a proliferative neural precursor cell to a post-mitotic neuron is a highly regulated step, which, in many instances, has been shown to involve a cascade of transcription factors that is triggered by pro-neural genes. Differentiation of neural progenitor cells requires withdrawal from the cell cycle, which is regulated by the expression of cell cycle inhibitors, such as p27 in Xenopus. Consistent with the increase in mitotically active cells, a reduced p27 expression was observed in znf367 morphants, thus raising the possibility that the neural progenitors are prevented from undergoing differentiation because they are not able to exit the cell cycle, remaining in an undifferentiated state. Znf367 morphants also expressed high levels of cyclin B1, which is required to drive cells into mitotic division, but that must be degraded to allow anaphase. Again, this datum corroborates our hypothesis that znf367 deficient neuroblasts could enter M phase, but they...
could not correctly exit mitosis and differentiate. Given the requirement of znf367 for both proliferation and neuronal differentiation of neuroectodermal cells, it is plausible that znf367 could be required to exit M phase or in the control of the spindle checkpoint that precedes anaphase. To have a wider view on the molecular mechanisms potentially regulated by znf367, we performed a correlation-based network analysis testing the conservation of gene co-expression networks between N. furzeri brain and human neuronal differentiation in vitro, identifying a conserved module that contains znf367. To this purpose we analysed the public RNA-seq database CORTECON. CORTECON reports transcript expression levels for all stages of in vitro differentiation from hESCs to cortical neurons, therefore capturing even the earliest stages of neurogenesis comparable to the stages analysed here in Xenopus.

We noted enrichment in genes involved in the regulation of the cell cycle and specifically in the progression of mitosis and mitotic spindle checkpoint point. The involvement of znf367 in the control of cell cycle is supported by functional studies that demonstrated its implication in regulating different aspects of cancer progression. Some of the genes, correlated to znf367 in our correlation analysis are both implicated in CNS development as well as in cancer initiation and/or progression. Among these, we decided to closely analyze the relation between znf367 and smc2, sk3 and fancd2. SMC2 is part of the condensing complex required for the structural and functional organization of chromosomes. Its role is crucial in the chromatin compaction in the prophase. In our functional study the loss of znf367 seemed to interfere with smc2 mRNA level, but even if smc2 mRNA seemed to be more abundant in znf367 morphants than in controls, the results are suggestive of a trend, but not statistically significant. Fancd2 is essential during zebrafish CNS development to prevent neural cell apoptosis during neuroblast proliferative expansion. FANCD2, when mutated, is one of the causative genes of Fanconi anemia, an inherited disorder characterized by developmental defects, progressive bone marrow failure, and predisposition to cancer. In particular, FANCD2 in potential and adult life is required for the functional maintenance of the hematopoietic stem cell pool. The link between znf367 and fancd2 seems therefore particularly intriguing since the znf367 function seemed to be required to repress fancd2 expression and allow cells to inactive the spindle checkpoint and proceed towards differentiation. The level of fancd2 mRNA is significantly up regulated in znf367 morphants. It is tempting to speculate that during primary neurogenesis in Xenopus znf367 could regulate fancd2 expression levels in order to define the pool of neuroblast and coordinate the cell cycle exit necessary for post-mitotic differentiation. SKA3 is one of the spindle assembly checkpoint proteins. SKA3 is strongly associated with kinetochores during prometaphase and metaphase, while being diminished during anaphase and lost in telophase. Its major role is to contribute to the silencing of spindle checkpoint during metaphase and to the maintenance of chromosome cohesion in mitosis. In our znf367 morphants, ska3 is upregulated, supporting the idea that znf367 could play a key role in the control of mitosis and in particular during metaphase. As ska3 has to be downregulated to allow the progression towards anaphase and telophase. A possible hypothesis we can formulate is that the loss function of znf367 could maintain abnormally high levels of ska3, keeping cells blocked in mitosis (metaphase). This analysis provided us with a deeper view of the possible action of znf367 during neurogenesis but deeper analysis, necessary to shed light on the direct targets of znf367 and their function, should be addressed in the future.

In conclusion, we unveiled a role for znf367 during neurogenesis in vertebrates. In particular, znf367 emerged as a key controller of the neuroblast cell cycle, and it seemed to act regulating the events that are strictly controlled during the metaphase to allow the progression of the cell cycle and the onset of anaphase. The observed age-related down-regulation of znf367 correlated with the age-related decline of quiescent aNSC to activate and give rise to new neural progenitors. Our data shed light on the role of znf367 during neural progenitors development giving proof of concept of the continuity of molecular control in developing and adult neurogenesis. It will be of interest for future studies to identify both the upstream regulators and the downstream effectors of znf367. This is important not only due to the requirement of znf367 during X. laevis neurogenesis, but more generally for the identification of the molecular factors involved in neuronal progenitors cell cycle exit and differentiation. If our findings will be validated also in adult neurogenesis they could represent the first step in defining new strategies to increase adult neurogenesis, leading to improved maintenance of functional aNSC.

