NOT YOUR FATHER'S PLANARIAN: A CLASSIC MODEL ENTERS THE ERA OF FUNCTIONAL GENOMICS

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Freshwater planarians were a classic model for studying the problems of development and regeneration. However, as attention shifted towards animals with more rigid developmental processes, the planarians, with their notoriously plastic ontogeny, declined in significance as a model system. This trend was exacerbated with the introduction of genetic and molecular approaches, which did not work well in planarians. More recently, the heightened interest in stem-cell biology, along with the successful application of molecular, cellular and genomic approaches in planarians, is re-establishing these fascinating organisms as models for studying regeneration and developmental plasticity.

PLURIPOTENCY
The ability of a cell to contribute to multiple tissues in a developing organism. If a cell is able to contribute to all tissues, it is said to be totipotent.

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DOI: 10.1038/nrg759

Among the recent triumphs of molecular biology are the elucidation of many of the basic mechanisms that underlie embryonic development, and the demonstration that these mechanisms have been strikingly conserved between widely divergent species. Given that Abraham Trembley's investigations of regeneration in *Hydra* (published in his Mémoires in 1744) launched the era of experimental biology[1], it is ironic that the problem of regeneration still awaits a satisfying mechanistic explanation. By cutting a *Hydra* in half and noting that each fragment was capable of regenerating complete animals, Trembley not only introduced the use of experimentation to the field of biology, but also discovered regeneration and asexual reproduction in animals. Regeneration of missing body parts from differentiated tissues has posed questions, such as those concerning the regulation of polarity, positional identity, and the scale and proportion of the regenerating tissues, that have remained largely unanswered for more than 200 years. More fundamentally, regeneration reflects either the inherent PLURIPOTENCY of differentiated cells and/or the maintenance of undifferentiated cells in the adult from which the lost tissues are to be regenerated, and is, therefore, keenly associated with the current interest in stem cells as a means to overcome many human frailties. The study of regeneration in simple organisms should therefore offer important insights into stem-cell biology and the emerging field of regenerative medicine.

The tremendous strides made in understanding embryogenesis have been driven largely by genetic approaches using model systems that are amenable to classical genetic analysis. Unfortunately, these model organisms have either limited regenerative abilities (*Drosophila* imaginal discs, mouse and zebrafish) or lack them entirely (*Caenorhabditis elegans*). Furthermore, many of the organisms that are commonly used in regeneration studies (such as Urodele amphibians) require more than one month to complete regeneration, have long life cycles that preclude the possibility of genetic analysis and have extremely large genomes, which seriously complicates molecular studies. However, more recently, the tools of functional genomics have been shown to be highly adaptable and can be applied to systems that are less tractable to genetic analysis. Therefore, we, and others[2], have sought to apply these techniques to the freshwater planarian, a classic subject of earlier regeneration studies, as a model for the functional analysis of the genes that are involved in regeneration. As we describe towards the end of this article, these techniques are showing signs of great promise.

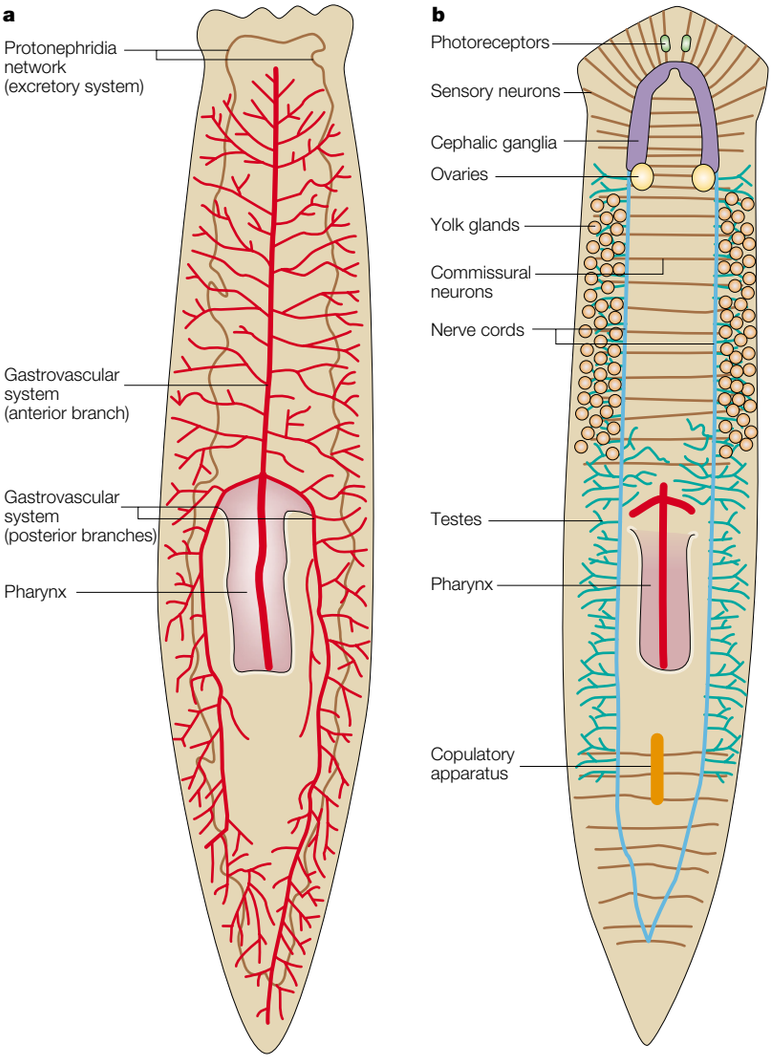

Figure 1 | **Diagrams of the major organ systems in freshwater planarians.** The figure illustrates two of the many morphologies found in the triclads[18]. **a** | *Dendrocoelum lacteum* depicts the gastrovascular and excretory systems (other organ systems not shown for simplicity). **b** | A representative of the genus *Schmidtea* in which the reproductive and nervous systems are shown. For clarity, the yolk glands are only shown in the anterior region of the animal so that the testes can be seen. The oviducts and sperm ducts are not shown. Adapted from the Wandtafeln (wall charts) of Rudolph Leuckart (http://hermes.mbl.edu.leuckart).

COELOM
A fluid-filled body cavity that is lined by mesodermal cells.

