Bridging the regeneration gap: genetic insights from diverse animal models

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Abstract | Significant progress has recently been made in our understanding of animal regenerative biology, spurred on by the use of a wider range of model organisms and an increasing ability to use genetic tools in traditional models of regeneration. This progress has begun to delineate differences and similarities in the regenerative capabilities and mechanisms among diverse animal species, and to address some of the key questions about the molecular and cell biology of regeneration. Our expanding knowledge in these areas not only provides insights into animal biology in general, but also has important implications for regenerative medicine and stem-cell biology.

Dedifferentiation
The process by which a terminally differentiated cell loses its tissue-specific characteristics and becomes undifferentiated. Dedifferentiated cells can either re-differentiate into cells of their original type or to a cell of different lineage.

Transdifferentiation
The process by which a terminally differentiated cell dedifferentiates and then re-differentiates to a cell of a different lineage, for example, the transdifferentiation of iris pigment epithelial cells to lens during newt lens regeneration.

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Regeneration — the regrowth or repair of cells, tissues and organs — is widely but non-uniformly represented among all animal phyla[1] (FIG. 1a). The diverse modes by which regeneration is accomplished is a topic of as much interest as its distribution among taxonomic groups[2]. Regenerative strategies include the rearrangement of pre-existing tissue, the use of adult somatic stem cells and the dedifferentiation and/or transdifferentiation of cells (FIG. 2), and more than one mode can operate in different tissues of the same animal. All these strategies result in the re-establishment of appropriate tissue polarity, structure and form. Therefore, the key questions in regeneration research relate to the evolutionary and biological reasons that distinct modes are used in different cases, the molecular pathways that are involved and the differences and similarities between regeneration and normal development.

As well as being a fascinating biological problem, regeneration has long attracted biomedical interest because of the potential of replacing old or damaged tissues with new ones. Most studies of regenerative biology that are aimed at biomedical applications have focused on stem cells *in vitro*. However, to gain a full understanding of regeneration, the processes that are involved must be studied *in vivo*, in the context of the complex interactions that take place within and among the different cell types that are involved. Model organisms are essential for such *in vivo* interrogations, and stand to provide us with the necessary knowledge to eventually manipulate and control regenerative properties. Moreover, understanding the modes and mechanisms that are involved in regeneration in diverse model systems is potentially advantageous for biomedicine. Understanding, for instance, why a particular regenerative process takes place in a model system but not in human tissues could provide new pathways to stimulating regeneration if endogenous pathways are unavailable.

Despite these clear reasons for gaining a detailed knowledge of the biology of regeneration, many of the fundamental questions that are outlined above remain unanswered. This has mainly been due to an inability to carry out genetic studies in the species that have traditionally been used to study regeneration. However, recent methodological advances are beginning to erode and overcome these daunting shortcomings. Such progress has also invigorated a more thorough investigation of the modest regenerative capacities of well-established genetic model systems. Here we discuss the diverse model systems that are currently being used to dissect the molecular and cellular bases of regeneration (FIG. 1b), with a focus on *in vivo* models. We examine the particular aspects of regenerative biology into which each model provides insights and highlight their relevance to human biology and medicine.

Regeneration in invertebrates

Invertebrate regeneration has been studied for more than 200 years[3]. Two classes of invertebrate have received the most contemporary attention: the diploblast *Hydra vulgaris*, and the triploblast, bilaterally symmetrical freshwater planarians such as *Schmidtea mediterranea* and *Dugesia japonica*.

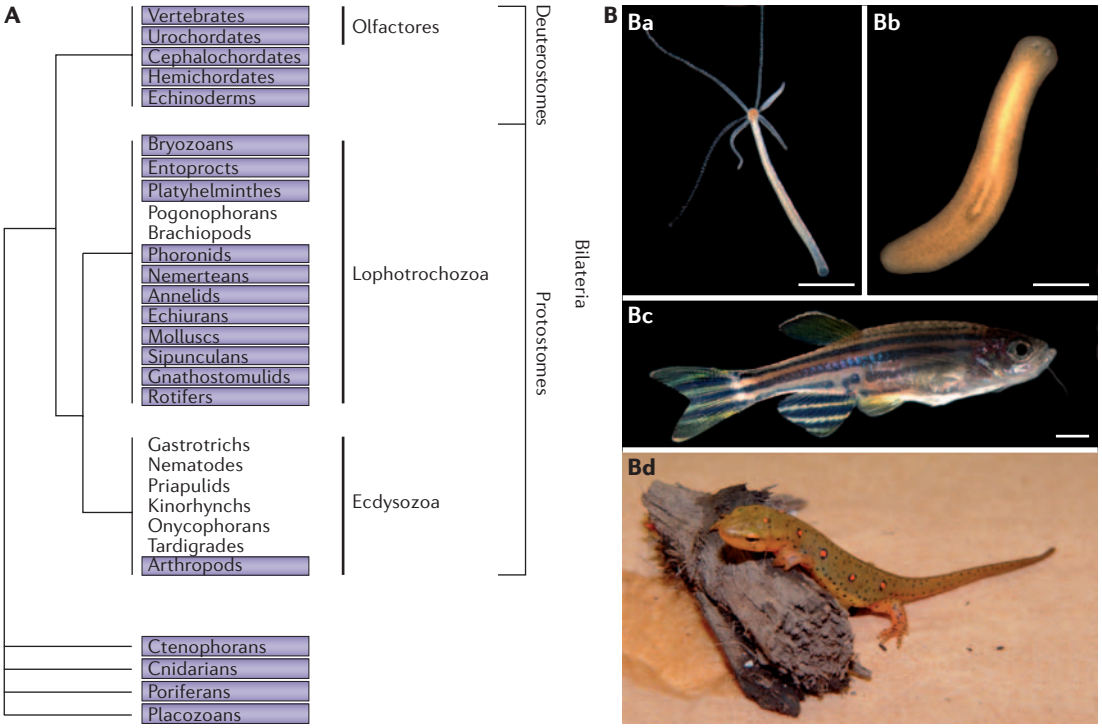


Figure 1 | **Regeneration in multicellular organisms — phylogenetic distribution and model species. A** | The phylogenetic distribution of regeneration in multicellular organisms. The taxa that contain animals that are capable of regeneration are shown on a purple background[1,2]. For the remaining taxa, either the absence of regeneration has been reported or its presence is unknown. The tree is modified from Adoutte *et al.*[107] to include the placozoans, which include the simplest known animal (*Trichoplax adhaerens*)[108], and the recently recognized olfactore clade, within which urochordates and not cephalochordates are more closely related to vertebrates[109]. **B** | Examples of organisms in which regenerative capacities are currently under molecular investigation. Panel **Ba** shows the cnidarian *Hydra vulgaris*; panel **Bb** shows the freshwater planarian *Schmidtea mediterranea* (a platyhelminth); panel **Bc** shows the zebrafish (*Danio rerio*); panel **Bd** shows the newt (*Notophthalmus iridescens*). Scale bars represent 2 mm.

