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Agr genes, missing in amniotes, are involved in the body appendages regeneration in frog tadpoles

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Previous studies have shown that *Agr* genes, which encode thioredoxin domain-containing secreted proteins, play a critical role in limb regeneration in salamanders. To determine the evolutionary conservation of *Agr* function, it is important to examine whether *Agrs* play a similar role in species with a different type of regeneration. Here, we refined the phylogeny of *Agrs*, revealing three subfamilies: *Ag1*, *Agr2* and *Agr3*. Importantly, we established that *Ag1* was lost in higher vertebrates, which correlates with their decreased regeneration ability. In *Xenopus laevis* tadpoles (anamniotes), which have all three *Agr* subfamilies and a high regenerating capacity, *Agrs* were activated in the stumps of tails and hindlimb buds that were amputated at stage 52. However, *Agrs* were not up-regulated when the hindlimb buds were amputated at stage 57, the stage at which their regeneration capacity is lost. Our findings indicate the general importance of *Agrs* for body appendages regeneration in amphibians.

Genes of the *Anterior gradient* (*Agr*) family were first identified in *Xenopus laevis* embryos, in which they are expressed in the anterior ectoderm beginning in the early gastrula stage and later in the cement and hatching glands[1,2]. *Agrs* encode for proteins belonging to the superfamily of protein disulphide isomerases (PDI), which contain the core thioredoxin domain and accelerate the folding of other proteins via the regulation of disulphide bond formation[3]. Among other members of this superfamily, the majority of which are located intracellularly, Agrs attract substantial attention because of their ability to be secreted from the cell and to regulate cell growth and differentiation, particularly in such processes as embryonic development and carcinogenesis[4–6]. In addition, the key role of one of Agrs, nAG/Agr2, as a regulator of limb regeneration in salamanders has recently been shown[7,8]. In newts, nAG interacts with the membrane receptor Prod1, which results in the activation of ERK1/2 MAP kinase signalling in blastema cells[9]. Curiously, however, no obvious homologs of Prod1 were found in other vertebrates. Accordingly, the question arises regarding whether the role of nAG as a regulator of regeneration may be universal for its homologs in other vertebrates. A promising way to answer this question is to examine the correlation between *Agr* gene expression and the processes of body appendage regeneration in other vertebrates.

As a suitable model for our present study, we chose *Xenopus laevis* tadpoles, which have a pronounced capacity to regenerate amputated hindlimb buds between stage 43 and stage 57[10,11]. Importantly, at later stages, this hindlimb regeneration ability disappears[11]. In addition, tadpoles can effectively regenerate their tails. Significantly, by contrast to the limb buds, tail regeneration is effective, except for a small refractory period between stage 46 and 47, throughout the tadpole's life, until the end of metamorphosis.

Before analysing *Agrs* expression during regeneration, we re-examined the phylogeny of these genes in vertebrates by using novel genomic data that recently appeared in the Ensembl and GeneBank databases. Such an analysis has been justified by the considerable confusion existing in the literature regarding the terminology and orthologous relationships between *Agrs* in different species. As a result, we established that there are three monogenic subfamilies of *Agrs* in vertebrates, *Ag1*, *Agr2* and *Agr3*, which are in two syntenic fragments, *Ag1* and *Agr2/Agr3*. Importantly, *Ag1* genes were lost in amniote ancestors; this fact correlates with a decreased ability to regenerate body appendages in higher vertebrates.

Using qRT-PCR, we have demonstrated that amputation of the hindlimb buds and tails causes a sharp increase in the expression of representatives of two revealed syngenic groups of *Agr*: *Xag2*, *Xagr2a and Xagr3a*. Importantly, the dynamics of this increase in expression correlate well with the dynamics of the regenerative capacity at each stage: increased *Agrs* expression is observed in the blastema cells of the hindlimb bud amputated

at stage 52; however, *Agrs* expression is not detected during the disappearance of the regenerative capacity at stage 57. At the same time, increased *Agrs* expression in the tail stumps was detected regardless of whether the amputation was performed at stage 52 or at stage 57. We also confirmed these findings by performing in situ hybridisation with *Xag2* and *Xagr2a* probes and by the in vivo monitoring of regeneration in transgenic tadpoles expressing EGFP under the control of the *Xag2* promoter. Our findings, together with the data obtained in salamanders, indicate that *Agrs* has a universal role in body appendage regeneration in amphibians.

Results

Phylogeny of Agrs. Currently, considerable confusion exists in the literature concerning the terminology and the phylogenetic relationships between *Agr* genes in different classes of vertebrates. Therefore, before beginning our expression study, we undertook a wide screening of the available on-line databases to clarify the evolutionary connections between different members of this family. In addition to ordinary screening of the genetic databases for sequences homologous to known *Agrs*, we also analysed local synteny of *Agrs*-containing loci in 17 amniotic and 6 anamniotic species with assembled genomes (available on www.ensembl.org site), paying special attention to ascertain the orthologous relationships between members of *Agr* family in different species. The following species were analysed in this way: fishes (*Danio rerio, Gasterosteus aculeatus, Oryzias latipes, Takifugu rubripes, Tetraodon nigroviridis*); amphibians (*Xenopus tropicalis*); reptiles (*Anolis carolinensis, Pelodiscus sinensis, Chrysemys picta bellii*); birds (*Gallus gallus, Meleagris gallopavo, Melopsittacus undulatus, Taeniopygia guttata*); and mammals (*Bos taurus, Gorilla gorilla, Homo sapiens, Ictidomys tridecemlineatus, Macropus eugenii, Monodelphis domestica, Mus musculus, Ornithorhynchus anatinus, Pan troglodytes, Sus scrofa*).