BILATERIA
Animals in which the right and left halves are mirror images. Bilaterians include most animals: chordates, arthropods, worms, molluscs and others.

PROTOSTOME
Bilaterian animals, including arthropods, molluscs and worms, whose mouth develops before the anus during embryogenesis.

Before beginning our discussion of current work, we provide some historical background, showing that the key questions concerning the biology of planarians posed by investigators more than a century ago remain unanswered. We then describe important aspects of planarian life history that make them ideal models for the study of regeneration and stem-cell biology. After discussing current work that is geared towards bringing large-scale functional genomic analysis to the study of planarians, we outline future directions for the field, with the hope of stimulating new interest in this classic problem of developmental biology. The topic of planarian regeneration has been reviewed well and often, and we refer readers who are interested in further information on the planarian system to other reviews that cover the many classic regeneration experiments[2–7].

A planarian primer

Planarians are free-living members (class Turbellaria) of the phylum Platyhelminthes, which are the flatworms. These animals are among the simplest organisms that have three tissue layers, bilateral symmetry and tissues with distinct organs (FIG. 1). These traits, combined with their lack of a COELOM and an anus, have led numerous evolutionary biologists to afford the Platyhelminthes an important position in the evolution of the Metazoa, as basal members of the BILATERIA[8–11]. In other words, planarians have key anatomical features (mesoderm, central nervous system (CNS) and excretory system) that might have been platforms for the evolution of the complex and highly organized tissues and organs found in higher organisms. However, the phylogenetic status of the Platyhelminthes has always been controversial: Libbie Hyman — one of the great invertebrate zoologists of the twentieth century — wrote that "throughout the nineteenth century the number of arrangements published was about equal to the number of interested zoologists"[12]. Reading the contemporary literature on the subject, a similar statement could be made now. So, it remains a point of contention whether flatworms represent basal bilaterians, basal PROTOSTOMES, derived protostomes or derived DEUTEROSTOMES[13–17].

The planarians that are most commonly used in regeneration experiments are freshwater representatives of the (sub)order Tricladida (the triclads; see REF. 18 for recent phylogenetic hypotheses about the Tricladida); this designation is based on the three main branches that comprise their digestive system. Flatworms lack respiratory and circulatory systems, and instead rely on diffusion to obtain oxygen. The excretory system consists of an elaborate network of FLAME CELLS that are connected by ciliated ducts, and is involved in both osmoregulation and the removal of waste products[19,20]. The planarian nervous system consists of bi-lobed cerebral ganglia at the anterior end and two longitudinal nerve cords that underlie the ventral body-wall musculature[21]. A sub-muscular NERVOUS PLEXUS runs beneath the body-wall musculature and connects to the main nerve cords. Sensory structures (photoreceptors[22] and chemoreceptors[23]) that are located at the anterior of the animal send projections to the cephalic ganglia, which then process these signals and direct the appropriate behavioural responses. Planarians also have several diverse sub-epithelial gland cells that are involved in producing the mucous secretions used by the animal for locomotion, protection, adhesion to substrates and capturing prey[12,24–26].

A brief history of regeneration

Trembley's discoveries of regeneration and asexual reproduction in *Hydra* stimulated many of his contemporaries to study the problem of regeneration and to identify other organisms with regenerative abilities similar to his famous POLYPS. Although a planarian is

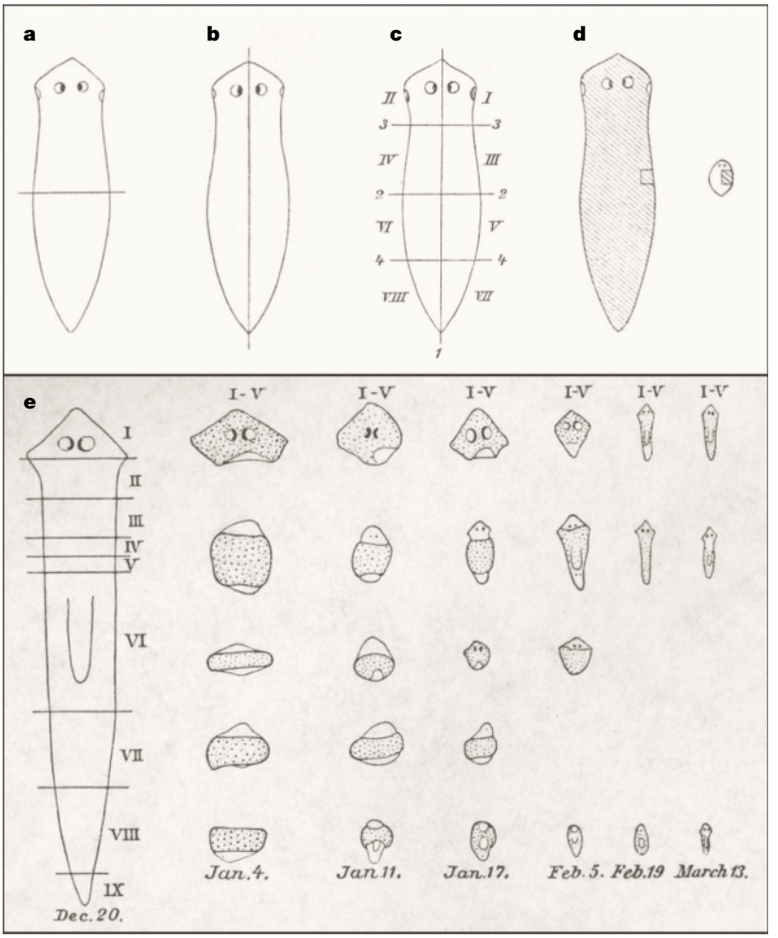

Figure 2 | **The planarian's regenerative and remodelling abilities.** The drawings illustrate the original work of Randolph[28] and Morgan[29], and show the extraordinary plasticity of planarians. **a** | Transverse cut. **b** | Longitudinal cut. **c** | Cut into eight fragments. The numbers indicate the order of the amputations. **d** | Fragment just visible to the naked eye. The shading represents the pigmentation of the intact animal and distinguishes regenerant from pre-existing tissue. All of these fragments can regenerate a complete worm. **e** | Restoration of appropriate proportion to regenerating fragments. Roman numerals I–V indicate amputation fragments depicted on the left. The date of observation is indicated for each column with fragment I at the top and V at the bottom. Parts **a**–**d** reproduced from REF. 28 and part **e** reproduced from REF. 29.