Hydra. The cnidarian *H. vulgaris* (FIG. 1Ba) is organized along a single oral–aboral axis, and is divided into three distinguishable anatomical parts: a foot used by the animal to adhere to substrata, a body column that serves as a gastric cavity, and a head region, which is decorated with a ring of tentacles surrounding a primitive mouth[4]. This simple body plan is composed of two germ layers, the ectoderm and endoderm, which are separated by an extracellular matrix known as the mesoglea, in which interstitial stem cells reside[5].

Hydra has the distinction of being the first animal in which regeneration was formally described[3]. Death due to the loss of essential body regions such as the head is prevented by regeneration in this species. Moreover, because hydra constantly replace the cells that are lost to normal physiological turnover, these animals can be considered negligibly senescent[6]. Within the first few hours after decapitation, regeneration proceeds without detectable cell proliferation[7]. Instead, the removal of the head resets positional values along the remaining body axis, which causes the cells in the gastric column to undergo determination and differentiation to replace the missing head[8]. Such remodelling of the pre-existing tissue into new structures during regeneration is not unique to hydra. For example, studies of the early stages of regeneration of the mammalian pancreas after acute pancreatitis[9], and recent evidence for the regeneration of β-producing cells from non-endocrine cells[10], indicate that the respecification of pre-existing tissues during regeneration in different species might share a common evolutionary origin. Another interesting property of hydra is its ability to re-form an animal from dissociated cells[11], a trait that could serve as a paradigm for understanding the molecular basis of *de novo* organizer formation during non-embryonic processes such as regeneration[12].

The regenerative plasticity that is seen in hydra has been the topic of many studies, which have focused on explaining the mechanisms by which polarity is regulated[12]. Hydra orthologues of key developmental regulators, such as Hox genes[13–15], *brachyury*[16], and *goosecoid*[17], and components of developmentally relevant signalling pathways (TABLE 1), such as Wnt[11,18] and FGF (fibroblast growth factor)[19], have been identified in these studies. For example, Wnt, β-catenin and Tcf, which are components of the Wnt signalling pathway, are upregulated during head regeneration[11]. Interestingly, Wnt and Tcf expression domains define head organizer regions during the *de novo* pattern formation that occurs in cell aggregates that are obtained from

Diploblast
An organism that is derived from two primary germ layers: the ectoderm and the endoderm.

Triploblast
An organism that is derived from three primary germ layers: the ectoderm, the mesoderm and the endoderm.

Organizer
The regions within an embryo that control development and differentiation.

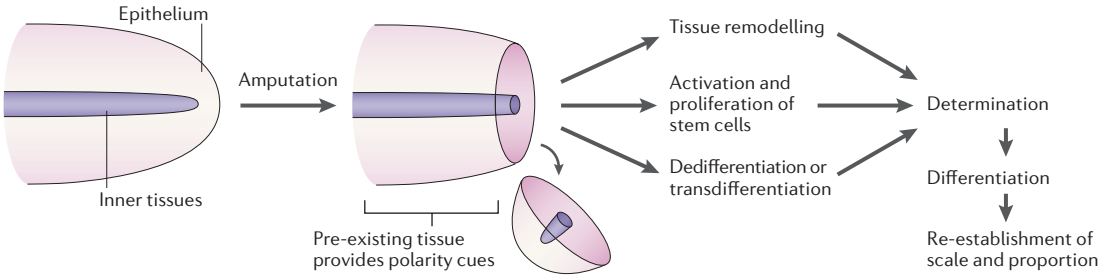


Figure 2 | **Basic mechanisms of regeneration.** After amputation, wound healing occurs. This is an injury response that is common to all animals, whether or not they can regenerate. After wound healing, if the resulting tissue stump is capable of regeneration, at least three processes can be activated, either independently or together. Hydra, for example, undergo remodelling of pre-existing tissues to regenerate amputated parts. Planarians undergo both tissue remodelling and proliferation of resident adult somatic stem cells; in vertebrates, both stem-cell proliferation and the dedifferentiation or transdifferentiation of the cells that lie adjacent to the plane of amputation take place. The cells that respond to the stimulus of amputation eventually undergo determination and differentiation, resulting in new tissues that must then functionally integrate with and scale to the size of the pre-existing tissues.

dissociated hydra[11]. More recently, the hydra orthologue of dickkopf (a Wnt signalling antagonist) was shown to be an early injury and regeneration responsive gene that is downregulated by Wnt/β-catenin signalling in the regenerating and developed head[18]. Although these studies point to an ancestral conservation of developmental signalling pathways, they also show that activation of these pathways can be triggered by processes that are not related to embryonic development, such as amputation and re-aggregation. Uncovering the mechanisms that are involved in this activation will shed light on the fundamental differences and similarities that must exist between embryonic development and regeneration.

The ability to carry out loss- and/or gain-of-function studies in hydra has been limited[20], which has hampered the systematic testing of genes and molecular pathways. In recent years, however, two key methods have been extended to the study of hydra biology: transgenesis and RNAi. The first successful demonstration of transgenesis in hydra was reported by Galliot and colleagues[21]. They demonstrated the feasibility of using cnidarian and bilaterian promoters to drive the expression of transgenic GFP. Another approach has recently been reported in which transgenic hydra lines were generated by embryo microinjection[22]. Transgenesis will allow both real-time determination of cellular dynamics and gain-of-function studies during regeneration.

RNAi studies have already begun to shed light on key aspects of hydra biology[23]. Silencing of the evolutionarily conserved serine-protease gene *kazal1* indicated that there is a role for the suppression of excessive autophagy in intact hydra as well as in cell survival after amputation and during the initial stages of regeneration. Remarkably, these functions are consistent with the pancreatic autophagy phenotype that is observed when the Kazal domain of the *SPINK1* (serine peptidase inhibitor, Kazal type 1) and *SPINK3* genes in humans and mice are mutated[23]. These results underscore the possibility that the molecular mechanisms that are activated during hydra regeneration might have relevance to tissue regeneration in mammals.

By extending to hydra the methods that have been used in *Caenorhabditis elegans* and the planarian *S. mediterranea* (see below) to deliver dsRNA by the uptake of bacteria, Galliot and colleagues have now opened up the possibility of carrying out large-scale RNAi-based screens in cnidarians. Although they are in their early days, these methods will allow for a much needed mechanistic interrogation of the cellular and molecular biology of hydra.

Planarians. Free-living freshwater planarians, which have been classic models of animal regeneration for more than 100 years[24], are also experiencing a renaissance in terms of regeneration research[24,25]. Unlike hydra, planarians regenerate missing body parts by first assembling a specialized structure known as the regeneration blastema, which arises from the proliferation of pre-existing somatic stem cells known as neoblasts (FIG. 3). This structure consists of an outer epithelial layer that covers the mesodermally derived tissue. It represents a canonical epithelial–mesenchymal interaction, a tissue relationship that is conserved among tripoblasts and is central to the development of complex structures in bilaterian embryos. The blastema seems to be a conserved structure that is used in the regeneration of all bilaterians that are known to possess this ability, including vertebrates, suggesting that the study of blastema formation, determination and differentiation in planarians could inform regenerative processes in other organisms.

