Table 3. Depiction of Unique Regeneration Characteristics in the *B. leachi* WBR Model System

Key Characters	Other Representative Models[a]	*B. leachi* WBR
Site for initial regeneration	Blastema	Regeneration niche
Number of regeneration sites	One per entity	Multiple
Type of participating cells	Noncirculating cells	Circulating blood cells
Restoration derivates	Soma (except for planaria, which is soma and germ)	Soma and germ
Intraorganismic hierarchy	No competition, a single regeneration site	Competition between regeneration niches, only a single survivor
Intraorganismic regulation	Centralized induction	Systemic induction

[a]Representative regeneration models include adult salamanders, zebrafish, planaria, and echinoderms [2, 63–67].
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ment of tissues and organs are one of the major objects in developmental biology. Redeployment of these embryonic signaling pathways at different biological contexts enables quicker responses by multicellular organisms to adverse environmental conditions, such as RA signaling in the regeneration of specific tissues and organs in various vertebrate model systems [26,49,50]. A functional example of redeployment of a developmental mechanism comes from the mammalian central nervous system. Embryonic central nervous system undergoes regeneration through RARβ2-stimulated RA signaling, unlike adult central nervous system, which does not express RARβ2. Redeployment of RA signaling through overexpression of RARβ2 indeed promotes functional regeneration of adult central nervous system [31].

It had already been shown that RAR is expressed in mesenchymal cells of developing buds in the colonial tunicate *P. misakiensis,* where it regulates developmental aspects in normal budding processes, including the induction of a secondary axis in developing buds [51–53]. Following the evaluation of the biological redeployment strategy, this study reveals, for the first time to our knowledge, the important roles of RA in botryllid ascidian WBR. We show that RAR is expressed during WBR, specifically in haemocytes within the vasculature. In contrast to tissue- and organ-specific expression patterns of RA receptors in vertebrates, RAR expression in botryllid regeneration does not follow developmental processes expressed in normal blastogenesis of tissue and organ structuring, but is expressed ubiquitously throughout the entire body. This observation suggests that in addition to its role in early stages of regeneration, RAR expression also represents other RA activities, such as maintenance or other yet unknown proceedings. We further show that the entire body of developing zooids is susceptible to RA signaling. The use of both DEAB, Citral, BMS-493, and RNAi-mediated knockdown of Bl-RAR to disrupt RA synthesis and RA receptor function, collectively result in WBR arrest and bud malformation. The administration of all-*trans* RA into fragments of blood vessels results in accelerated regeneration and the unusual development of multibuds, leading to restored colonies with multiple functional zooids. This observation could indicate that in regular *Botrylloides* WBR, the systemic developmental inhibition of all buds but one, could be achieved by controlling the levels of RA transcriptional cascade. We found, during early stages of WBR, that highly Aldedh-positive macrophages form foci of expression that correspond to foci of multiple initiations of regeneration, strengthening the notion that RA is required for the early stages of this process [54,55].

Regeneration as a central discipline in biology holds great promise not only for the understanding of species-specific developmental issues, but also for deduction of its evolutionary roots and for medical applications [56]. In the past few years, ascidians have become model organisms for the study of a wide variety of biological phenomena, including developmental processes, embryogenesis, stem cell biology, and immunology [57–60]. This group of organisms demonstrates basic mechanisms of biological phenomena, similar to those observed in vertebrates. In light of the unique regenerative power of botryllid ascidians, WBR in *B. leachi* may serve as a model system for studying the evolutionary roots of organ regeneration, lost in most vertebrate taxa.

Materials and Methods

Animal husbandry and colony dissection. Colonies of *B. leachi* were collected from underneath stones in shallow waters along the Mediterranean coast of Israel. The colonies, with thin layers of stony material attached to them, were carefully pealed off from the substratum using industrial razor blades and individually tied with fine threads onto 5 × 7.5-cm glass slides. Colonies were cultured on slides placed in 17-l tanks of standing seawater system [33]. Within several days of culture, ampullar contractions and expansions resulted in complete or partial sliding of colonies from their natural calcareous substrate onto the glass slides. Colonies were cleaned weekly by carefully removing debris (empty substrates and other settled organisms) from the glass slides with industrial razor blades and fine brushes.

Isolation of marginal ampullae and blood vessel fragments was performed under a dissecting microscope using an industrial razor blade and a fine tungsten needle. Next, the dissected colonies were removed from the glass slides onto other slides, and the colonial fragments were cut further into smaller fragments using a fine tungsten needle. Blood vessel fragments were left to regenerate in 17-l tanks and were monitored daily by observing slides under a dissecting microscope. Whole fragment pictures were taken with a Supercam camera (Applitec, http://www.applitec.co.il).

Histology and immunohistochemistry. Immunohistochemistry and general histology were performed as described by Lapidot [61]. Dissected blood vessels were fixed in Bouin's solution for 1 h, dehydrated in 70% ethanol, and embedded in paraffin wax. For general histological observations, we used the regular hematoxylin-eosin staining protocol. For immunohistochemistry, serial 5-μm thick sections were prepared, attached to SuperFrost Plus microscope slides (Menzel-Glaser, http://www.menzel.de), dewaxed, and antigen retrieval was performed by microwaving (480 W) the sections for 30 min in 10-mM citrate buffer (600 ml, [pH 6], 30 min). After a 5-min cooling period, up to 1 l in volume distilled water was added, and the slides were incubated for additional 10 min at room temperature, followed by several washes with TBS. Endogenous peroxidase activity in the sections was blocked by incubation in Dako EnVision$^+$ System Peroxidase block (AEC, catalogue number K4005, http://www.dako.com,) for 6 min, and the slides were washed with water. Nonspecific binding sites were blocked by incubation in 1% BSA (Sigma, http://www.sigmaaldrich.com) in 50 mM TBS–0.1% Tween 20–0.01% Triton × 100 for 1 h at room temperature. The slides were washed with TBS