As a result, we established that there are three subfamilies of *Agrs*, *Ag1*, *Agr2* and *Agr3*, whose nearest homologs are genes encoding non-secreted PDI of the TLP19 subfamily (see the alignment and phylogenetic tree of these proteins for selected species of the main classes of vertebrates in **Fig. 1 and 2A,** respectively). Members of two subfamilies, *Agr2* and *Agr3*, are present only in amphibians and higher vertebrates and are located close to one another in the same syntenic fragment with such genes as *Snx13* (*sorting nexin 13*), *Ahr* (*aryl hydrocarbon receptor*), *Tspan13* (*tetraspanin 13*), *bzw2* (*basic leucine zipper and W2 domains 2*) and *Ankmy2* (*ankyrin repeat and MYND domain containing 2*). Notably, there is only one *Agr2/3* gene in fish genomes; this gene is most homologous to *Agr2* of higher vertebrates (**Fig. 2B**).

Genes of the third *Agr* subfamily, *Ag1*, are located within another syntenic fragment with such genes as *Usp40* (*ubiquitin specific peptidase 40*), *Trafi31* (*TNF receptor-associated factor 3 interacting protein 1*), *Asb1* (*ankyrin repeat and SOCS box containing 1*), *Hdac4* (*histone deacetylase 4*) and *ANM2* (*DIP2 disco-interacting protein 2 homolog A*). Surprisingly, orthologs of Ag1 are seemingly absent in reptiles, birds and mammal but are present in fishes and amphibians, i.e., in species that are characterised by highly regenerative abilities (**Fig. 2B**). Indeed, all the amniotic sequences that were found during our screening and that demonstrate protein homology with Agrs equal to or higher than any member of the TLP19 family ($\geq$ 35%) are firmly clustered either with Agr2, Ag3 or TLP19 branches, but not with Ag1. Accordingly, in all tested amniotic species whose genomes were assembled, all such sequences (having protein homology with Agrs $\geq$ 35%) are in *Agr2*, *Ag3* or *TLP19* syntenic loci. All amniotic protein sequences demonstrating homology with Agrs less than 35% appeared to be those that were previously described as belonging to other families not related to Agrs. Finally, as we revealed, none of the analysed amniotes with assembled genomes have genes that would be homologous to Agrs in the genomic region syntenic with the *Ag1*-containing regions of anamniotic-assembled genomes. Notably, in some amniotic species (in *Meleagris gallopavo* and *Homo sapiens*, for example), the region syntenic to the *Ag1*-containing locus of anamniotes still contains fragmentary sequences that are disrupted by stop codons and homologous to *Agrs*. Obviously, this fact could be considered to be further evidence in favour of our hypothesis that functional *Ag1* genes are not present in amniotes.

Study of *Xag2* and *Xagr2a* expression by qRT-PCR. In considering the data from our phylogenetic analysis, it would be important to study the expression of representatives of both syntenic groups of *Agr* genes during regeneration in *Xenopus laevis* tadpoles. There are three pairs of extremely homologous pseudoalleles of these genes in the tetraploid *Xenopus laevis* frog: *Xag1*/*Xag2*, *Xagr2a*/*Xagr2b* and *Xagr3a*/*Xagr3b* (more than 90% identity between pseudoalleles in each pair). Therefore, to conduct the expression analysis in the most economical way, we have chosen only three of these genes for our study, namely, *Xag2*[1] and *Xagr2a*[2] and *Xagr3a*, which represent both syntenic subgroups of the *Agr* family.

Pieces of stumps of hindlimb buds and tails of tadpoles that were amputated at stage 52 and at stage 57 were analysed by qRT-PCR to determine the expression of these *Agr* genes, as shown in **Fig. 3A**. We established that the expression of all *Agrs* sharply increased in the distal parts of stumps of both hindlimb buds and tails amputated at stage 52 at the first day post-amputation (1 dpa) (**Fig. 3B**). Notably, a decrease in this high expression was observed at 2 dpa, followed by expression equal to the control background value at 5 dpa (**Fig. 3B**). Similar dynamics were revealed for the expression of *Xag2* and *Xagr2a* in the tail stumps. By contrast, we were unable to detect any increase in *Xagr3a* expression in the tail stumps. It should be also noted that the expression level of *Xag3a* was much lower than that of *Xag2* and *Xagr2a*. Thus, whereas the PCR efficiency of all three *Agrs* tested was approximately equal and close to 2 (see Methods), the signal generated by *Xagr3a* cDNA appeared, on average, 6 cycles later (at 27–30 cycles) than that generated by *Xag2* and *Xag2a* cDNA (at 21–24 cycles).

Importantly, whereas substantial activation of expression of *Xag2* and *Xagr2a* was still observed in stumps of tails amputated at stage 57, no such activation was observed in the hindlimb bud stumps amputated at this stage (**Fig. 3B**).

Study of *Agrs* expression by whole-mount in situ hybridisation. To verify the results of qRT-PCR analysis by another method, we assessed the expression of *Xag2, Xagr2a* and *Xagr3a* by whole-mount in situ hybridisation.

The expression of *Xag2* and *Xagr2a* was detected at a low level in the epidermis of the control hindlimb buds (**Fig. 4A and F**) and tails (not shown). Simultaneously, an obvious increase of the hybridisation signal was observed at 1 dpa in the distal part of stumps (**Fig. 4B and G, D and I**). By contrast, no expression of *Xagr3a* was detected even at the 4th day of staining in the intact or in the amputated hindlimb buds and tails (**Fig. 4K and L, N and O**). Obviously, these results correlate well with the data from our qRT-PCR analysis, which revealed low expression levels of *Xagr3a* (see above). Moreover, *Xag3a* transcripts were revealed in the cement gland and otic vesicle at the late tailbud stage, which confirms the operability of the *Xagr3a* probe (**Fig. 4P and Q**).

Notably, as was revealed by histological sections, an increased concentration of *Xag2* transcripts was observed both in cells of the epidermis and in the distal blastema cells located beneath the epidermis (**Fig. 4C and E**). Simultaneously, a high level of *Xagr2* transcripts was observed primarily in cells of the distal epidermis (**Fig. 4H and J**). No expression was observed with *Xag2* and *Xagr2* sense probes (**Fig. 4R–V and not shown**).