Recently, two planarian species have received the most molecular attention: *D. japonica* and *S. mediterranea* (FIG. 1). A clonal strain of *D. japonica* (GI) has been used to study regeneration for over a decade[26]. These studies have generated large amounts of molecular information, including a cohort of genes that are coordinately activated during the regeneration of the CNS after decapitation[27,28]. *In situ* hybridizations have implicated roles for developmental pathways in this process (for example, the FGF and Wnt signalling pathways), and other genes that are conserved in mammals but have unknown functions[28]. However, the fact that *D. japonica* is mixoploid and harbours large numbers of expressed retrotransposons[29]

Autophagy
A nutritionally and developmentally regulated process that is involved in the intracellular destruction of endogenous proteins and the removal of damaged organelles.

Mixoploid
An organism that contains cells which are of different ploidy, for example, diploid and polyploid.

Argonaute/PIWI family
Members of this protein family contain PAZ and PIWI domains, which are involved, respectively, in binding small RNAs and mediating silencing, either by cleavage of mRNAs or through inhibition of translation.

has hampered genome sequencing efforts and positional cloning, preventing a more detailed characterization of genetic and molecular mechanisms.

A clonal line of *S. mediterranea* (CIW4) has also been established, and expression patterns for a large number of genes have been characterized[30]. *Schmidtea mediterranea* is more amenable to functional genomic studies than *D. japonica* as it is a stable diploid and lacks the large numbers of expressed retrotransposons[30,31] that are seen in *D. japonica*. Furthermore, it exists in exclusively asexual and sexual biotypes that allow for a direct comparison of regeneration and embryogenesis[25]. Sequencing of the *S. mediterranea* genome is nearing completion, and putative planarian orthologues for all of the main signalling pathways that are used during animal embryogenesis have been identified (TABLE 1).

RNAi screening has begun to provide molecular insights into planarian regeneration[32]. In *D. japonica*, this approach uncovered a role for the FGF signalling pathway in the regulation and maintenance of cephalic structures. Silencing of this pathway results in the production of ectopic brains, indicating that FGF has a role in the *de novo* formation of cephalic structures[33], which is consistent with the role of this pathway in hydra[23]. More recently, RNAi was used to carry out an unbiased screen of over 1,000 genes for their involvement in regenerative processes[34]. This screen was the first of its kind in an animal model of regeneration, uncovering 240 genes that, when silenced, yield phenotypes that relate to all aspects of regeneration (FIG. 4). These genes encode molecules with roles in both embryonic- and non-embryonic-specific growth, and phenotypes relating to wound healing and blastema establishment, maintenance and differentiation were uncovered. Of the genes that were identified, several are predicted to encode proteins that are similar to FKBP-like immunophilin, chondrosarcoma-associated protein 2 (CSA2), nucleostemin (a neurogenic stem-cell marker in mammals) and SMAD4.

Given that 85% of the genes that yielded regeneration phenotypes in the *S. mediterranea* RNAi screen are conserved in other animals, the results of this first regeneration screen in planarians could serve as a good point of departure to determining the extent of conservation of regeneration mechanisms among the Metazoa. The product of *smedwi2*, a member of the argonaute/PIWI family of proteins, which are involved in silencing by small interfering RNAs and microRNAs, represents one such highly conserved gene that was identified in the *S. mediterranea* RNAi screen. Interestingly, in all organisms in which these proteins have been studied, they have been implicated in the regulation of stem cells, including germ cells. In *S. mediterranea*, *smedwi2* is expressed in dividing stem cells[35]. Although the loss of function of this gene has no effect on stem-cell maintenance in *S. mediterranea*, it renders stem cells unable to produce progeny that can make regeneration-competent blastema cells, and prevents stem-cell progeny from replacing aged differentiated cells in intact animals[35]. These results are consistent with the finding that the PIWI-related mouse proteins MIWI and MILI are expressed in the testes and are needed for the completion of spermatogenesis, but not for primordial germ-cell formation[36,37]. The biological functions that have been identified for PIWI proteins in planarians and other animals indicate that these proteins might be universal regulators of the production of stem-cell progeny that are competent for performing differentiated functions[35]. More importantly, these findings indicate that planarians will be a useful *in vivo* model system for studying how stem cells function in regeneration.

Other invertebrates. Although the most exhaustively studied members of the Ecdysozoa (*Drosophila melanogaster* and *C. elegans*) do not have robust regenerative capacities, they are likely to be the exception rather than the rule, as other members of this group, such as cockroaches[38], spiders[39] and mites[40], can regenerate lost body parts. However, it is worth pointing out that transplanted fragments of *D. melanogaster* imaginal discs have been put forward as a model of regeneration[41]. This approach demonstrates that *D. melanogaster* larval tissues can regenerate in some conditions. However, how this phenomenon relates to regeneration in other animals remains unclear, and whether it can actually be considered regeneration

Table 1 | **Extensive conservation of major signalling pathways involved in cellular differentiation**

Signalling pathway	Species or group		
	Hydra magnipapillata	*Schmidtea mediterranea*	Vertebrates
TGFB	Yes	Yes	Yes
Notch	Yes	Yes	Yes
Wingless	Yes	Yes	Yes
Hedgehog	Yes	Yes	Yes
JAK/STAT	Unknown	Yes	Yes
EGF receptor	Yes	Yes	Yes
FGF receptor	Yes	Yes	Yes
Toll/NFκB	Unknown	Yes	Yes

Data for hydra were obtained from ESTs that are available at GenBank. Data for *S. mediterranea* were obtained from analyses of genome trace reads that were deposited in GenBank by the Washington University Genome Sequencing Center. EGF, epidermal growth factor; FGF, fibroblast growth factor; JAK, janus kinase; NFκB, nuclear factor κB; STAT, signal transducer and activator of transcription; TGFB, transforming growth factor-β.

