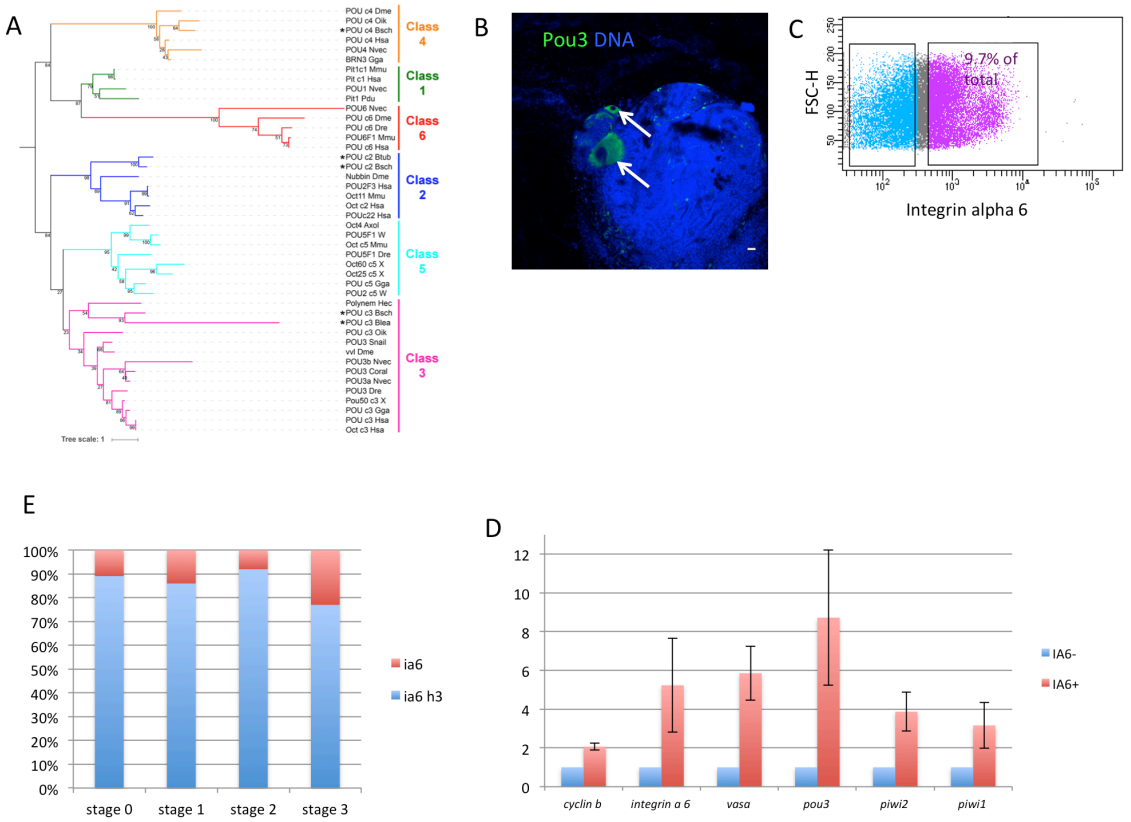

Figure S1: A: Maximum likelihood phylogenetic analysis of POU family members. Phylogenetic tree was constructed with RAxML using a maximum likelihood method, the JTT substitution matrix, and empirical frequencies. Botryllid POU proteins identified in this study are marked by asterisks. Families of POU proteins are labeled and indicated by vertical lines. Nodes are labeled with bootstrap values in units of percentage. The scale bar for branch lengths indicates the mean number of inferred substitutions per site. B: FISH for pou3 (green) on whole colonies. Pou3 is expressed in germ cells associated with the secondary bud (arrows). C: Fluorescence-activated cell sorting (FACS) of Integrin-alpha-6-positive cells. Integrin-alpha-6-negative cells were gated based on isotype-control staining. D: quantitative-real-time-PCR analysis of gene expression in FACS-sorted IA6+ cells. Data are expressed as averages from 4 experiments, normalized to IA6- cells. E: Percentages of ia6/h3 double positive cells (blue) and ia6 single positive cells (red) for each stage. Single positive (*ia6* or *h3*) and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from 4 independent samples were counted.