DEUTEROSTOME
Animals, including chordates and echinoderms, whose mouth develops after the anus during embryogenesis.

FLAME CELL
A cell that is distinguished by a tuft of beating cilia (resembling a flame) and that filters waste materials into the excretory system.

NERVOUS PLEXUS
A bundle or collection of nerves.

POLYP
A sessile form of an animal, such as a *Hydra*, that is attached to a substrate.

actually pictured in Trembley's memoirs (plate 7, figure 9 in REF. 1), he apparently did not attempt to determine its regenerative abilities. So, the first description of planarian regeneration was not published until more than 20 years later, by Peter Simon Pallas in 1766 (REF. 27). Subsequently, numerous investigators were attracted to study these organisms that were considered to be "immortal under the edge of the knife" (John Graham Dalyell; see REFS 5,28 for historical reviews). FIGURE 2a–d depicts images from Harriet Randolph's classic paper[28] that show this regenerative potential. Planarians can regenerate completely when cut in half transversely (FIG. 2a) or longitudinally (FIG. 2b), as can fragments that are derived from cutting the worm into eight pieces (FIG. 2c). Randolph also showed that a piece just visible to the naked eye could regenerate into a complete planarian (FIG. 2d). Randolph was a student of E. B. Wilson at Bryn Mawr College, and Thomas Hunt Morgan credited her systematic experiments as the "starting point" for his own work on planarians[29].

Before his work on the problem of inheritance in *Drosophila*, Morgan was an experimental embryologist, publishing prolifically on a wide range of experimental subjects[30], including planarians. Building on Randolph's work, Morgan showed that a fragment as small as 1/279 of a planarian could still regenerate a complete animal[29]. Morgan also tackled the problem of polarity: how does a tail fragment 'know' to make a new head and a head fragment 'know' to make a new tail[31–33]? Morgan provided the first suggestion of a MORPHOGENETIC GRADIENT to account for this process in earthworms[34–37]. The remodelling of tissue that occurs when small fragments regenerate was another topic of considerable interest to Morgan. The top row of FIG. 2e (taken from Morgan's 1898 paper) illustrates these regulatory changes in shape. Amputation of the head (I) generates a relatively short and wide triangular head-piece, and over the next several weeks this fragment remodels itself, becoming proportionately narrower and longer; within two months, a well-proportioned little planarian is formed. Morgan coined the word 'morpholaxis' (changed to the etymologically more correct 'morphallaxis'[38]) to describe the transformation that takes place in the old tissue, which results in the restoration of proportion; this remodelling takes place "without proliferation at the cut surfaces"[38].

One of the most striking examples of this form of developmental plasticity is the ability of the planarian to grow and 'de-grow' depending on nutritional status[39–43] (FIG. 3). When food is plentiful, planarians grow until they reach a maximum size. However, during periods of prolonged starvation, planarians can shrink; during the course of many months, a full-grown adult (~20 mm in length) can shrink to a size that is smaller than when it hatched from the egg capsule (~1 mm). Many investigators have proposed that de-growth leads to a reversal of the ageing process and the rejuvenation of the individual[39,44,45]. Growth and de-growth in planarians arise largely from changes in cell number[43,46] rather than from changes in cell size. Because cell proliferation continues even when the animal is shrinking[47], it is thought that the ratio of cells born by proliferation to cells lost by apoptosis is altered during starvation[43]. De-growth raises numerous questions: How is the planarian counting its cells? How is it maintaining proportion during shrinkage? How do different organs retain their function while the organism is shrinking? Some of the tools that we describe below should allow these questions to be addressed experimentally.

Reproductive modes and plasticity

Freshwater planarians reproduce either asexually, by transverse fission, or sexually, as cross-fertilizing hermaphrodites[12]. Several planarians use exclusively one mode of reproduction; others might alternate between them depending on the season; and some asexuals can be made to switch to sexual reproduction experimentally[48]. In the asexual mode of reproduction, the tail of

Figure 3 | **Growth and de-growth in response to food availability.** Animals that are starved will reduce their size, while maintaining their form and function. Feeding will reverse this condition and return the animals to their original size. The square represents 1 mm^2.

the worm adheres to the substrate while the anterior part pulls away. The worm stretches itself, becoming longer and thinner, until the fission event occurs in the posterior two-thirds of the animal. Two fragments are generated, each of which will regenerate the missing tissue, thereby producing two planarians.

Many cues determine whether or not the planarian will undergo fission; these include population density, temperature, size of the animal and light–dark cycles[49–51]. Fission is stimulated under conditions of low population density and inhibited at high population density (social control); fission is also favoured at higher temperatures and in larger animals; and occurs predominantly in the dark. Amputation of sensory structures at the antero-lateral margins relieves the social control of fission, such that amputated flatworms undergo fission in crowded conditions[50]. Because amputation of the head stimulates fission, it is thought that the planarian cephalic ganglia produce inhibitory signals that prevent fission[49,52].

These asexually reproducing worms are, in principle, immortal. Numerous clonal lines derived from many different species have been propagated in laboratories around the world for many years (for example, 15 years for *Phagocata vitta*[5]). However, it is unclear if anterior and posterior fission fragments derived from a single individual are identical with respect to this apparent immortality. Studies that compare long-term viability of anterior versus posterior fission fragments have not been done in planarians; however, Sonneborn's work on the microturbellarian *Stenostomum* shows that fission progeny are not necessarily identical[53]. *Stenostomum* is a catenulid, one of the basal orders of Platyhelminthes, which also reproduces asexually. Unlike most planarians, which undergo fission and then regenerate the missing structures (architomy), *Stenostomum* first differentiates the new structures and then undergoes fission (paratomy). Sonneborn showed that lines generated from successive anterior fragments ultimately senesced and were unable to propagate further, whereas lines generated from successive posterior fragments could be propagated without senescence[53]. Given that asexual cultures of *Stenostomum* have been maintained for more than a thousand generations in the laboratory[54], it might be concluded that these posterior fragments are immortal. It seems as if the process of forming a brain *de novo* somehow revitalizes the posteriorly derived fission fragments, rendering them refractory to the effects of ageing and senescence.