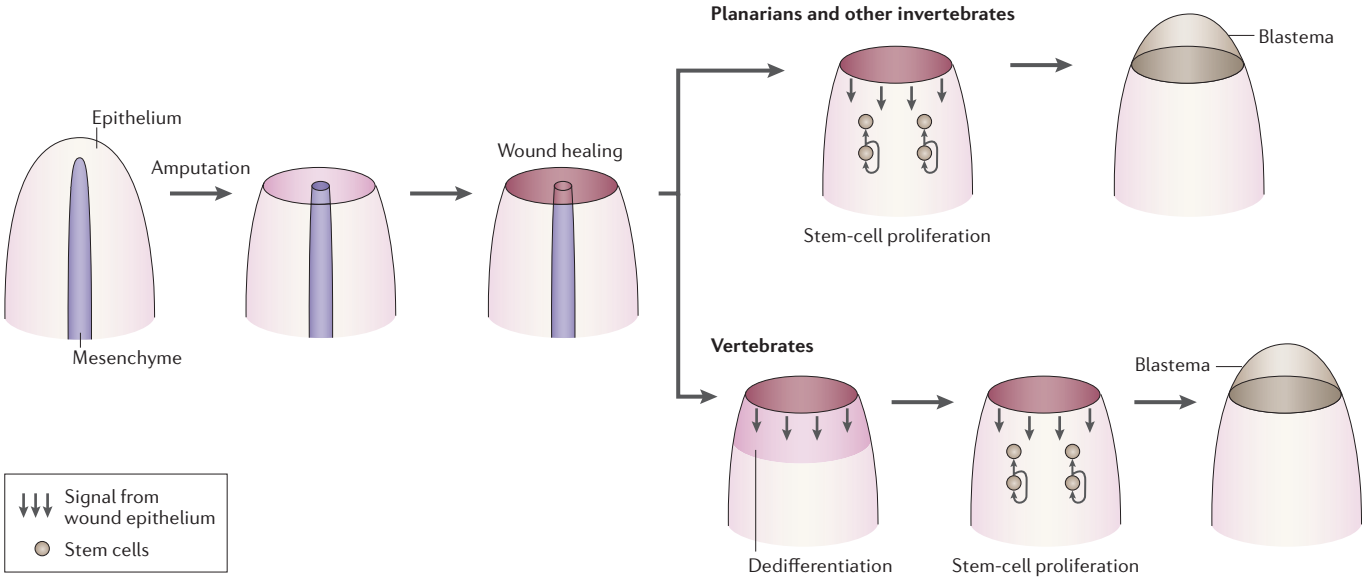


Figure 3 | **Basic steps in the formation of regeneration blastemas in vertebrates and invertebrates.** In vertebrates, there is evidence that both stem cells and cell-dedifferentiation processes have a role in blastema-mediated regeneration. In invertebrates such as planarians, stem-cell proliferation seems to have a pivotal role.

rather than tissue regulation has also been questioned, because the amputation of imaginal discs without transplantation results in polarity defects[42].

Besides planarians, many members of the Lophotrochozoa display robust regenerative abilities. Examples are provided by the polychaete[43,44] and oligochaete[45,46] worms, both of which can regenerate heads after decapitation. Some deuterostome invertebrates also regenerate missing body parts. Echinoderms such as the sea cucumber dispose of their entire digestive system as a decoy to predators, and proceed soon after to regenerate it completely[47]. Solitary (*Ciona intestinalis*) and colonial (*Botryllus schlosseri*) ascidians are also known to regenerate. Adult *C. intestinalis* can regenerate its siphon after amputation[48], and *B. schlosseri* undergoes cyclical regeneration and degeneration of its organs[49]. However, most studies of these processes are descriptive in nature, and these mechanisms await systematic molecular interrogation. Nevertheless, a major source of regenerated tissues for all the invertebrates that have been studied so far is provided by resident populations of somatic stem cells. This shared attribute suggests that understanding regeneration in invertebrates will probably necessitate a better understanding of the maintenance and regulation of their somatic stem cells. Given the wide phylogenetic conservation of PIWI proteins and their involvement in the regulation of stem cells from plants to humans[50], defining the biological function of these molecules in as many invertebrates as possible could be a fruitful way to understand the role of stem cells in animal regeneration.

Regeneration in vertebrates

Several vertebrate species have noteworthy regenerative capacities. Newts and salamanders are perhaps the most remarkable in this respect, followed by fish and then mammals, which by comparison occupy a fairly distant third place. It is not clear why such a wide gamut of regenerative capacities exists in the vertebrates. Our lack of understanding has been exacerbated by the fact that an inversely proportional relationship exists between models that are good regenerators and the ability to investigate molecular pathways using traditional genetics (TABLE 2). Given that experimentation and manipulation at the genetic level are crucial to dissect the mechanisms of regeneration, we focus here on vertebrate species in which traditional genetics and new genetic tools are being deployed.

Amphibia

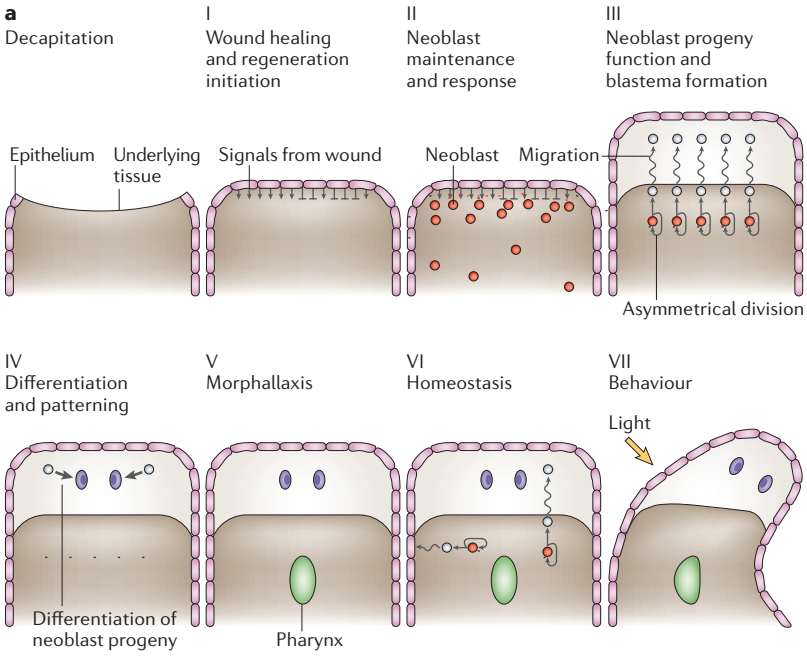
Regeneration in amphibians is thought to be mediated mainly by extensive cellular transdifferentiation. Terminally differentiated cells at the site of amputation dedifferentiate and then re-differentiate to form the lost part. This is in contrast to mammals, in which transdifferentiation has been observed in only a few cell types, such as the endothelial cells of the pancreas[10] and the Schwann cells of the peripheral nervous system[51]. Recently, a role for stem cells in amphibian regeneration has also been uncovered[52], suggesting that regenerative capacities in these animals might involve both differentiated and undifferentiated cell types.

Among amphibians, and in the vertebrates in general, the newt is generally regarded as the champion of regeneration. As an adult it can regenerate many organs, including limbs, the tail, the brain and spinal cord, hair cells, the lens and retina, the jaws and the heart[53]. However, newts are difficult to breed under laboratory conditions and, as such, they have not been accessible to traditional genetics. Notwithstanding these problems, functional genetic analyses in newts and salamanders (which are closely related) are becoming possible. A mass of tools, including ESTs and robust transgenesis[54–56], has begun to accumulate for the axolotl,

Schwann cells
Non-neuronal cells that mainly provide myelin insulation to axons in the peripheral nervous system of jawed vertebrates.

a neotenous salamander that is amenable to routine breeding and can regenerate limbs, the tail and the spinal cord. Another amphibian for which transgenesis is possible and ESTs are available[57] is *Xenopus laevis*. It too can regenerate, but only as a tadpole during pre-metamorphic stages, effectively limiting the scope of questions that can be studied in this animal (for example, the regeneration of adult organs and appendages cannot be studied). Nevertheless, it is expected that with the ongoing sequencing of the *Xenopus tropicalis* genome, and the ability to carry out loss- and gain-of-function assays, this organism will begin to rise in prominence in the study of vertebrate regeneration.