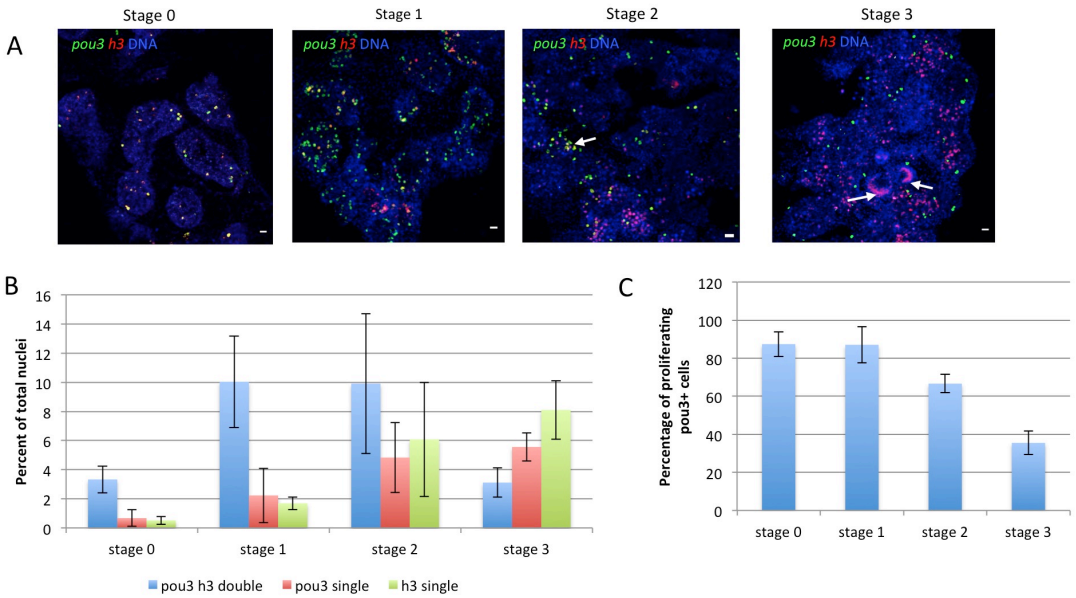

Figure S2:A) FISH showing expression of *pou3* (green) and *histone 3* (h3, red) during stages 0, 1, 2 and 3 of WBR. DNA was stained with Hoechst (blue). Scale bars 20um.Arrows in stage 3 indicate the beginning of double vesicle formation. B) Single positive (pou3 or h3) and double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from 4 independent samples were counted, comprising a total of 2500 - 3000 cells for each sample. Graph shows percentages of pou3/h3 double positive and pou3- or h3- single positive cells among all Hoechst-positive nuclei. Error bars show standard deviation. C) Averages of percentages of proliferating pou3+ cells among all pou3+ cells. Double positive cells were counted using the cell counter feature in FIJI, and for each stage, 4 images from 4 independent samples were counted, comprising a total of 2500 - 3000 cells for each sample. Error bars represent standard deviation.