***In vivo* tracing of EGFP expression under the control of *Xag2* promoter during regeneration in transgenic tadpoles.** Using transgenic tadpoles expressing fluorescent proteins (FP) under the control of promoters of the genes of interest is an effective approach

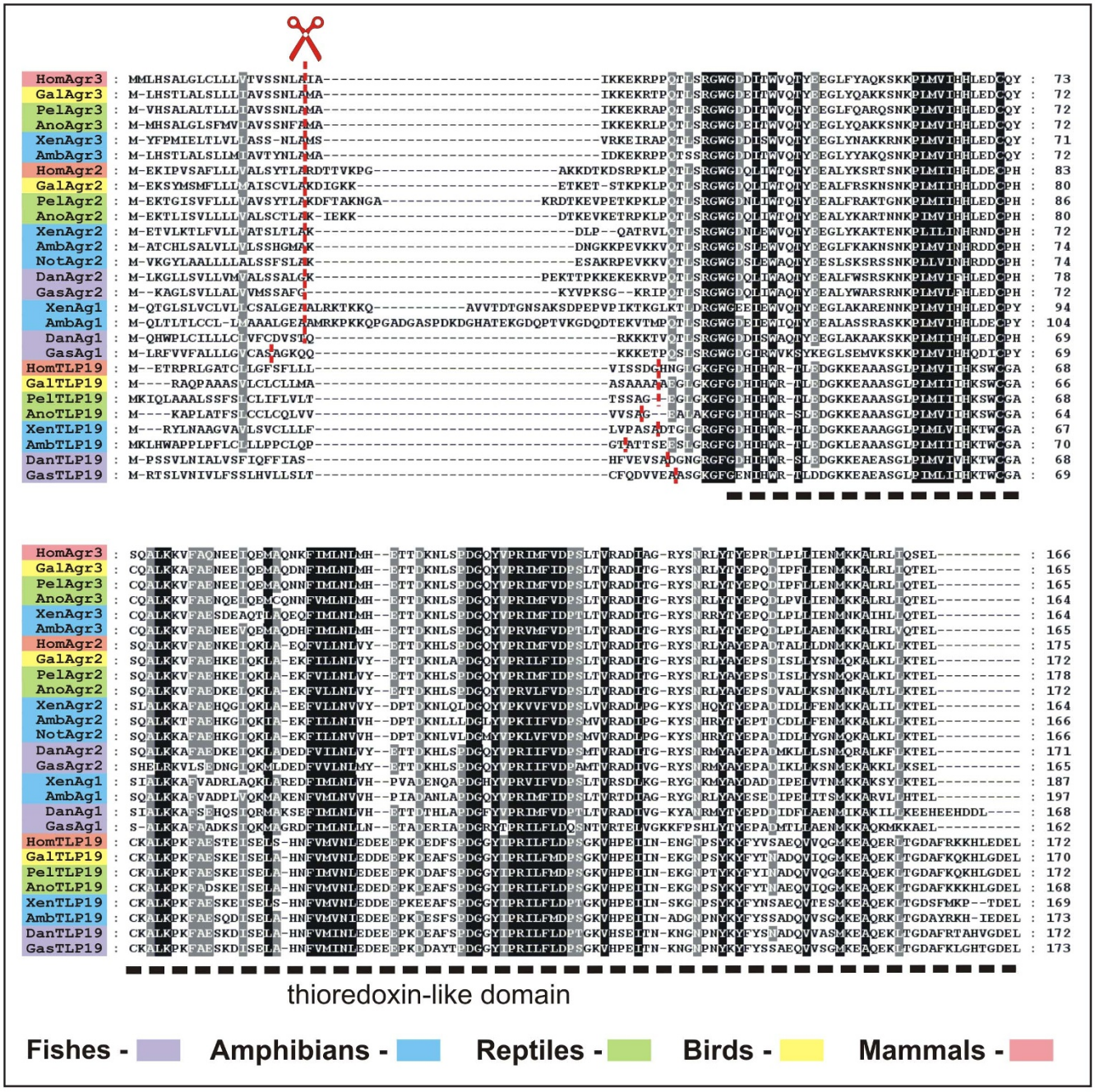

Figure 1 | **Clustal alignment of Agr genes.** Putative cleavage sites for signalling peptides predicted by Signal-P program (http://www.cbs.dtu.dk/services/SignalP/) are indicated by a red dotted line. Highly conservative aa positions are marked by black, and less conservative aa positions are marked by grey shading. Different classes of vertebrates, whose proteins were aligned, are highlighted by different colours (see decoding of colours at bottom). Abbreviations: **Amb** – *Ambistoma maculatum* (salamander) (GeneBank accession numbers: Ag1 - KC253227, Agr2 - KC253228; Agr3 - KC253230; TLP19 - KC253229); **Ano** – *Anolis carolinesis* (lizard) (GeneBank accession numbers: Agr2 - JX854521; Agr3 - JX854522; TLP19 - JX854523); **Dan** –*Danio rerio* (fish) (GeneBank accession numbers: Ag1 - JX566722, Agr2 - AY796218; TLP19 – DQ28661); **Gal** – *Gallus gallus* (chick) (GeneBank accession numbers: Agr2 - XM418698, Agr3 - NM001199613, TLP19 – XM001235372); **Gas** –*Casterosteus aculeatus* (fish) (GeneBank accession numbers: Ag1 - JX854524, Agr2 - JX854525; TLP19 – JX854526); **Hom** – *Homo sapiens* (GeneBank accession numbers: Agr2 - BT007048, Agr3 - AY069977, TLP19 – BC008913; **Not** – *Notophthalmus viridescens* (newt) (GeneBank accession numbers: Agr2 - EF667357); **Pel** – *Pelodiscus sinensis* (turtle) (GeneBank accession numbers: Agr2 - JX854518, Agr3 - JX854519, TLP19 – JX854520); and **Xen** – *Xenopus tropicalis* (frog) (GeneBank accession numbers: Ag1 - NM213699, Agr2 - NM001016627; Agr3 - NM001006908; TLP19 - CT030507).

that allows one to study gene expression dynamics in vivo. In addition, as this approach is based on a different principle than that of qRT-PCR or in situ hybridisation, it can be used as an independent control for these traditional methods.