Here we outline the insights that have been gained so far from the regeneration of several body parts in vertebrates, and how recently developed methods are contributing to studies of these processes.



b

	Candidate functions include:		Example (gene or predicted product)
I	Wound healing	*n*=1	One novel gene
	Regeneration signalling; blastema formation	*n*=25	*smad4*
II	Basal neoblast functioning during regeneration	*n*=30	Phosphatidylinositol-transfer protein
III	Neoblast progeny functioning during regeneration	*n*=13	Spastic paraplegia
	Blastema formation; differentiation	*n*=38	Gene encoding a DEAD box protein
IV	Blastema shape or morphology	*n*=51	*bmp1*
	Patterning (cellular level)	*n*=40	Cyclin fold-protein
	Blastema maintenance	*n*=5	FK506 binding protein
V	Changes in pre-existing tissue	*n*=11	Nuclear distribution C (*nudC*)
VI	Functioning of differentiated cells	*n*=62	Casein kinase
	Differentiated cells, not needed in neoblasts	*n*=29	Tubedown-100
	Basal neoblast functioning in cell turnover	*n*=43	Chromobox protein
	Neoblast progeny functioning in cell turnover	*n*=14	Striatin
VII	Behaviour	*n*=44	Rootletin

Novel | Specific; e.g. signalling | Basal; e.g. translation

Figure 4 | **An RNAi screen for genes with functions in planarian regeneration.** **a** | Seven stages of planarian head regeneration are shown. Stage V indicates the regeneration of organs (pharynx formation) in pre-existing tissues. **b** | Gene identities and candidate functions that were revealed in an RNAi screen for defects in the regeneration stages that are shown in part **a**. Some genes are present in multiple categories. Genes were classified as specific if they are predicted to encode proteins that have roles in signal transduction, transcription, cell adhesion, neuronal functions, disease, RNA binding, channel or transporter function and cytoskeletal regulation. Genes were classified as having basal roles if they are predicted to encode proteins that are involved in translation, metabolism, RNA splicing, proteolysis, protein folding, vesicle trafficking, the cell cycle or the cytoskeleton machinery. Modified with permission from REF. 34 © (2005) Elsevier Science.

Limb regeneration. Limb regeneration in newts and axolotls requires the formation of a blastema. Detailed analyses of this structure have provided insights into the important signalling processes. After amputation, the wound is covered by a specialized epithelium that is required for regeneration to proceed. It is thought that this epithelium provides signals to the underlying cells of the stump to dedifferentiate and/or maintain cell proliferation. One early signal that is associated with cell-cycle re-entry in cultured newt myofibres and during lens regeneration (see below) seems to be provided by thrombin[58,59]. Inhibition of Msx1 (msh homeobox homologue 1) also prevents cellularization of newt myofibres[60]. In addition, a much sought after nerve-dependent trophic factor is suspected to have a role in regeneration, as denervated limbs fail to regenerate, and cell proliferation, cell survival and the expression of growth and differentiation factors in blastemas are affected by the nerve supply[61]. Glial growth factor (GGF) and transferrin have been proposed as possible candidate factors[62,63]. However, limbs might be 'addicted' specifically to nerves, because limb regeneration in aneurogenic animals is normal[64]. Other experiments have suggested that, with denervation, Schwann cells release an inhibitor of cell cycling[65]. Other factors might also be required, because frogs, which can regenerate limbs as well as tails and lenses, lose this ability after metamorphosis[66].

Among other suspected cell-proliferation and regeneration-inducing factors for the blastema are FGFs and their receptors (FGFRs), which are expressed during limb regeneration[67]. For instance, *Fgf10* is expressed in blastemal mesenchymal cells and is correlated with regeneration in *Xenopus*[68]. Furthermore, the introduction of FGF10 into regeneration-deficient *Xenopus* limb buds can stimulate the expression of *Shh* (sonic hedgehog), *Msx1*, and *Fgf10*, and the concomitant production of new limb structures[69]. FGF2 is suspected to be the elusive neurotrophic factor, because it is expressed in the nerves and epidermis of limb buds and blastemas and is also an endogenous mitogenic factor that is responsible for blastema formation in frogs. Denervation of axolotl limbs during regeneration results in the epidermal downregulation of *Fgf2* and *Dlx3* (a homologue of the homeobox-containing gene *distal-less*); whereas FGF2 replacement re-establishes the normal expression of *Dlx3* (REF. 70).

Table 2 | **Model systems in regeneration research, and their genetic and genomic tools**

Species or group	Regenerative capabilities	Microarray	Transgenesis	Knockout/knock down	Genome sequenced
Invertebrates					
Hydra	All tissues and organs	No	Yes	RNAi	No
Planarians	All tissues (neurons, muscles, epithelia) and organs (brain, sensory organs, digestive system, musculature)	Yes	No	RNAi	Yes
Ascidians	All tissues and organs	Yes	Yes	Morpholinos	Yes
Vertebrates					
Newts	Limbs, tail, heart, lens, spinal cord, brain, jaw, retina, hair cells of the inner ear	Yes	Yes	Morpholinos	No
Axolotls	Limbs, tails, heart, spinal cord, brain	Yes	Yes	Morpholinos	No
Frogs	Pre-metamorphic limbs, tail, retina, lens, hair cells of the inner ear	Yes	Yes	Morpholinos	Yes
Zebrafish	Fins, tail, heart, liver, spinal cord, hair cells of inner ear, lateral line	Yes	Yes	Mutagenesis, morpholinos	Yes
Chicks	Hair cell of the inner ear	Yes	Yes	Morpholinos	Yes
Mice	Liver, digit tips	Yes	Yes	Mutagenesis, homologous recombination	Yes

Tail, spinal cord and brain regeneration. The amphibian tail is composed of tissues that are similar to those found in the limbs, with the prominent exception of the spinal cord. Whereas cellular dedifferentiation has a role in limb regeneration, stem cells seem to have a key role in CNS and tail regeneration. Therefore, amputation and regeneration of the tail provides a model for vertebrate injury and regeneration of the CNS. For instance, the frontal lobe of salamanders and *Xenopus* regenerates after removal, a process that seems to be mediated by the proliferation of the ependymal epithelial cells that line the ventricles of the brain. The mechanisms that are used by these cells to completely restore an amputated frontal lobe have not been conclusively elucidated[71]. However, in salamanders, the ependymal cells that line the central canal of the spinal cord are considered to be CNS stem cells, and have also been shown to participate in the regeneration of the spinal cord. Furthermore, the ependymal cells migrate to the surrounding tissues during tail regeneration and form cartilage and muscle, thereby switching from ectodermal to mesodermal lineages[72].