In the sexual mode of reproduction, hermaphroditic planarians mate with a partner, cross-fertilize and then deposit egg capsules, in which 1–20 planarian embryos develop, depending on the species. Planarians have paired ovaries that are situated anteroventrally, in a region behind the cephalic ganglia; numerous testes are located dorsolaterally (FIG. 1). One interesting feature of the planarian reproductive system is the separate vitelline glands, which deposit yolk on the outside of the egg as it moves through the oviduct. The result is a highly modified embryogenesis, in which the yolk cells reside outside the embryo proper (ectolecithal); as the embryo develops, it forms a provisional pharynx that ingests the surrounding yolk cells, bringing them into what will become the digestive system. The provisional pharynx is lost during embryogenesis and is replaced by the definitive pharynx that will be used for food intake by the small planarian that emerges from the egg capsule[55].

The planarian reproductive system is intriguing because the germ-cell lineage does not seem to be segregated during embryogenesis; rather, it is only when the planarian has attained an appropriate size that ovaries, testes, accessory glands and the copulatory apparatus are formed *de novo* in the appropriate parts of the animal[56]. Morgan showed that the germ line could be reconstituted from the soma by amputating heads that are anterior to the position of the ovaries and the testes. After regeneration, these head fragments, which were completely devoid of germ-line structures, could reform functional gonads[32]. Interestingly, when sexually mature planarians de-grow during starvation, the reproductive system is resorbed[40,41]. When growth is resumed, the structures are reformed; so, as in the soma, the planarian germ line is highly plastic.

Little is known about the mechanisms that lead to germ-cell formation in planarians, or the signals that instruct ovaries, testes and the complicated reproductive apparatus to be formed in the appropriate place in the flatworm. At the molecular level, the first germ-line markers to be identified in planarians were a receptor tyrosine kinase (*DjPTK1*)[57] and two *vasa*-like genes (*DjvlgA* and *DjvlgB*) from *Dugesia japonica*[58]. *vasa* was initially identified in *Drosophila* as a maternal-effect mutation that results in progeny that failed to form germ cells and abdominal segments[59]. The Vasa protein was shown to be a component of POLAR GRANULES in *Drosophila*[60]. Subsequent work has shown that *vasa* homologues are expressed in the germ cells of numerous organisms[61–66] and, in sexual strains of *D. japonica*, both homologues are expressed in the ovaries and the testes; in the testes, *DjvlgA* is expressed in spermatogonia,

MORPHOGENETIC GRADIENT
A progressive increase or decrease in the concentration of molecules that cause cells to adopt different developmental fates at different concentrations.

POLAR GRANULE
A cytoplasmic organelle that is associated with the germ plasm (germ-line material) in *Drosophila*.

spermatocytes and spermatids, whereas *DjvlgB* is expressed only in the spermatocytes. At this time, the functional role of these genes in the planarian germ line remains to be shown. However, the expression of *vasa*-like genes in the planarian germ line indicates that it shares mechanisms of germ-cell formation with those in other organisms, although the timing of their deployment seems to have been shifted later in development in the planarian.

A model to study stem-cell biology

The developmental plasticity described in the preceding sections is based on a population of stem cells that is maintained in the planarian throughout adult life. These cells are referred to as neoblasts (a word initially coined by Randolph when referring to cells thought to give rise to mesoderm in ANNELIDS[67]), and they are the only proliferating cells in the planarian[47]. In intact planarians, neoblasts are scattered throughout the PARENCHYMA, and their division progeny generate replacements for cells lost during the course of physiological cell turnover. When a planarian is injured, the neoblasts are stimulated to proliferate[68]; as the neoblasts migrate towards the wound epithelium, they give rise to the regeneration BLASTEMA — the structure in which the missing parts will be regenerated (FIG. 4).

Several classic experiments indicated that neoblasts are planarian stem cells[69–71], but the key experimental demonstration of this point came from experiments by Baguñà *et al.*[72]. It has long been recognized that X-irradiation of a planarian results in a loss of the proliferative cells, an inability to regenerate and death of the organism in several weeks[73]. Baguñà and co-workers used serial filtration to prepare cell fractions that were highly enriched in neoblasts. This methodology consists of passing a suspension of cells obtained from disaggregated planarians through sieves of progressively smaller pore diameters, therefore enriching for cells with a diameter of less than 10 μm, such as neoblasts. Introduction of these neoblast fractions into irradiated worms restored both regenerative abilities and long-term viability. Moreover, by using sexual and asexual strains of *Schmidtea meditteranea* (see below), they showed that neoblasts from a sexual strain, when introduced into an irradiated asexual strain, could form functional gonads and copulatory apparatus in previously asexual organisms, which shows that these neoblast fractions contained stem cells that could give rise to both soma and germ line.

The mechanism by which wounding stimulates neoblast proliferation and regenerative outgrowth is unknown, but a recent series of experiments sheds some light on this issue. Following classic grafting experiments, which showed that reversal of the dorsoventral axes of grafted tissue could result in the formation of aberrant outgrowths[74–76], Kato *et al.*[77] showed that blastema-like outgrowths were produced at the junction of dorsoventral discontinuities. By using molecular markers, including homologues of *orthodenticle* and the Hox gene family, to examine the patterning and polarity of these outgrowths, the authors concluded that a new dorsoventral axis was established. In subsequent work, doing similar grafts with irradiated tissue[78], they concluded that the neoblasts responded to positional information provided by the differentiated cells. This was not unexpected given the results of Baguñà *et al.* that injection of neoblasts can restore regenerative abilities and viability[72]. From the above experiments, it seems clear that wounding alone will not stimulate blastema formation and proliferative outgrowth by neoblasts. Rather, the neoblasts are probably responding to specific signals from the wound epithelium; these signals can be generated at positions of dorsoventral discontinuities.