In the present work, we analysed the expression pattern of EGFP driven by *Xag2* promoter during the regeneration of the hindlimbs and tails of transgenic tadpoles. The line of frogs bearing this and other transgenic constructs expressing green and red FPs under the

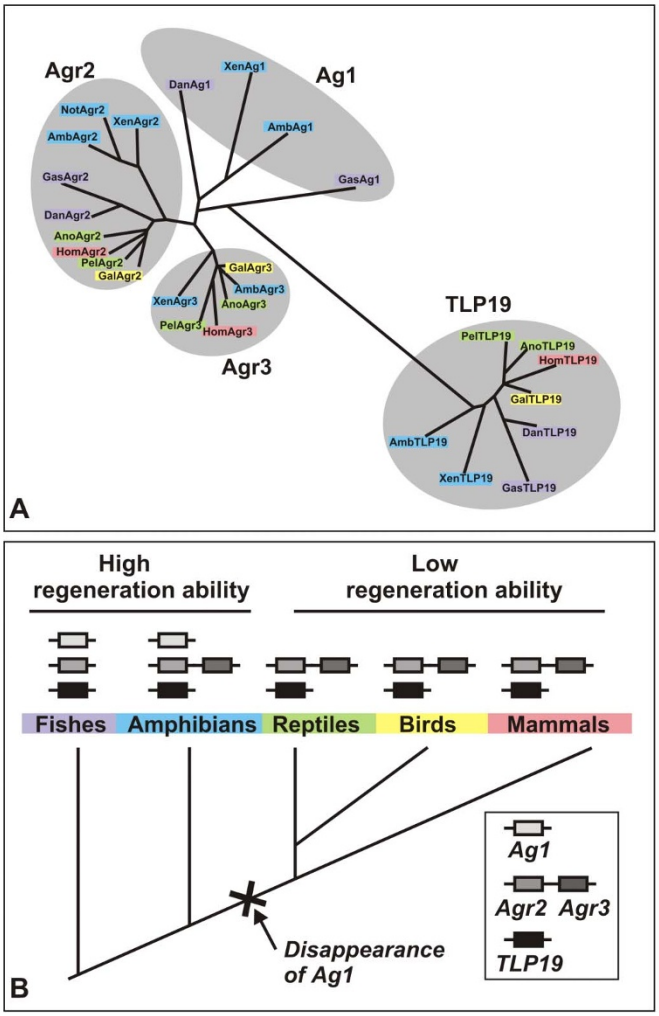

Figure 2 | Phylogeny of Agr genes. (A). Phylogenetic unrooted tree with branch length for Ag1, Agr2, Agr3 and TLP19 proteins shown in Fig. 1. (B). Evolution of different subfamilies of *Agrs* in vertebrates.

control of *Xag2* promoter was generated previously and maintained in our laboratory[12].

As shown in **Fig. 5A and A′**, *Xag2-EGFP* transgene expression appeared to be activated already in the wound epidermis at the distal parts of the hindlimb bud and tail stumps 12 hours after amputation. Notably, single cells expressing EGFP were also observed in the tail outside the regenerating blastema (**Fig. 5C and C′**). No such effects were observed in the intact appendages (**Fig. 5B and B′**). The EGFP signal was still strong in the distal parts of stumps during the next 2 days of regeneration; however, the signal entirely disappeared in cells of the regenerating hindlimb and tail by 7th day post-operation (not shown).

Taking advantage of the fact that the regenerative capacity of the hindlimb bud is lost by stage 57, whereas it continues to be effective in the tail (Beck et al., 2009), we performed a series of amputations specifically at this stage to verify a possible correlation between the expression of *Xag2-EGFP* transgene and the ability of appendages to regenerate.

By contrast to the operation performed at the stage of 52, EGFP signal was not observed on either the 1st or 2nd day when the hindlimb bud was amputated at stage 57 (**Fig. 5D and D′**). Notably, as in the previous series of experiments, expression of EGFP was detected in the blastema and the adjacent tissues of the tail stumps already at the first day after surgery (**Fig. 5F and F′**). The dynamics of *Xag2-EGFP* transgene expression correlated well with the previously described dynamics of the loss of regenerative capacity in the hindlimbs, and with conservation of this capacity in the tail[11].

Discussion

In this study, we demonstrated by three independent methods that the expression of three representatives of *Agr* family genes, *Xag2*, *Xagr2a* and *Xag3a*, is activated in regenerating hindlimb buds and tails of *Xenopus laevis* tadpoles. We have also shown that the age dynamics of the loss of the ability to activate *Agrs* expression are in parallel with the loss of the ability to regenerate amputated appendages. Thus, the elevation of *Agrs* expression can be observed when amputation is performed during the period when a given body appendage is able to regenerate (hindlimb bud at stage 52 and tail at stage 52 and stage 57); however, no increase in *Agrs* expression is observed when the regeneration capacity ceases (hindlimb at stage 57).

Remarkably, *Agrs* have demonstrated increased expression already at 1 dpa in both limb buds and tails. Moreover, in transgenic embryos expressing EGFP under the control of *Xag2* promoter, the EGFP signal was observed in the epidermis covering the regeneration blastema, even after 12 h post amputation. Given that EGFP in *Xenopus* embryos could be visible for the first time only 6–10 h after the beginning of translation of its mRNA (Ermakova and Zaraisky unpublished), one may conclude that activation of *Agrs* expression is indeed an early event, immediately following amputation. In turn, this indicates a possible important role of these proteins in regeneration.