Molecular insights into tail regeneration have been provided by transgenic *Xenopus*. Tail regeneration in *Xenopus* is suppressed in developmental stages 45–47, providing a context in which to test the ability of signalling molecules to induce regeneration. Expression of the constitutively active bone morphogenetic protein (BMP) receptor *Alk3* during this period results in regeneration of all tail tissues. Similar results were obtained with an activated form of *Msx1*, whereas expression of a constitutively active *Notch* intracellular domain (NICD) resulted in imperfect regeneration, mostly owing to significantly hampered muscle regeneration. These data indicate that BMP acts upstream of *Notch*, and exerts an independent effect on muscle regeneration. When BMP or Notch signalling are inhibited at regeneration-permissive stages 50–52, inhibition of tail regeneration is observed[73]. Similar studies of other molecules should further delineate the mechanisms of CNS and tail regeneration in amphibians and other vertebrates.

Lens and retina regeneration. Regeneration of the newt lens occurs by transdifferentiation of the pigment epithelial cells (PECs) at the tip of the dorsal iris. These cells dedifferentiate to form a vesicle, which in turn differentiates to form the lost lens. The ventral iris PECs are not able to transdifferentiate to lens, even though they are morphologically indistinguishable from their dorsal counterparts[74]. The newt retina is also able to regenerate, and does so through the dedifferentiation of the retinal pigment epithelium (RPE). In this case, one daughter cell of the dividing RPE cells contributes to regeneration and the other replenishes the RPE[74].

The differential regenerative ability of the dorsal and ventral iris provides an experimental paradigm to define the molecular factors that are involved in lens regeneration. Comparisons between the two cell types have pinpointed possible regulators of lens regeneration: *Six3* (sine oculis homeobox homologue 3), retinoic acid and BMP[75]. *Six3* is a transcriptional regulator of eye and lens development that operates in a feedback loop with the eye-specific gene *Pax6* (paired box 6). The function of this gene in eye regeneration might be ancestral, as several members of the six family are also upregulated during eye regeneration in jellyfish[76]. When *Six3* is overexpressed in the ventral retina in the presence of retinoic acid, ventral iris PECs transdifferentiate into lens cells. Inhibition of BMP also promotes the transdifferentiation of ventral iris cells. This genetic regulatory network is similar to the situation during eye development, when inhibition of BMP is upstream of the *Pax6*/*Six3* loop[77]. Because *Six3* is

Neotenous animals
Animals that, as adults, retain traits that are usually seen only in juveniles.

normally expressed in both the dorsal and the ventral iris, induction of regeneration could be attributed to either an elevation of levels over particular thresholds, or to an additive effect of retinoic-acid-regulated factors. Interestingly, neither the lens nor the retina regenerate in the axolotl, but newt dorsal iris does transdifferentiate to lens tissue when transplanted to the axolotl eye, indicating that no inhibitory signals exist in the axolotl to prevent lens regeneration. This provides a unique experimental opportunity to molecularly dissect the role of evolutionarily conserved molecules that are involved in lens and retina regeneration in vertebrates.

Little is known about the molecular mechanisms of newt retina regeneration, but a key event must be upregulation of *Pax6*. The only other model for *in vivo* retina regeneration by transdifferentiation is the chick embryo. Here the RPE transdifferentiates to retinal tissue under the influence of FGF. Inhibition of *Mitf* (microphthalmia-associated transcription factor) or *Shh* (which induces *Pax6*) increases the domain of transdifferentiation[68,78]. The drawback of the chick system is that the retina is regenerated at the expense of the RPE, which transdifferentiates to retinal tissue and does not replenish itself. This is in contrast to the newt, in which both tissues are restored during regeneration.

Other tissues. Regeneration of the heart has been reported in newts. After removal of part of the ventricle, and following wound healing by clotting and contraction, adult cardiomyocytes re-enter the cell cycle and eventually repair the damaged heart[78]. Regeneration of hair cells of the inner ear in both salamanders and frogs has also been described, and occurs through transdifferentiation of the supporting cells[79]. Functional assays such as transgenesis and loss of function by morpholino electroporation[56,80] should provide us with a more detailed understanding of how these and other regenerative properties are regulated in the amphibia.

Fish

In general, fish have good regenerative capabilities, and the zebrafish is beginning to provide an excellent opportunity to study regeneration in a lower vertebrate[81]. Zebrafish are easily reared in the laboratory, their developmental time is short and genetic screens have produced numerous mutants, including some that affect regeneration. Also, the genome is now mapped, microarray analyses are possible, and transgenesis and knock-down technology using morpholinos is readily available. Furthermore, chemical mutagenesis and small-molecule screens have provided both developmental and regeneration mutants[82].

Fin regeneration. As in amphibian limb regeneration, fin regeneration in zebrafish is mediated by the formation of a blastema at the site of amputation. Interestingly, several aspects of fin regeneration are similar to amphibian limb regeneration, such as the establishment of the wound epithelium, blastema formation and response to retinoic acid[83]. By testing candidate genes in loss- and gain-of-function assays and examining the proliferation properties of blastema cells, blastema production has been shown to be associated with FGF signalling and the expression of the transcription factor *msxB*[84]. Both *fgfr1* and *msxB* transcripts are found in the mesenchymal cells of the blastema, and an Fgfr1-specific inhibitor blocks both cell proliferation and *msxB* expression, and prevents further growth[85]. Similar results have been obtained by knocking down expression of *fgfr1* and *msxB* using morpholinos[86]. This is consistent with findings in amphibians, in which FGF signalling and *msxB* have key roles in regeneration.

Genetic screens have increased our understanding of the role of FGFs in fin regeneration. The *dob* (devoid of blastema) mutant, forms an abnormal regeneration epithelium without a blastema and ultimately fails to regenerate. The mutation has been mapped to *fgf20a*, which is expressed early at the epithelial–mesenchymal boundary, and later in a pattern that overlaps with the blastema marker *msxB*[87]. Defects of epithelial–mesenchymal interactions have also been proposed to be the cause of the loss of limb regeneration that occurs in the *short toes* mutant[88], implicating a role for the FGF pathway in effecting these interactions.

Genetic screens have also identified unexpected regeneration regulators in fish. For example, *nbl* (no blastema), was found to encode heat-shock protein 60 (Hsp60). Its expression is increased in blastema cells and its dysfunction due to mutation affects the mitochondria and leads to apoptosis[89]. Whether these and other molecules that have been uncovered by conditional screens in zebrafish are in fact regeneration-specific or also have a role in normal development remains to be determined.