Another unresolved issue relates to the heterogeneity of the neoblast population. What percentage of cells that are morphologically defined as neoblasts are truly totipotent, and how many of them represent lineage-restricted descendants? The identification of neoblast markers, such as homologues of *MCM2* (mini-chromosome maintenance)[79] and *PCNA* (proliferating cell

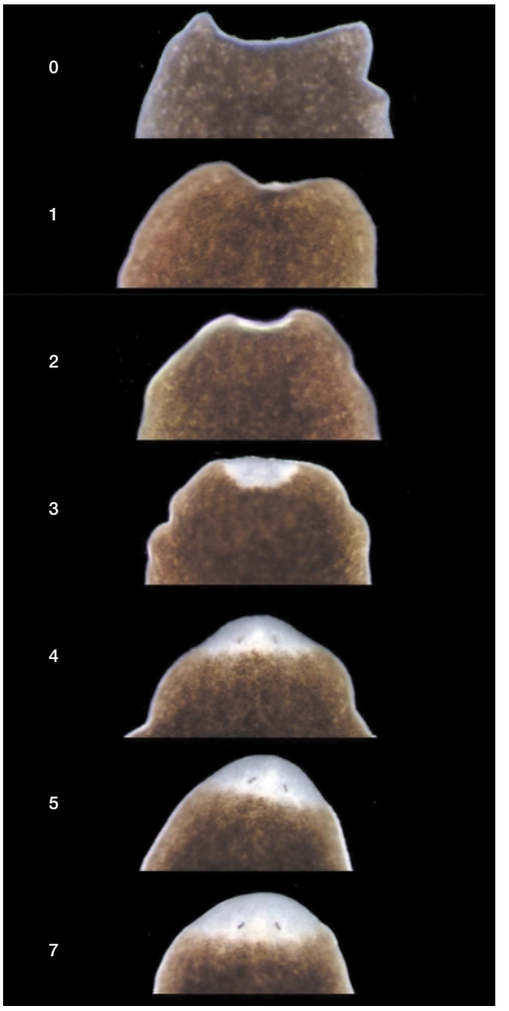

Figure 4 | **Regeneration in *Schmidtea mediterranea*.** Time-course of the same organism undergoing cephalic regeneration. The blastema is unpigmented. Numbers refer to days after decapitation.

ANNELID
The phylum of segmented worms.

PARENCHYMA
Mesodermal tissue that fills the space between the epidermis and the gut in acoelomates (animals that lack a coelom).

BLASTEMA
A specialized structure that is composed of an epithelial layer and mesodermally derived, undifferentiated cells.

nuclear antigen)[80] should help to address these questions, because such markers might potentially be used to identify different subsets of neoblasts. For example, PCNA protein persists long enough in neoblasts that have left the cell cycle and are in the process of differentiating to allow their migration into the regenerating pharynx to be visualized[80]. The planarian *vasa* homologue *DjvlgA* that is expressed in the ovaries and the testes of sexual planarians is also detected in asexual planarians, in mesenchymal cells that are located along the entire length of the planarian, as well as in the pharynx[58]. *DjvlgA*-expressing cells are detected in regeneration blastemas and there is a marked decrease in *DjvlgA*-expressing cells after irradiation, which led Shibata *et al.*[58] to suggest that this gene is expressed in neoblasts and is involved in regulating their totipotency. However, *DjvlgA*-expressing cells are abundant in all regions of the animal, including regions that lack stem cells (see below), so there are other possible interpretations of these data. Perhaps *DjvlgA* is expressed in the lineage-restricted or committed daughters of neoblast division or in cells that are in the process of differentiating, and therefore is involved not in regulating totipotency but rather in activating differentiation.

Because the neoblasts are the only proliferating cells in the planarian, they can be specifically labelled with the thymidine analogue bromodeoxyuridine (BrdU)[81], which is incorporated into DNA during the S phase of the cell cycle[82]. Shortly after a pulse of BrdU, the neoblasts can be detected throughout the mesenchyme, surrounding the gastrovascular system (FIG. 5). Notably, BrdU-labelled neoblasts are not detected in the region anterior to the photoreceptors or in the highly differentiated pharynx, both of which are post-mitotic structures that are incapable of regenerating a complete animal when isolated from the worm[29]. BrdU labelling has revealed several features of neoblast behaviour during regeneration: neoblast migration is an active process, not passive cell spreading due to proliferation; BrdU-labelled neoblasts contribute to the regeneration blastema; and the differentiation of neoblasts into epithelial cells occurs in both the intact animal and during regeneration. The ability to specifically label the S-phase neoblasts paves the way for a detailed analysis of the planarian stem cells, with respect to studying both the heterogeneity of the population and the control of stem-cell proliferation in the context of the whole organism. For instance, the extent of molecular differences that might exist in the morphologically homogeneous population of planarian neoblasts remains unknown, and the mechanisms that regulate the cell cycle during regeneration, growth and de-growth have yet to be discovered.

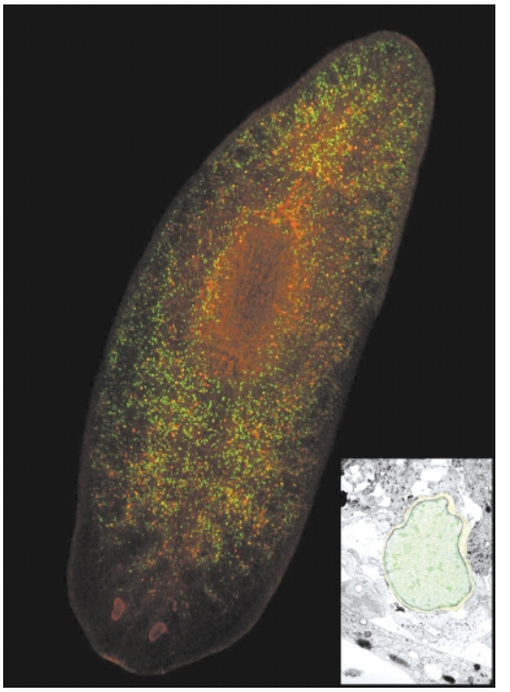

Figure 5 | **Distribution of proliferating cells in the intact planarian.** Confocal projection of a planarian fixed 8 h after a single pulse of bromodeoxyuridine (BrdU) and stained to detect BrdU (in green) and phospho-histone H3, a marker of mitosis (in red). The anterior of the animal is at the bottom of the image. The inset is a pseudocoloured electron micrograph of a neoblast (nucleus in green, cytoplasm in yellow) of ~8 μm in diameter near the wound epithelium 30 min after amputation.