The Xagr2a/b ortholog, nAG (Agr2), was recently shown to have a key role in the limb regeneration of the newt[7]. Like *Agrs* in *Xenopus laevis*, *Agr2* in the newt and in the spotted salamander *Ambystoma maculatum* is also expressed at a low level in the intact limbs; however, its expression increases dramatically after amputation[7,8]. Additionally, as the membrane receptor of Agr, Prod1, has been found only in salamanders, but is absent in other vertebrates[9], one may suppose that the details of the mechanism by which Agrs are involved in the regeneration processes in the newt and frog might be different.

In support of this, *Agr2* in salamander regenerating limbs demonstrates expression dynamics that are different from *Xagr2* in *Xenopus*. In the newt, nAG is first induced by nerves on the 5 dpa in Schwann cells that surround regenerating axons. Thereafter, by 10 dpa, nAG induces its own expression in single gland cells underlying the wound epidermis[7]. Notably, although nerves are able to up-regulate nAG in Schwann cells during regeneration, they cause down-regulation of nAG in the epidermal cells during development[8].

By contrast, the expression of *Xag2* and *Xagr2a* in the regenerating hindlimb buds and tails of the *Xenopus laevis* tadpoles are activated in all cells of the wound epidermis at much earlier stages, and such expression is seemingly not preceded by activation of these genes in Schwann cells. The independence of *Agrs* epidermal expression on innervation in *Xenopus* is also confirmed by the data of Kumar et al., 2011, who demonstrated that the presence of nAG ortholog, Xagr2, could be revealed in epidermal cells by specific antibodies irrespective of their innervation. On the basis of these data, the authors concluded that the nerve dependence of *Agrs* expression is not a general feature of vertebrate limb development and that it may be specific only for salamanders.

At the same time, despite the difference in the expression patterns of *Agrs* during regeneration in salamanders and in *Xenopus*, the obvious involvement of these secreted proteins in the regeneration process in both anurans and urodeles models allows one to conclude that further investigation of the function of these genes could be a promising way to unravel the basic principles of organ repair.

One more important outcome of our work is that there are seemingly no orthologs of *Ag1* genes in higher vertebrates. Given this fact, along with the established correlation of *Ag1 (Xag2)* expression with the regenerative capacity of *Xenopus laevis* tadpoles, it would be attractive to speculate that the disappearance of *Ag1* through evolution might be one of the events that led to the reduced ability of

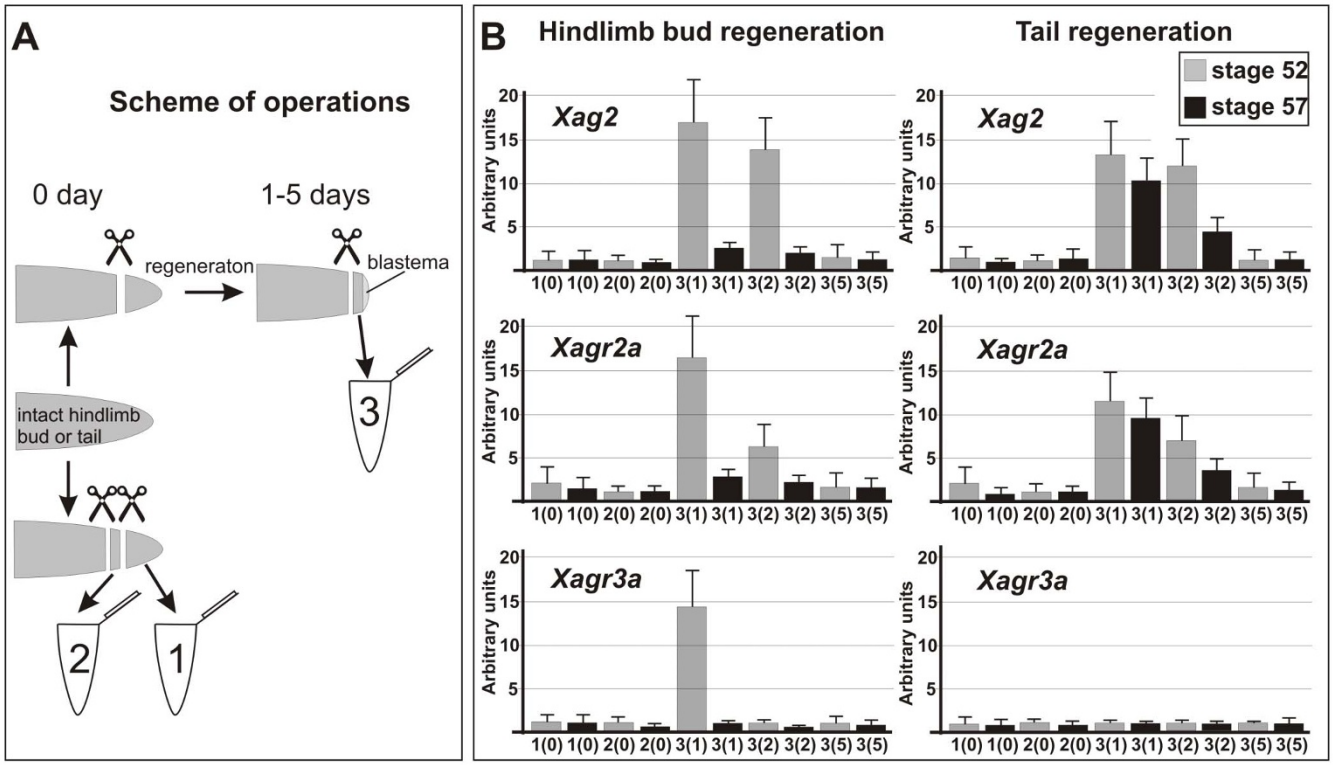

Figure 3 | Analysis of *Agrs* expression by qRT-PCR. (A). Scheme of experiments. (B). QRT-PCR analysis of *Xag2*, *Xagr2a* and *Xagr3a* expression in tissue samples of 1st -3rd types as indicated in (A). Days after amputation are in brackets. All graphs represent means of triplicate experiments. Bars indicate standard deviations. The geometric mean of expression of *ornithine decarboxylase* (ODC)[16] and *elongation factor 1alpa* (*EF-1alpha*)[16] was used for normalisation of experimental values. The value of normalised PCR signal in sample 2(0) in each series was taken as an arbitrary unit.

higher vertebrates to regenerate body appendages. In turn, it would be interesting in future research to test whether the experimentally induced down-regulation of *Ag1* may elicit diminished regenerative capacity in modern amphibians. If so, one may further suppose that the observed restoration of regenerative ability by the transfection of an *Agr2*-expressing plasmid in salamander aneurogenic limbs could result from induction by *Agr2* of not only its own expression[7,8] but also the expression of salamander *Ag1*.