Other tissues. In the future, the zebrafish could provide an important model for the regeneration of other tissues. Of particular interest to regenerative medicine, adult fish can regenerate spinal cord and brain tissue. The adult rainbow fish *Lebistes reticulates* can regenerate the forebrain within 2 months, in a process that seems to require ependymal cells[71]. If frontal-lobe regeneration also occurs in the zebrafish, genetic analyses of this process would allow us to learn much about the regeneration of complex neuronal systems in vertebrates.

Although some fish can regenerate the lens from the dorsal iris, the zebrafish cannot (P.A.T., unpublished observations). However, it can achieve some regeneration of the retina when lesions are introduced. *pax6*-positive cells from the inner nuclear layer seem to contribute to regeneration[90]. Moreover, *six3* can induce ectopic lens formation in fish when it is supplied exogenously[91].

More remarkable is the ability of the zebrafish to regenerate its heart. When up to 20% of ventricular resection is performed, cardiomyocytes at the leading epicardial edge of the regenerating myocardium proliferate and replace the lost part within 2 months[92]. Interestingly, it seems that the process involves upregulation of *notch1b* and *deltaC,* preceding that of *msxB* and *msxC* genes[93]. These genes are also upregulated during fin regeneration, and could be part of a shared regenerative response mechanism.

Morpholino
A chemically modified oligonucleotide that behaves as an antisense RNA analogue and can therefore be used to interfere with gene function.

Forebrain
The rostral-most portion of the brain.

Mammals

Compared with other vertebrates, regeneration of missing body parts in mammals is modest at best. However, there is a continuous renewal of tissues in mammals as part of tissue homeostasis, for example, in haematopoiesis, gametogenesis, and intestinal-tract epithelium and skin renewal. Such tissue homeostasis is achieved primarily by the activities of multipotent stem cells that reside in the renewing tissues. Therefore, most studies of the regenerative capacities of mammals have focused primarily on the role of stem cells (somatic, fetal and embryonic) in tissue repair. Nevertheless, examples of mammalian regeneration of missing body parts are known to occur.

Digit-tip regeneration in mice and humans. If left untreated, an amputated digit tip can regenerate. This has been observed in children and in experiments with mice. The amputation has to be distal to the first phalange. Curiously, regeneration cannot take place if the amputation is more proximal or if the wound is covered with an epithelial flap. As in the newt, blastema-like cells seem to mediate regeneration. In mice, the capacity for digit-tip regeneration has been correlated with BMP signalling and *Msx1* expression. *Bmp4* is expressed in the mesenchyme that underlies the epithelium, and is restricted to a domain that extends up to the first phalange. In *Msx1* mutant mice, *Bmp4* expression and regeneration are impaired. However, when BMP4 is experimentally introduced in such mice, regeneration is restored. Likewise, when BMP signalling is inhibited, wild-type animals fail to regenerate[94]. This action and requirement of BMP signalling is reminiscent of the similar requirement for BMP in tail regeneration in frogs[73]. The involvement of *Msx1* in digit-tip regeneration could also be mediated through its ability to induce dedifferentiation in mammalian myotubes[95]. Mouse digit-tip regeneration, therefore, is accessible to genetic interrogation and has the potential to become an informative experimental paradigm to study regeneration in mammals.

Liver regeneration. The digestive system in many animals, including humans, has remarkable regenerative abilities. Among the organs that comprise this system, the intestine and liver show great potential for renewal. The pattern of cell turnover and the proliferation of stem cells in the epithelium that lines the small intestine have been extensively studied as a paradigm for tissue turnover. However, amputation of the intestine does not result in its regeneration in mammals. By contrast, after partial hepatectomy, the remaining lobes of the liver enlarge to replace the missing mass of the organ. The enlargement of the remaining lobes is achieved through the proliferation of all mature cell types comprising the intact liver, in a specific order, without apparent dedifferentiation or transdifferentiation. Several signalling molecules are known to be involved in inducing and regulating this proliferation, including molecules with established roles in development (for example, endothelium and hepatocyte growth factors) and those with no known developmental roles (such as the complement system component C5)[96–98]. There is also evidence for the existence of stem cells in the liver; for instance, hepatic oval cells might be stem cells for hepatocytes. It is not clear, however, what the role of these cells might be during either the maintenance or the regeneration of liver[99,100]. Identifying a genetic model system such as the zebrafish to study liver regeneration would significantly aid in resolving these issues.

Nervous system and other tissues. There is extensive evidence for olfactory-neuron regeneration in mammals, and the generation of new neurons in the adult mammalian brain has been detected in the dentate gyrus of the hippocampus and the lateral ventricles of the forebrain[101]. Attempts to induce regeneration after spinal-cord injury have mainly focused on the use of growth factors and adhesion molecules that are capable of modulating the induction of axonal growth, such as nogo[102], FGF[103], and integrin[104]. Nevertheless, complete neuronal regeneration of CNS components such as the spinal cord has yet to be reported. Numerous experiments have shown that adult or embryonic stem cells can repopulate sites of damage in tissues that include the heart, brain and retina. However, given the complexity of stem-cell regulatory systems and the tissues and organs they differentiate into, direct transplantation of stem cells has proved inefficient at restoring missing or damaged tissues[105]. Therefore, studying how invertebrates and lower vertebrates effectively deploy stem cells to repair and regenerate tissues is likely to significantly inform our understanding of why these processes are not readily activated in mammals.

Emerging themes and outstanding questions

The extension of genetic tools to traditional animal models of regeneration, and the addition of genetically amenable models to the list of species in which regeneration is studied, has begun to answer some of the key questions about the biological basis of regeneration. A role for known developmental signalling pathways in regeneration is being uncovered in organisms from hydra to mammals. Given that the regenerated body parts are essentially identical to the original parts that were made during development, this is to be expected, as all animals studied so far share a finite pleiotropic set of developmental pathways[106]. However, tissue induction and interactions during development bear little resemblance to those that occur during regeneration in adult animals. For example, during vertebrate development, the lens is induced by the interactions between the surface ectoderm and the optic cup, whereas during regeneration, the lens arises from PECs of the dorsal iris (or from the cornea in frogs). This suggests that some of the mechanisms that are used to form a body part during embryogenesis are accessed again, through mechanisms that are so far poorly understood, during the regeneration of such parts in the adult animal.

Intriguingly, WNT-, BMP/transforming growth factor-β (TGFB)- and FGF-signalling pathways are repeatedly encountered during many instances of regeneration, not just as modulators of differentiation of the regenerating tissues, but also as active participants in the induction of regeneration (FIG. 5). A fruitful line of

Complement system
A biochemical cascade that is involved in innate immunity: the first line of defence that helps to clear pathogens from an organism.

Hippocampus
A part of the brain that is located inside the temporal lobe. It forms part of the limbic system and has a role in memory and spatial navigation.

investigation, therefore, might be found in unravelling the mechanisms that activate such pathways after injury and/or amputation. Axolotls, which are closely related to newts, can regenerate limbs and tails, for example, but they are unable to regenerate the lens and retina. Understanding these restrictions could help to identify the mechanisms by which reactivation of signalling pathways for regeneration is regulated in animals. It will also be important to determine whether the molecular trigger that activates such pathways after amputation is shared or unique to each tissue.