Schmidtea mediterranea as a model planarian

Given that there are hundreds of species of planarians, the selection of any single species as a representative model for molecular analysis is fraught with potential difficulties. Ideally, a species would be chosen on the basis of several properties: relative ease of culture in the laboratory; developmental plasticity (including asexual and sexual modes of reproduction); and a comparatively small, diploid genome. Because most freshwater triclads are easily reared in the laboratory and show extraordinary developmental plasticity, the issues of genome size and ploidy become crucial for selection of a model species. Many species of planarian exist either as MIXOPLOIDS or as polyploids[83]. For example, attempts to study the arrangement of Hox clusters in *Girardia tigrina*[84] and *Dugesia japonica*[85] have been hampered because both of these species are mixoploids ($2n = 16$; $3n = 24$)[85,86] that have undergone genome duplications. This complicates the assignment of homologous genes into orthologous and paralogous groups. Furthermore, *G. tigrina* carries thousands of copies of *MARINER* transposable elements in its genome[87], and *D. japonica* harbours numerous expressed retrotransposons, which hinders attempts to carry out chromosome walks and assembly of contiguous genomic clones.

In contrast to the above species, *S. mediterranea* (FIG. 6) is a stable diploid ($2n = 8$)[88] with a haploid genome of ~7×10^8 bp (REF. 89). *S. mediterranea* lacks detectable *mariner* elements in its genome[87], and a recent EST (expressed sequence tag) project carried out in our laboratory (~8,000 total cDNA clones sequenced) did not identify large numbers of expressed retrotransposons (A.S.A. and P.A.N., unpublished data).

MIXOPLOID
Containing cells that are of different ploidy — for example, diploid and polyploid.

MARINER
A transposable element that was originally discovered in *Drosophila* and has since been shown to be present in the genomes of diverse species.

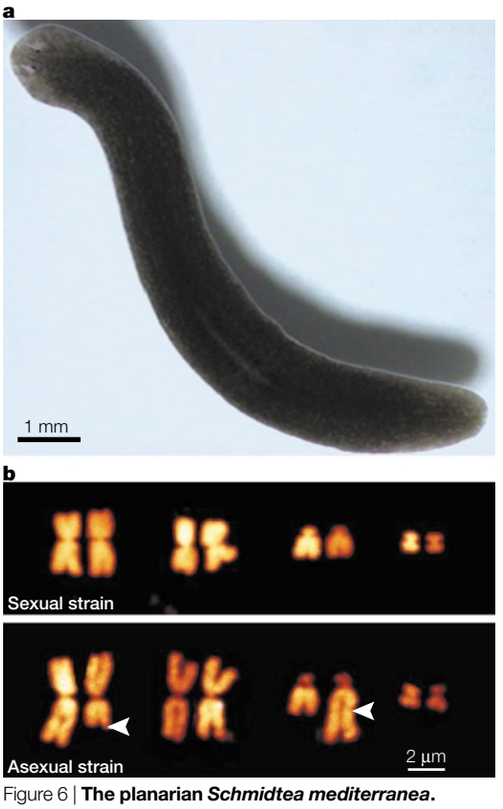

Figure 6 | **The planarian *Schmidtea mediterranea*.** **a** | Individual of the *S. mediterranea* asexual strain. **b** | Metaphase chromosome spreads of sexual and asexual strains. Arrowheads indicate the sites of the translocation that are associated with the asexual mode of reproduction.

Furthermore, sexual and asexual strains of this species can be distinguished by a chromosomal translocation that is present only in the asexuals[88] (FIG. 6). Individuals that harbour this translocation reproduce by transverse fission and do not differentiate germ line or the somatic copulatory apparatus; individuals that lack this translocation are hermaphroditic and do not reproduce asexually. These chromosomal differences provide points of entry for studying the genetic regulatory mechanisms that underlie sexual and asexual modes of reproduction. For example, BAC (bacterial artificial chromosome) libraries from both sexual and asexual strains are being generated and mapped in the hope of identifying and testing the complement of genes that are disrupted by the translocation.

The regenerative abilities of both the sexual and asexual strains have allowed us to generate clonal lines of *S. mediterranea* derived from single animals. Such lines are desirable because they provide a uniform genetic background to minimize experimental variability and they reduce sequence polymorphisms that are normally encountered in wild-type populations. The clonal asexual lines generate hundreds to thousands of fission progeny each week, depending on the feeding regimen, which paves the way for high-throughput molecular analyses (see below). On the basis of all of the properties outlined above, we believe that *S. mediterranea* represents the most tractable planarian available for the molecular analysis of regeneration and developmental plasticity. However, comparative studies that use planarians with limited regenerative abilities (*Dendrocoelum lacteum*, FIG. 1a), as well as good regenerators such as *S. mediterranea* and *D. japonica*, should also offer important insights into the mechanisms of regeneration. Such studies will be invaluable in trying to identify the signals and to dissect the mechanisms that drive growth, development and regeneration in planarians.

Molecular tools

The recent application of a range of molecular biological methodologies to the study of planarians has the potential to transform our understanding of regeneration in these organisms. One of the most significant advances has been the development of whole-mount *in situ* hybridization techniques for analysing gene-expression patterns[90]. Although whole-mount hybridization techniques are now routine for most model organisms, planarian tissue is notoriously difficult to fix properly[91], which has led numerous investigators to the mistaken belief that the planarian parenchyma is a syncytium[12]. Furthermore, many common fixatives extensively crosslink the mucous coating that protects the animal, rendering the planarian impermeable to nucleic acid or antibody probes. So, the painstaking work carried out by Agata and his collaborators to develop appropriate fixation and hybridization conditions[90] represented an important turning point for localizing gene-expression patterns in the flatworm. Whole-mount *in situ* hybridization has now been used for several purposes: to define numerous cell-type-specific markers; to examine the expression patterns of planarian homologues of conserved developmental regulators[85,92,93]; and to re-explore the patterning events induced by classic grafting experiments[77,78,94]. Furthermore, CNS markers have been used to show the surprising degree of regionalization in the planarian cephalic ganglia[90,95] and that these ganglia might be structurally distinct from the ventral nerve cords[96].