Methods

Phylogenetic analysis. A translated BLAST program (tblast) was used to screen the available on-line nucleotide databases (all GeneBank: http://blast.ncbi.nlm.nih.gov/Blast.cgi; Ensembl: http://www.ensembl.org/index.html and *A. mexicanum* transcriptome: http://www.ambystoma.org) for sequences encoding proteins homologous to previously described Agr and TLP19 family proteins. The affiliation of each given gene to one of three syntenic loci (*Ag1*, *Ag2/Agr3* or *TLP19*) was checked manually by comparing the genomic environment of a given gene, revealed by BLASTing the gene on the Ensembl database, with the genetic environment characteristic for each of the loci (*Ag1*, *Ag2/Agr3* or *TLP19*). Multiple alignments and building of an unrooted phylogenetic tree with branch length were performed by using the standard CLUSTALW algorithm with the on-line program: http://www.genome.jp/tools/clustalw/.

Manipulations with tadpoles. *Xenopus laevis* tadpoles, raised from embryos obtained by the standard in vitro fertilisation procedure, were anesthetised in 1 : 7000 MS222 (Sigma-Aldrich) on 0,1 MMR. Hindlimb buds at the presumptive shank level[13] and tails were amputated at stage 52 and stage 57[14] by micro scissors and were further processed according to the schema shown in Fig. 1B.

Cloning of *Xag3a* cDNA. To generate the template for the synthesis of the *Xagr3a* probe, a DNA fragment corresponding to the protein coding region of *Xag3a* cDNA was obtained by RT-PCR from the first strand obtained from the total tailbud stage RNA with the following primers:

Xagr3a forward: 5′-ATGAATTCTTATGAAATTCTGAAGACA,
Xagr3a reverse: 5′-GAGTCGACGATCAGAGTTCAGTCTGC.

The obtained DNA fragment was cloned in pGEM-T (Promega) vector, and the correct clone was selected from sequencing five random clones.

qRT-PCR, in situ hybridisation and histological sections. For the extraction of total RNA, the amputated pieces of hindlimb buds and tails (see the scheme of operation in **Fig. 3A**) were cut by eye scissors and disrupted in Trizol® Reagent (Ambion) (20 µl per unit) by successive thorough pipetting and vortexing, for 30 sec each. For each type of sample shown in **Fig. 3A**, three replicates, each containing RNA from five amputated pieces of the same type, were prepared in this manner. The total RNA was extracted according to the Trizol® Reagent manufacturer's guidelines (Ambion), precipitated by standard EtOH-based procedure in the presence of glycogen (Fermentas) (20 µg/tube) and dissolved in 20 µl of RNAse-free water. The concentration of the extracted RNA was measured using a Qubit® fluorometer (Invitrogen), whereas RNA integrity was checked visually by gel electrophoresis. Then, 250 ng of total RNA that was extracted from each sample was reverse transcribed in a 20 µl final volume by M-MLV reverse transcriptase (Promega) in the presence of 10 pmol of oligo-dT primer (Evrogen), according to the manufacturer's guidelines (Promega) (+RT sample). In parallel, the identical reaction was assembled in each case without the addition of M-MLV reverse transcriptase (-RT control). For the qPCR reaction, which was performed on an ANK-32 (Syntol), 2 µl of +RT and -RT solutions of each type were mixed in parallel tubes with qPCRmix-HS SYBR (x5, Evrogen), corresponding primers (5 pmol each) and milli-Q water until the final volume equalled 25 µl. Primers were designed in such a way that the resulting PCR product would span at least three predicted exons. This approach allows one to avoid a contaminating signal that could be generated by traces of genomic DNA. The relevance of this approach was confirmed by the fact that when making qPCR with –RT samples, we were able to detect a signal at 8-10 cycles later than in the corresponding +RT samples. The following pairs of primers were used (length of each PCR product is indicated in brackets):

Xag2 forward: 5′-TGCTGCCAAGTCTGAGCCTGC and
Xag2 reverse: 5′-TCCTGAGCCAGTTTCTGTGCCA (227 bp);
Xagr2a forward: 5′-TGGCCAGTATGTTCCCAAGGTTGT and
Xagr2a reverse: 5′-CATCACTTTAGCATACACCTCCGC (238 bp);
Xagr3a forward: 5′-ATCCGGTCACCACAGACACTATC and
Xagr3a reverse: 5′-TTGGCAATACTGACACTCTTCTA (144 bp);
EF-1alpha forward: 5′-TCATACAGCTCATATTGCTTGTAAGT and
Ef-1alpha reverse: 5′-CAAGTGGAGGATAGTCTGAGAA (175 bp);
ODC forward: 5′-TCCATTGAGAGCGTAGGACTTG and
ODC reverse: 5′-GAGGCTCGCCGGTGAAATA (60 bp).