The study of regeneration in diverse organisms has also revealed a range of modes by which cells are replaced following damage, including dedifferentiation and/or transdifferentiation and the use of stem cells. Future studies will need to address the similarities and differences between these mechanisms. For instance, are stem-cell-maintenance factors also used during dedifferentiation? Are these factors involved in the induction of regeneration? Answers to these questions will not only enable us to form a general framework of the cellular biology of regeneration, but will also help to

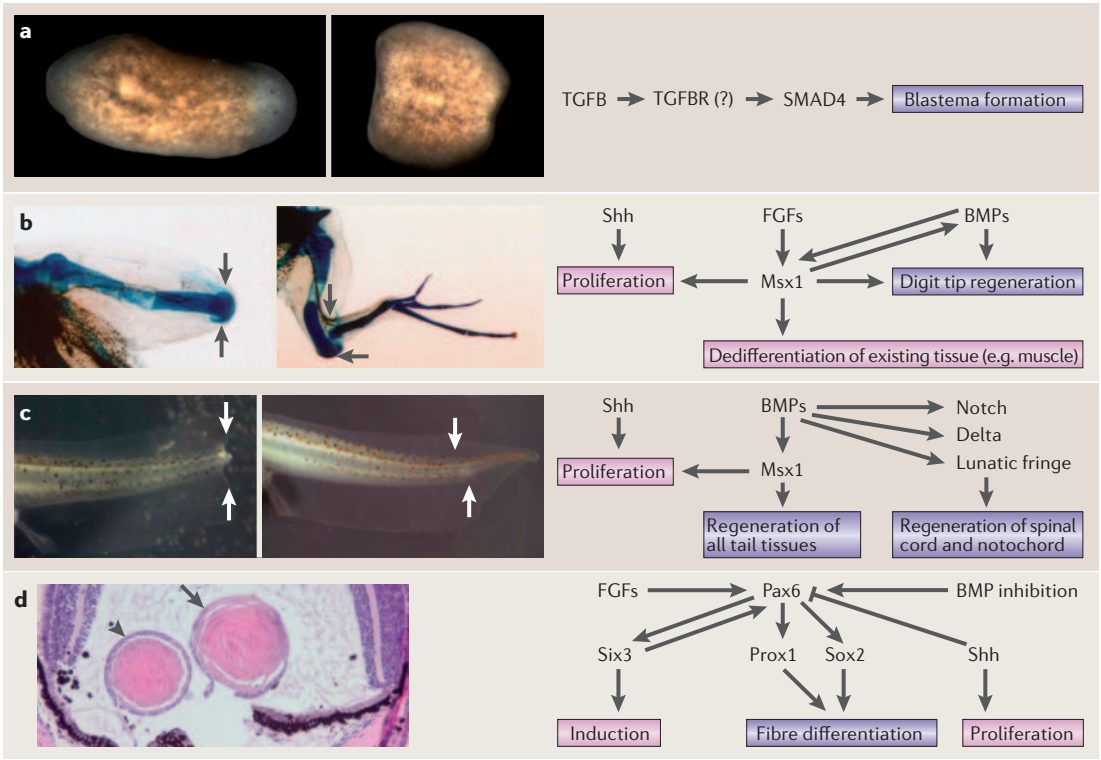


Figure 5 | **Common signalling pathways in the induction of regeneration in diverse species.** Examples of induction of regeneration are shown in the images on the left. The relevant regulatory pathways that are thought to be involved are shown on the right. **a** | Cephalic and caudal regeneration in planarians is inhibited by the abrogation of the transforming growth factor-β (TGFB) pathway. For example, this can be achieved by silencing the expression of *smad4*, a core component of the signal transduction mechanism of all known TGFBs[34]. The images show animals in which both the head and tail have been amputated. The image on the left shows a control animal 7 days after amputation; here, regeneration has taken place successfully. The image on the right shows abrogation of head and tail regeneration in animals in which the expression of *smad4* is knocked down by RNAi . TGFBR, TGFB receptor. **b** | Induction of *Xenopus laevis* limb regeneration by FGF10. The image on the left shows an animal in which the limb was amputated at the knee level at stage 56 of development. Amputation (indicated by arrows) was followed by the implantation of a bead that was soaked in a control buffer solution. No regeneration resulted. The right image shows limb regeneration, including the digits, after application of FGF10-soaked beads[69]. The part of the signalling pathway that is involved in this process that comprises msh homeobox homologue 1 (Msx1) and bone morphogenetic proteins (BMPs) is shared with mouse digit-tip regeneration. Shh, sonic hedgehog. **c** | Induction of *Xenopus laevis* tail regeneration by activation of the BMP pathway. The left image shows the result of amputation of a wild-type tadpole during a regeneration-refractory stage of development. The right image shows that tadpoles at the same stage that express a transgenic copy of the gene that encodes the BMP receptor Alk3 undergo tail regeneration. Arrows indicate the point of amputation[73]. **d** | Induction of lens regeneration in newts[75]. Ventral iris cells are normally incompetent to regenerate a lens. However, when they are treated with chordin (a BMP inhibitor) and implanted in the lentectomized eye of the newt, a second lens arises from these cells (indicated by the arrowhead), in addition to the host lens that is regenerated from the dorsal iris (indicated by the arrow). Pax6, paired box 6; Six3, sine oculis homeobox homologue; Prox1, homeobox prospero-like protein 1; Sox2, transcription factor Sox2. Panel **b** is reproduced with permission from REF. 69 © (2001) Elsevier Science; **c** is reproduced with permission from REF. 73 © (2003) Elsevier Science; and **d** is reproduced with permission from *Nature* REF. 75 © (2005) Macmillan Publishers Ltd.

inform more fundamental aspects of poorly understood metazoan cellular activities.

The use of models such as planarians promises to identify important aspects of the *in vivo* contexts in which somatic stem cells operate[35], and could ultimately help in the design and implementation of stem-cell therapies in mammals. Furthermore, delineating the molecular activities that underpin the capacity for cellular dedifferentiation and transdifferentiation in amphibians might uncover methods by which mammalian cells could be instructed to do the same, opening new therapeutic avenues for treating traumatic injury and degenerative disorders. Likewise, uncovering the differences between wound healing in mammals and fish could help explain why regeneration occurs in some animals but not in others. Because fish do not scar, but rather deploy apoptotic strategies to remove damaged tissues, investigating the molecular basis of this key difference between mammals and teleosts might identify cell-death pathways that could be manipulated in mammals to accomplish regeneration of organs like the heart and brain. All the recent advances that have been reviewed here have allowed genetic experimentation in lower organisms, and the current rate of progress suggests that research in regeneration stands to yield fundamental insights for our understanding of metazoan biology, and to expand the repertoire of therapeutic possibilities in the fields of reparative biology and medicine.

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Competing interests statement
The authors declare no competing financial interests.

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