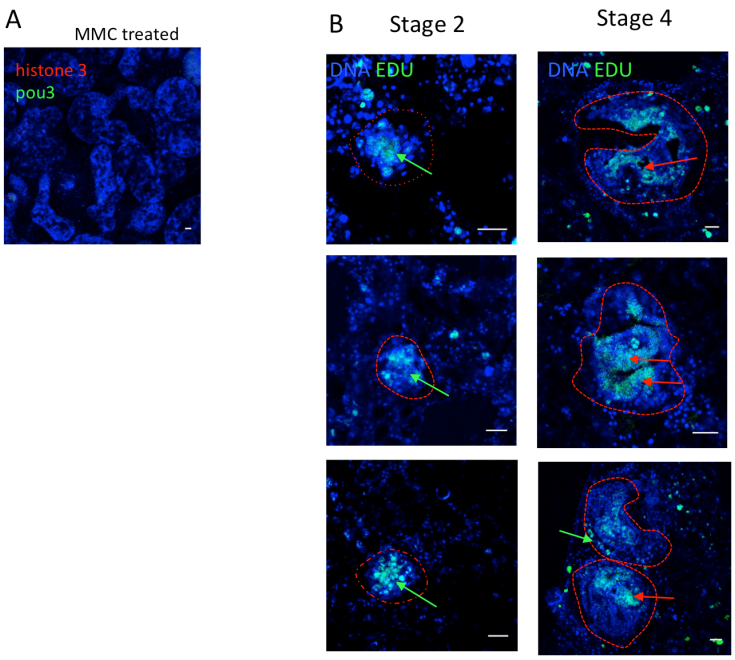

Figure S3: A: FISH for pou3 and histone 3 5 days after treatment with Mitomycin C. Scale bar 20µm. B: IA6+ cells were isolated from Edu-treated animals and injected into MMC treated vessel fragments. Overlay of nuclear staining (Hoechst, blue) and Edu (green) shows Edu-positive cells giving rise to regeneration foci at late stage 2 (white arrows). Red dotted lines outline regeneration foci. Scale bars 20µm. Images are representative of 10 samples from two independent experiments. Several samples from the same experiment as in A were followed to stage 4 (organogenesis). Red arrows: Edu-positive cells are present in several layers of differentiating tissue (red arrows) and occasionally in the outer epithelial internal tissue layers. Green arrows: Edu-positive epithelial layer. Red dotted lines outline regeneration niches. Scale bars 20µm.

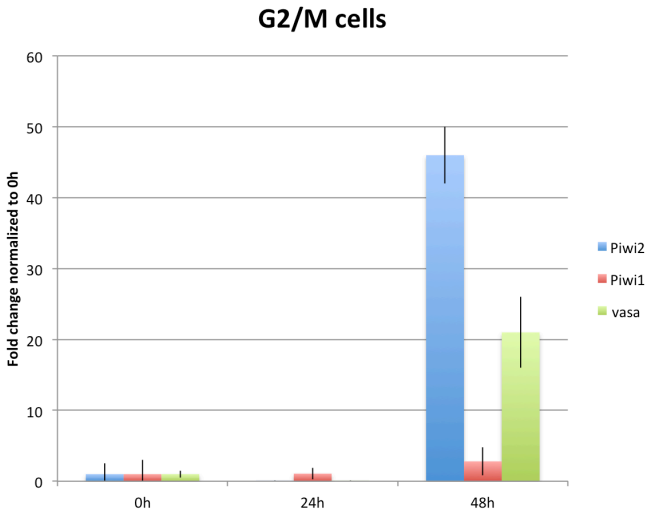

Figure S4: Q-PCR analysis of gene expression in cycling G2/M cells at different time points of WBR. Data are expressed as averages of 3 experiments, normalized to 0h. Error bars show standard deviation.

Pou3-phylogenetic analysis – protein sequences

Botryllus schlosseri Class 4 POU (POU_c4_Bsch):
FESLTLSHNNMVALKPILTTWLELAEEEYRRKMEQSGLAEKKRKRTSIAAPEKRSLEAYFLVQPRPSSEKIAAI
AEKLDLKKNVVRVWFCNQRQKQKRMKFSAFNGENGGM