**Methods**

**Synteny analysis of znf367.** Synteny analysis was performed using the NCBI GeneBank for the following organisms: *Xenopus laevis* znf367a (NP_001085362.1); *Xenopus laevis* znf367b (XP_018114684 PREDICTED); *Homo sapiens* ZFF29A (AY554164.1) and *Homo sapiens* ZFF29B (AY554165.1); *Nothobranchius furzeri* (HADY0111608.1).

**Embryo preparation.** Animal handling and care were performed in strict compliance with protocols approved by Italian Ministry of Public Health and of the local Ethical Committee of University of Pisa (authorization n.99/2012-A, 19.04.2012). *Xenopus laevis* embryos were obtained by hormone-induced laying and in vitro fertilization then reared in 0.1 X Mar’s Modified Ringer’s Solution (MMR 1×: MgCl₂, 5 mM HEPES pH 7.5) till the desired stage according to Nieuwkoop and Faber.

**Morpholino oligonucleotides, cloning and microinjections.** ZNF367 antisense Morpholino oligonucleotides (MO) and a standard Control MO were provided by Gene Tools, Philomath, OR, USA. ZNF367 MO sequence: 5′-CACGCTATCTGACATTTGTTACTAC-3′. Co MO sequence: 5′-CCTCTATACCTACGGTACAATTTATA-3′. Microinjections were performed as described previously. Injected MO amounts were: 9 ng ZNF367 MO and 9 ng Control MO. Correct injections were verified by co-injected of 250 pg of GFP mRNA and using a fluorescence microscope. The un-injected side represents an internal control in each embryo. For functional rescue experiments, the open reading frame of *X. laevis* znf367 (XM_018259195.1 PREDICTED) was cloned into the pCS2+. For Rescue experiments, 9 ng ZNF367 MO and 500 pg full-length znf367 mRNA were co-injected. Higher doses of morpholino, 12 or 15 ng per embryos, were
tested. Morphants injected with 12 or 15 ng of morpholino showed the described phenotype of altered neurogenesis but also defects in the neural tube closure that were not efficiently rescued by the injection of znf367 mRNA (not shown). For this reason all the experiments showed in the results section were performed at the 9 ng ZNF367 MO dose. Capped znf367 mRNA was obtained using the MegaScript in vitro transcription kit (Ambion), according to manufacturer’s instructions.

In situ hybridization (ISH) experiments. Whole mount in situ hybridization (WISH) approaches was performed as described42. BM purple (Roche) was used as a substrate for the alkaline phosphatase; digoxigenin-11-UTP-labelled sense, and antisense RNA probes were generated via in vitro transcription. After color development, embryos were post-fixed and bleached over light to remove the pigment. For ISH on cryosections (12 μm), embryos were fixed in 4% paraformaldehyde (PFA) in PBS, cryoprotected with 30% sucrose in PBS and embedded in Tissue-Tek O.C.T. compound (Sakura, 4583). ISH on cryosections was performed as described42. Unpublished new plasmids for in situ hybridization were generated as follows: X. laevis znf367 EST clone (ID: 6637026) was cloned in pBSK-vector. X. laevis-Ska3 (NM_001127749), fancd2 (NM_AY633665) and smc2 (NM_001087904) were obtained by RT-PCR and cloned in pGEM-T vector. The following plasmids were used for preparation of antisense RNA probes, enzyme used for linearization and polymerases are indicated: X. laevis znf367 EST-pBSK- (Xhol, T7); pcna-pBSK (SalI, T7); sox2-pCS2+ (EcoRI, T7); n-tubulin-pBSK (NotI, T3); elrC-pBSK (NOTI, T7); elrD-pBSK (Xhol, T3); rxi42; nrg1-pBSK (BamHI, T3); p27-Pbsk (BamHI, T7). X. laevis fancd2-pGEM-T (Clal; Sp6); X. laevis ska3-pGEM-T (Ncol; Sp6); X. laevis smc2-pGEM-T (Ncol; Sp6).