To compare the efficiency (*E*) of qPCR with primers to *Agrs*, we performed PCR with the following DNA-templates dilutions (for the preparing of templates, the coding region of cDNA for each *Agr* was initially obtained by PCR and purified by

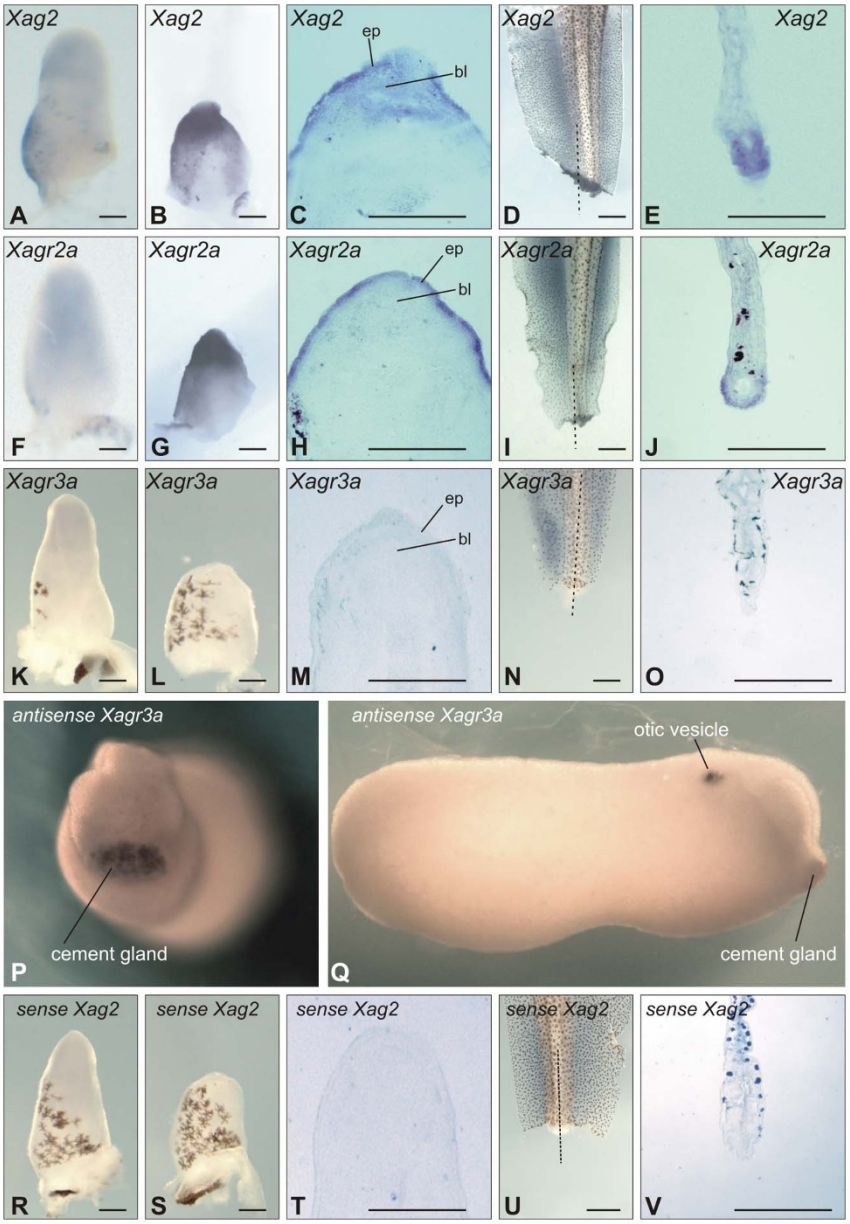

Figure 4 | Analysis of *Agrs* expression by whole-mount in situ hybridisation. (A) and (B), (F) and (G), (K) and (L). Intact hindlimb buds and hindlimb buds amputated at stage 52 and hybridised at 1 dpa with *Xag2*, *Xagr2a* and *Xagr3a* probes, respectively; distal to the top, ventral side to the right. (C), (H) and (M). Sagittal sections of amputated hindlimb buds shown on (B), (G) and L; ep – epidermis, bl – blastema. (D) and (E), (I) and (J), (N) and (O). Left side view and frontal sections (the level of section is indicated by dotted lines on (D), (I) and (N)) of tails amputated at stage 52 and hybridised at 1 dpa with Xag2 and Xagr2a probes; distal to the bottom, ventral side to the left. (P) and (Q). Stage 25 tailbud embryo hybridized with *Xag3a* antisense probe. Frontal and right side view respectively. Dorsal to the top. Anterior on B to the right. (R) and (S). Intact and amputated hindlimb buds operated at stage 52 and hybridized at 1 dpa with *Xag2* sense (control) probe; distal to the top, ventral side to the right. (T). Sagittal sections of amputated hindlimb buds operated at stage 52 and hybridized at 1 dpa with *Xag2* sense (control) probe. (U) and (V). Left side view and frontal sections (the level of section is indicated by dotted line on (U) of amputaited tail operated at stage 52 and hybridized at 1 dpa with *Xag2* sense (control) probe; distal to the bottom, ventral side to the left. Bars – 250 microns.

Promega Wizard columns): 4×10^{-4} ng, 5×10^{-5} ng, 6, 2×10^{-6} ng, and 7×10^{-7} ng. The averages of Ct data of each dilution from three replicates of qPCR with each pair of primers (Xag2, Xag2a, Xagr3a) were used to determine the slope and to calculate the *E* value ($E = 1/10^{-\text{slope}}$)[15]. The mean *E* values for the Xag2, Xagr2a and Xagr3a pairs of primers were almost identical: 1,821, 1,821 and 1,859.

A standard 40-cycle PCR program with a hot start was used in the main series of experiments; the annealing temperature was 59°C, elongation temperature was −72°C, and the melting temperature was 95°C, all lasting for 25 seconds. The PCR data were imported into Microsoft Excel and analysed by using the $\Delta\Delta C_t$ method[15]. The geometric mean of expression of two reference housekeeping genes, *ornithine decarboxylase* (ODC)[16] and *elongation factor 1alpa* (*EF-1alpha*)[16], was used for the normalisation of the target genes expression values. The value of normalised PCR signal in sample 2(0) in each series (see **Fig. 3A**) was taken as an arbitrary unit. Three replicates of each type of samples (see **Fig. 3A**) were taken for qPCR analysis. The qPCR for each replicate was performed three times. Mean values and standard deviations shown in **Fig. 3B** were calculated by Excel.