Botryllus schlosseri Class 2 POU (POU_c2_Bsch):
MLQYQQHRMFEDRRHSGGEFIHARPPSPGMHPGISQSPXHQSDYEETSSKRQRYEDEASELESLERFAKDFK
QRRIKMGFTQGDVGVAMGRFYGNDFSQTTISRFEALNLSVKNMGKLKPLLERWLIDVDRAISTGERSEGRPV
LSQPMMAMNSQSCAGRKRKKRTSIPTEGKSRLEDAFLKNPKPTTEEIGKFSEDLNMDREVVRVWFCNRRQK
QKRIATQQQRYVGEQHSPSSPAGFNEPHSVEGQRSPISSEDENYGSHHLSDRPPVHQAIEHSEMGTLPPQLPP
RPGIIQLKLSESHQMQFGRPPNYSSHSSLMGGNSAI

Botryllus schlosseri Class 3 POU (POU_c3_Bsch):
VEFYEMLSQVPNGELAYGSPLQDGCGKYSSHSEVGKCRNKTRIHENPHMPNHSPVMSYSTPGLSTYACLDSQ
PPIRSDTSQEQESFTKISDEHYRPYPNGYQFSNHCYQFNQSGYHRALPIPSLQEQLYSVHDSPRQRIPSRSPHTS
QVTEDFVIKESPAYTNDANVQSWHSFVHSAREISESSRLNTTSNAGCYPIANSVCASDKAGCVYSYQNQPGQY
PYCYSRNYYPASTNLQNRTWNPRPIDLSLKQDHCDGYGEMDYQPTHFTSYSPLRIDERNLLSDERTLNKLPC
DDMSLNGWTEEDMRQFSKVFKHRRTKLGYTQSDVGTSLGELYGSVFSQTTICRFEAQQLSLKNMCKLRPLLS
RWLQHKDNKHETLTPDIDQIDSENGPGRKRKKRTSIEAEVKAVLEKHFKLKPKPMTQEIVSIAEQLSLEKEVV
RIWFCNRRQKEKKVNEQVMRSQNPT

Botryllus tuberatus Class 2 POU (POU_c2_Btub):
LQGKEQKQKKLDYSQEDDRFAPAPGFHERHFVRQPSDFDVDPRQIRPDFAIRHSMLSHPVLLDNRRCSEEN
MSPPGPESPTVTPANMARHIPQSFSSDDQCDYDEIPKKRRMYDDEASELEDLEQFAKDFKQKRIKMGFTQGD
VGVAMGKFYGNDFSQTTISRFEALNLSVKNMCKLKPLLERWLIDVDRAISDRGEGGRLAISQPVIPMHPQQCT
GRKRKKRTSIPTEGKTRLEDAFKKNPKPTTEEICKFSENLKMDREVVRVWF

Botrylloides leachii Class 3 POU (POU_c3_Blea):
NNFYAHVSLTNENGRSVIAQGNECSGKQSPEYKEYETPIKELTYMSLHPRTVTSNGYYSQSSTFGENFRHFPT
QSPYTGHDHNPGYNYNSYPPTLIPADCLQGSLQSRHSFNSTFAPESYASEQNSDHPRISRSAPPNVTITECSDT
HYNANSVKCFSDRHSEYSYPHLPSPANMGVVCKREITTPSPQLRHEISSSWRGQELCPPSYLHQDTPQYRYSY
QANYWPLSPANSTSCSYQKASSNRFVKQERFQDYQTNTPMQKLPFRNFCTAAERGFSNETQFEQKFTSDES
MIGTESSDDMRIFANVFKARRIKLGFTQHDVGLDLKKFQGSAFSQTTICRFEAGGLSIKNMNRLKPLLTMWL
RHNDTEHISTLRDTDRSPLDNATTRKRKKRTCIEPQTKLALEEKFRNDQKPTTVQIAKIAEELSLDKEVVRIW
FCNRRQKEKKATVEIVQRDVA

Pou-Phylogenetic analysis

Protein sequences used for phylogenetic analysis were downloaded from the NCBI database or determined in this study. POU protein sequences were aligned using the ClustalW algorithm in the MEGA7 application [1]. Phylogenetic analysis of POU family members was performed with the software RAxML using a maximum likelihood method, the JTT substitution matrix, and empirical frequencies [2]. RAxML software was accessed using the CIPRES Science Gateway (Creating the

CIPRES science gateway for inference of large phylogenetic trees. In: Proceedings of the gateway computing environments workshop (GCE), New Orleans, LA, USA; 2010. p. 1–8.) and trees were visualized using the Interactive Tree of Life website (Letunic I and Bork P (2006) Bioinformatics 23(1):127-8 Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation).

1. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets.* Mol Biol Evol, 2016. **33**(7): p. 1870-4.
2. Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics, 2014. **30**(9): p. 1312-3.

qPCR primers:

actin Forward: AACAAGAAATGCAGACCGCC
actin Reverse: GGCCGATTCCATACCCAAGA
PCR product length: 146

cyclin b Forward: CCCCGCATGAGAACCATACTT
cyclin b Reverse: GCAAGCTGTAACTTGGTGCG
PCR product length: 146

Integrin-alpha-6 Forward: GGGATAGTAGTGCTGACGGC
Integrin-alpha-6 Reverse: CAGCGATGTCGGGATAACCA
PCR product length: 156

Pou3 Forward: CGATCTGTCGATTCGAGGCT
Pou3 Reverse:GGTTGCGTTGTCTAATGGCG
PCR product length: 146

Vasa Forward: ACTGTTGGATCTCAGCCGTG
Vasa Reverse:TCTGGCTCAAAGCCCATGTC
PCR product length: 153

Notch 1 Forward: GAACTCACTGGAAGGTCGCA
Notch1 Reverse: ACACTCCACCAACAGCGATT
PCR product length: 145

Notch2 Forward: TGCATGAGTAGCCTGGAACG
Notch2 Reverse: AGGATCTTGTACGCCATCAGC
PCR product length: 158

Hes1 Forward: ACTTGGATACGGGTGTGTGC
Hes1 Reverse: GTCAACGTGTTTCCGCCATC

PCR product length: 155

Frizzled 5/8 Forward: GCGGAACAAAACCTGCAAGA
Frizzled 5/8 Reverse: TTTCTTCCAAGGCAGGGCAA
PCR product length: 156

Dishevelled Forward: AACCTCGCTATCGCCACAAA
Dishevelled Reverse: TGATGCATGTTGCCATTCGT
PCR product length: 154

Beta catenin Forward: AAGATGACGTAGAGCGCACA
Beta catenin Reverse: GTGCTGCTGGGATTCTGAGT
PCR product length: 158

piwi1 Forward: ACCAGGAAGCGACTGACTTG
piwi1 Reverse:GGAGCTCGTTGTCCCAAGAA
PCR product length: 156

Piwi2 Forward: TAGGGCGTGGGACACTTTTC
Piwi2 Reverse: GAGTGCAGTTCCACTCGACA
PCR product length: 149

FISH probe cloning primers:

Integrin alpha 6 Forward: TGGCGTTGTAATCGTCGTGA
Integrin alpha 6 Reverse: TGAGCAGTGCATTTCCCGAT
PCR product length: 764

Vasa Forward: TGTCCCGTGACTGTCCTAGT
Vasa Reverse: TGCAAACTTGCGAGCCTCTA
PCR product length: 833

Histone 3 Forward: GCGGTCGTCTACACACTTCG
Histone 3Reverse: CACAAGGATTGGGTGGCTCT
PCR product length: 565

Notch1 Forward: AGGCACTTGCATTGACGGTA
Notch1 Reverse: CCTTCACACCTTGGTCCCTC
PCR product length: 792

Notch2 Forward: AAGTGCCTCCGTTCAAGCAA
Notch2 Reverse: CCCTGGTTACACCGGCATTA
PCR product length: 653

***B. leachii* Piwi mRNA sequences:**

B. leachii piwi1
GATCTGTTGTATTAAGGTTGCTGCAGAAGTATATGTAAAGTAAGCAGTTGTTTTATGGT
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B. leachii piwi 2:
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