The ability to analyse gene-expression patterns in the whole organism encouraged us, as well as Agata and his colleagues, to embark on independent, small-scale, EST projects. So far, ~2,000 non-redundant ESTs have been characterized from *D. japonica* (K. Agata, personal communication), and ~3,000 ESTs from *S. mediterranea* (A.S.A. and P.A.N., unpublished data). Nearly two-thirds of all non-redundant cDNAs from both species shared significant similarity with entries in public databases. Many of these conserved sequences (at least 60%) had higher similarities to deuterostome than to protostome sequences, and numerous homologues of mammalian genes that are not present in the *Drosophila* or *C. elegans* genomes have been identified in planarians. The analysis of the expression patterns of each of the *S. mediterranea* ESTs is also now under way. Taking advantage of the large amount of tissue produced each week by the fission process, we have adapted the whole-mount *in situ*

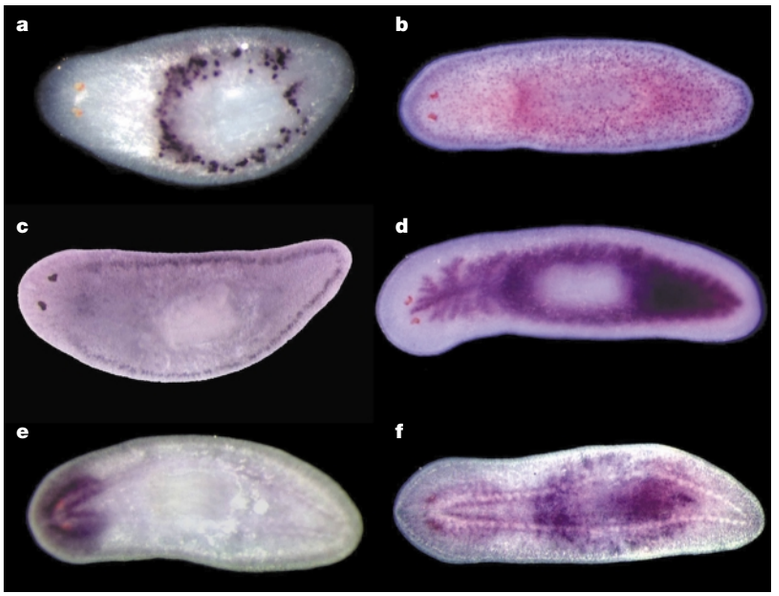

Figure 7 | **Representative whole-mount *in situ* hybridizations.** The images were obtained using the following labelled cDNA probes and an automated hybridization procedure (A.S.A. and P.A.N., unpublished data). **a** | Similar to *Drosophila melanogaster* CG6763 gene product (hypothetical zinc metalloproteinase; BLASTX = $3e^{-30}$). Expressed in central secretory cells. **b** | Similar to *D. melanogaster* CG10854 gene product (peroxisomal membrane protein; BLASTX = $2e^{-13}$). Expressed in a subset of epithelial cells. **c** | Similar to *Mus musculus* purine-rich binding protein (BLASTX = $2e^{-12}$). Expressed in subepidermal marginal adhesive gland cells. **d** | Similar to *Locusta migratoria* apolipophorin precursor (BLASTX = $6e^{-16}$). Expressed in gastrovascular system. **e** | No GenBank/dbEST match. Expressed in cerebral ganglia. **f** | No GenBank/dbEST match. Expressed in cerebral ganglia, nerve cords and central secretory cells.

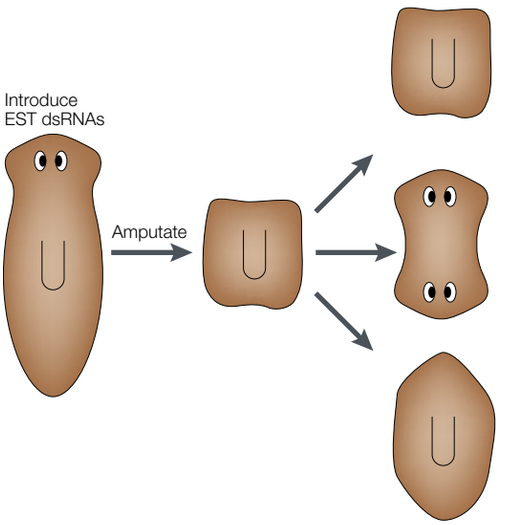

Figure 8 | **Scheme for using RNA interference to identify genes that are required for regenerative processes.** Double-stranded RNA (dsRNA) corresponding to EST (expressed sequence tag) clones is introduced into planarians. These animals are amputated and allowed to regenerate. Disruptions in the process might be scored by arrest or delay of regeneration, polarity defects, or defects in specific cell types (not shown).

hybridization protocol for use in a commercially available liquid-handling robot, allowing high-throughput mapping of expression patterns (FIG. 7; A.S.A. and P.A.N., unpublished data). This initial analysis has defined molecular markers for most of the cell types in the planarian.

Although potentially quite informative, the sequencing of ESTs and the determination of their expression patterns in specific cell types and/or tissues only hint at gene function. Because, ultimately, we would like to understand the molecular mechanisms that are involved in regenerative processes, a direct test of gene function is required. Planarians are not amenable to classical genetic analysis, so other methods for studying gene function are necessary. The discovery that introduction of double-stranded (ds)RNA can specifically inhibit gene expression in *C. elegans*[97] provided us with the impetus to extend this methodology to studies of planarian regeneration. Microinjection of dsRNA has been used successfully to inhibit several planarian genes[98]. For example, it has been shown that a planarian homologue of *sine oculis* — a gene that is required for eye formation in *Drosophila*[99,100] — is required for photoreceptor regeneration[101]. The large collections of available ESTs, the determination of their spatial patterns of expression in intact and regenerating planarians, and the ability to specifically inhibit the functions of these genes, promise to revolutionize our understanding of the mechanisms that underlie the developmental plasticity shown by these fascinating organisms.

Future directions

At the molecular level, the problem of regeneration still represents largely uncharted waters. So far, most molecular studies of planarian regeneration have followed in the wake of model genetic systems, and have been limited to studies of homologues of previously identified developmental regulatory genes. This approach will surely be informative, but given how little is known about the similarities and differences that exist between regeneration and embryogenesis, more unbiased methods for studying the problem are also necessary. Two experimental approaches can be taken to identify important components in the regenerative process: microarray analyses and large-scale RNA interference (RNAi)-based screens.