Dig-labelled RNA antisense probes for whole-mount in situ hybridisation were synthesised by T7 polymerase from *Xag2*, *Xagr2a* and *Xag3a* cDNA containing pGEM-T (Promega) linearised by NotI. For whole-mount in situ hybridisation, the hindlimbs and tail were cut off from the body of the tadpole by micro scissors and processed according to the previously described protocol[17]. Some of the samples after in situ hybridisation and staining in whole-mount were embedded in Paraplast by standard protocol and sectioned into serial 10 μm slices that were mounted on glass slides, freed from Paraplast by xylene, embedded in Moviol (Sigma) and photographed on a transmission Polyvar (Reihert-Jang) microscope.

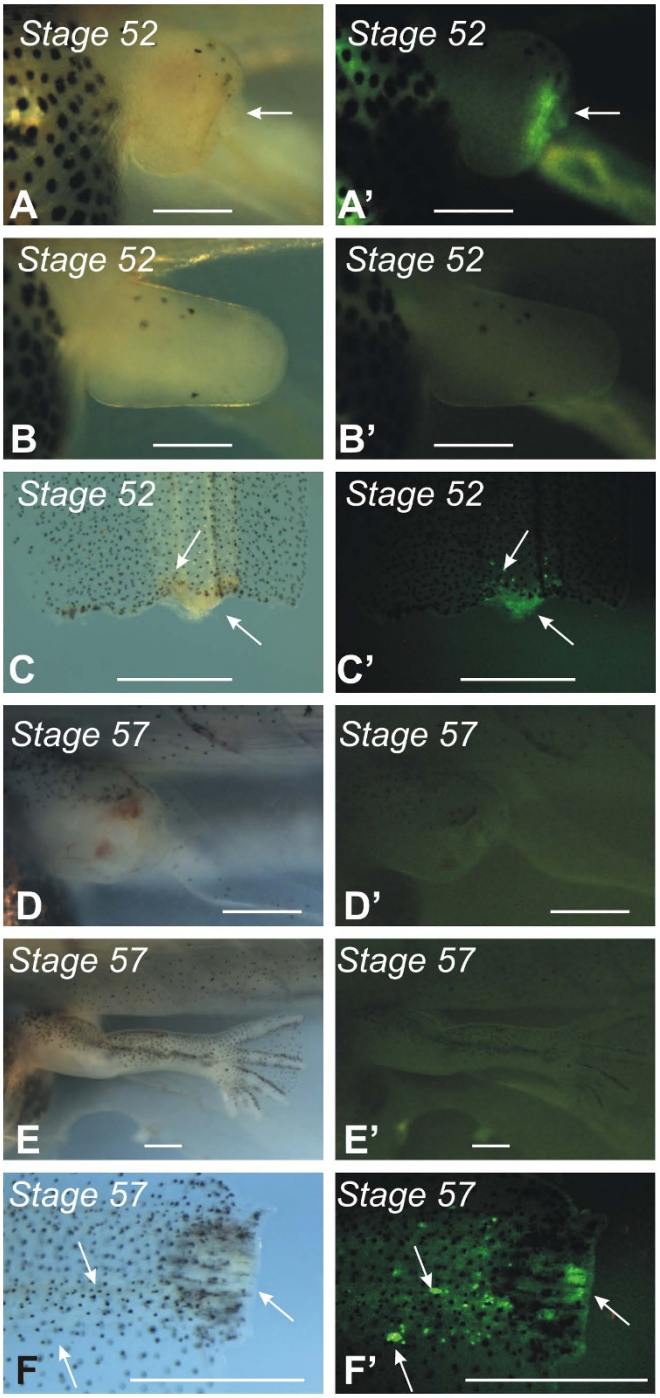

Figure 5 | Analysis of *Xag2* expression by transgenic tadpoles. (A) and (A′), (B) and (B′). Amputated and intact hindlimb buds of the same transgenic embryo, 12 h post-amputation at stage 52; distal to the right, dorsal to the top. (C) and (C′). Amputated tail, 12 h post-amputation at stage 52; distal to the bottom, ventral to the right. (D) and (D′), (E) and (E′). Amputated and intact hindlimb buds of the same transgenic embryo, 1 dpa at stage 57; distal to the right, dorsal to the top. (F) and (F′). Amputated tail, 1 dpa at stage 57; distal to the right, ventral to the bottom. Arrows indicate cells expressing *EGFP*. Bars – 500 microns.

Transgenic frogs.. Transgenic frogs expressing EGFP and different variants of RFP under the control of *Xag2* promoter were generated previously and maintained in our lab[12]. Briefly, a 1295 nucleotide fragment of *Xag2* promoter from *Xenopus laevis* genomic DNA was obtained by PCR with Tersus Taq polymerase (Evrogen) and the following pair of primers:

DirXag2-SalI 5′-AATTGTCGACCTCACCTATTAAGCTCACTATAGACA and *RevXag2-KpnI* 5′-CCGCGGTACCAGGTAGCTTCAAATCAGCTCCT (*Xag2* promoter sequences are underlined). The obtained PCR fragment was cloned, specifically, into pEGFP-1 plasmid by SalI and KpnI restrictases and checked by sequencing. Strains of transgenic *Xenopus laevis* frogs bearing EGFP cDNA under the control of a 1.3 kb promoter of the *Xenopus laevis Xag2* were generated by the REMI technique, as previously described[18,19]. Embryos were obtained from these transgenic frogs by a standard in vitro fertilisation procedure and were grown to the desired stages, and then the limbs were amputated and photographed using white light and EGFP filter sets on a Leica M250 stereomicroscope.

Ethics statement. All experiments were approved by Mestkom and Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry Ethics Committee.

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Author contributions

A.S.I. and M.B.T. performed cloning, qRT-PCR, in situ hybridization and transgenic experiments. G.V.E. has generated transgenic line of frogs and participated in conducting experiments with transgenic tadpoles. V.V.B. inspired initial idea that led to this work. A.G.Z. performed the phylogenetic analysis, designed the project and wrote the manuscript.

Additional information

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