TUNEL and PH3 staining in Xenopus. TUNEL (TdT-mediated dUTP-dig nick end labeling) and PH3 (phospho histone 3) staining was performed at neurula stage according to established protocols41,43. TUNEL and PH3 positive cells were counted within defined areas in control and injected side of each manipulated embryo. P-values were calculated by paired Student’s T-test using GraphPad Prism 6 software (San Diego, CA, USA). Statistical significance was indicated as: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from 30 Xenopus morphants (at stage 18) using Nucleospin RNA (Macherey-Nagel) according to the manufacturer’s instruction. cDNA was prepared by using iScript cDNA Synthesis Kit (Bio-Rad) and quantitative real-time PCR was performed using GoTaq qPCR master mix (Promega) according to the manufacturer’s instruction. Relative expression levels of each gene were calculated using the 2−ΔΔct method44 and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used to perform qRT-PCR: pcna45 (Forward: 5′-CCTCGGCTAGGATACCCAATCCTTT-3′; Reverse: 5′-TGCACACAGGGGCAGCAGAG-3′); N-tubulin and sox2 (2′ (De Robertis’ lab, web site: http://www.hhmi.edu/derobertis/); elrC46 (Forward: 5′-GCTTTCATGCCCTCCAGGTT-3′; Reverse: 5′-GCAACAGGAGACTCTCATC-3′); bryt (Forward: 5′-TGAACGGGCCTTCAATGTGTCG-3′; Reverse: 5′-TGACCTCCTAGGGCAGAGA-3′); sox2 (De Robertis’ lab, web site: http://www.hhmi.edu/derobertis/); elrC46 (Forward: 5′-GCTTTCATGCCCTCCAGGTT-3′; Reverse: 5′-GCAACAGGAGACTCTCATC-3′); bryt (Forward: 5′-TGAACGGGCCTTCAATGTGTCG-3′; Reverse: 5′-TGACCTCCTAGGGCAGAGA-3′); sox2 (De Robertis’ lab, web site: http://www.hhmi.edu/derobertis/); elrC46 (Forward: 5′-GCTTTCATGCCCTCCAGGTT-3′; Reverse: 5′-GCAACAGGAGACTCTCATC-3′); bryt (Forward: 5′-TGAACGGGCCTTCAATGTGTCG-3′; Reverse: 5′-TGACCTCCTAGGGCAGAGA-3′); sox2 (De Robertis’ lab, web site: http://www.hhmi.edu/derobertis/); elrC46 (Forward: 5′-GCTTTCATGCCCTCCAGGTT-3′; Reverse: 5′-GCAACAGGAGACTCTCATC-3′); bryt (Forward: 5′-TGAACGGGCCTTCAATGTGTCG-3′; Reverse: 5′-TGACCTCCTAGGGCAGAGA-3′).

Statistics. Statistical analysis for qRT-PCR experiments were performed by Student’s T-test using GraphPad Prism 6 software (San Diego, CA, USA). The levels of mRNA expression for individual genes were evaluated for Control-Morpholino or ZNF367-Morpholino injected embryos by qRT-PCR. The results obtained in three independent experiments were normalized to the expression of the housekeeping gene, gapdh. The mean of the Control-Morpholino was set 1. The relative mRNA levels were calculated using the comparative Ct method (2−ΔΔct). Statistical significance was indicated as: *p ≤ 0.05.

Statistical analysis for the phenotypes observed after the injection of the Control-Morpholino or the injected ZNF367-Morpholino were performed by Student’s T-test using GraphPad Prism 6 software (San Diego, CA, USA). We compared the percentage of the embryos with altered markers gene expression between the Control-Morpholino injected embryos and the ZNF367-Morpholino injected embryos. Statistical significance was indicated as: ***p ≤ 0.001, ****p ≤ 0.0001.

WGCNA (Weighted Gene Co-expression Network Analysis). Network analysis was performed using WGCNA method45. Samples used for the workflow were derived from two independent datasets, one from Nothobranchius furzeri’s brain, comprehensive of two strains (MZM-04010 and GRZ), six different time points and five replicates per time point20, and the other one from human embryonic stem cells. In particular the latter was obtained from a cerebral-cortical developmental experiment performed on hESC with 9 different time points20.

Network analysis was performed through different steps:

- Setting of the soft threshold, coefficient necessary for the adjacency matrix construction, as shown in the formula:

\[ a_q = |\text{cor}(x_q, x_i)|^\beta \]

- Adjacency matrix and TOM (Topological Overlap Matrix), defined as:
Network construction was done in two independent analyses: the first one only on *Nothobranchius furzeri* dataset, while the latter using a consensus network obtained matching the two datasets. As soft threshold, we chose $\beta = 6$ for both the analyses to obtain the correspondent adjacency matrix and TOM, and significant modules negatively correlated with *N. furzeri* brain aging were selected. The genes contained in the selected modules were then tested for GO analysis using WebGestalt software, and then visualized using Cytoscape. Finally, the overall module membership of the genes contained in the "turquoise" module (as specified above, and only for the second analysis) was plotted on the ranked genes for both the killifish and the human data.

Data availability. All data generated or analysed during this study are included in the manuscript.

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Author Contributions

V.N., S.M., D.C. performed all the Xenopus experiments. M.T.M. and A.C. performed WGCNA (Weighted Gene Co-expression Network Analysis), contributed in the manuscript discussion and writing. M.O. contributed to conceptualization, provided necessary financial resources, experimental supervision, data analysis and discussion, writing.

Additional Information

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