The large collections of cDNAs obtained from *D. japonica* and *S. mediterranea* are now being used to generate microarrays for determining the gene-expression profiles that define regenerative processes and for identifying CNS-specific genes (M. Nakazawa *et al.*, unpublished data; A.S.A., unpublished data). These studies will allow the identification of genes, the transcription of which is regulated during various stages of regeneration, from wound healing and proliferation to patterning. Such candidate molecules can then be studied in more detail: by examining the spatio-temporal pattern of gene expression using whole-mount *in situ* hybridization; and by using RNAi to find out if inhibition of that gene results in a disruption of the regenerative process. Such analyses are likely to uncover roles in

regeneration for genes, the functions of which have been characterized in other contexts, and might also identify novel genes, the functions of which are unknown.

Recent work from Timmons and Fire has shown that, in *C. elegans*, ingestion of bacteria that express dsRNA results in specific gene inhibition that is comparable with that obtained by microinjection[102,103]; these results paved the way for large-scale chromosome-wide screens using RNAi in a manner analogous to chemical mutagenesis[104]. We can foresee similar RNAi-based screens being done to identify genes with roles in regeneration, patterning and proportion regulation in planarians (FIG. 8). As more cell-type-specific markers become available in planarians, the specificity of such screens will be greatly increased, thereby allowing the dissection of the pathways that specify neoblasts to generate the 25–30 different cell types in the planarian.

Before the full potential of the planarian as a model system can be realized, however, several challenges still have to be met. For instance, optimal conditions for the *in vitro* culture of neoblasts have yet to be defined[105]. Efforts that are now under way to isolate neoblasts using fluorescence-activated cell sorting should aid the systematic characterization of both neoblast heterogeneity and the culture conditions that are necessary to maintain, expand and differentiate neoblasts *in vitro* (K. Agata, personal communication). As neoblasts are totipotent, it is conceivable that in the future these cells might be used as vectors to introduce DNA into irradiated planarians for the generation of transgenic animals[6]. Development of an accessible gain-of-function assay for planarians, such as transgenesis, will complement the tools already available and allow the development of further research avenues.

Given that the traditional experimental subjects used for the study of blastema-based regeneration, such as salamanders and axolotls, are not readily amenable to the study of gene function, flatworms are a particularly attractive model to dissect the molecular control of metazoan regeneration. The stem-cell population that is responsible for their regenerative prowess also renders them useful for studying the control of cellular pluripotentiality and stem-cell proliferation. The molecular tools that are now being applied to studies of this classic model organism provide hope that the developmental plasticity that seemed so insoluble to Morgan might now be addressed experimentally.

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One of the first systematic examinations of neoblast behaviour in culture.

Acknowledgements

We thank S. Robb and R. Juste for their outstanding contributions to the *S. mediterranea* EST project; M. Sepanski for electron microscopy; M. Pala for her generous gift of the sexual strain of *S. mediterranea*; K. Agata and the members of his lab for sharing unpublished results; N. Oviedo for sharing *in situ* results before publication; anonymous referees for their helpful suggestions; and the National Institutes of Health, National Institute of General Medical Science for supporting our work on planarians.

Online links

DATABASES

The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
MCM2 | *PCNA* | *sine oculis* | *vasa*

FURTHER INFORMATION

Alejandro Sánchez Alvarado's lab:
http://planaria.neuro.utah.edu
Phillip Newmark's lab:
www.life.uiuc.edu/csb/faculty/newmark.html
Planarian papers published in the 18th–19th centuries in Europe: http://www2u.biglobe.ne.jp/~gen-yu/pla_classic_e.html
Planarian resources on the Web:
http://www2u.biglobe.ne.jp/~gen-yu/plaweb_e.html
Access to this interactive links box is free online.

- The planarian was established as a system for the study of regenerative development more than 200 years ago. As attention shifted towards organisms with more rigid patterns of development, and molecular and genetic techniques gained popularity, planarians fell out of favour.
- Planarians show remarkable developmental plasticity. For example, a planarian can regenerate from a piece of tissue that represents less than 1/279 of the adult organism.
- They also de-grow in nutritionally limiting conditions and can shrink to a size that is smaller than their size at hatching. De-growth is accompanied by loss of reproductive structures, which re-form when nutrition becomes plentiful again.
- Developmental plasticity is dependent on a population of pluripotent cells called neoblasts. Neoblasts are thought to represent stem cells and are stimulated to migrate, grow and divide by discontinuities in the adult structure. Planarians therefore have great potential as a model system for studying stem-cell biology.
- Genetic markers have recently become available that will help to study neoblasts, and to determine, for example, whether the cells are a homogeneous population or consist of a collection of lineage-restricted cells.
- Further technological improvements in this system have been the development of methods for *in situ* hybridization, the successful application of RNA interference and an accumulation of genomic resources.
- The current fascination with stem-cell biology, along with the technological advances in planarians, has set the stage for a resurgence of interest in these organisms. Planarians could provide important insights into the mechanisms that underlie regeneration and development.

Phillip Newmark received his B.A. in Biology from Boston University and his Ph.D. from the Department of Molecular, Cellular and Developmental Biology at the University of Colorado at Boulder. He began his work on planarians as a Postdoctoral Fellow of the Cancer Research Fund from the Damon Runyon-Walter Winchell Foundation. This work was carried out in the laboratory of Jaume Baguñà at the Department of Genetics, University of Barcelona. He continued his postdoctoral work in the laboratory of Alejandro Sánchez Alvarado at the Department of Embryology, Carnegie Institution of Washington, Baltimore. He is now Assistant Professor in the Department of Cell and Structural Biology at the University of Illinois at Urbana-Champaign.

Alejandro Sánchez Alvarado graduated from Vanderbilt University in 1986 and received his Ph.D. in 1992 from the Department of Pharmacology and Cell Biophysics at the University of Cincinnati School of Medicine. From 1994 to 1996, he was a postdoctoral fellow in the laboratory of Donald D. Brown at the Carnegie Institution of Washington, Department of Embryology. In 1996, he was appointed Staff Associate at the Department of Embryology, where he and Phil Newmark began to explore planarians as a model system to dissect the molecular basis of regeneration. He is now Associate Professor in the Department of Neurobiology and Anatomy at the University of Utah School of Medicine, where he and his laboratory continue their work on these fascinating organisms.

URLs
LocusLink
MCM2
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=4171
PCNA
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=5111
sine oculis
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=35662
vasa
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=34884

Further information
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