Regenerative responses following genotoxicity vary along the anterior/posterior axis in *Schmidtea mediterranea*

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Summary statement: This report shows different stem cell responses against a genotoxic agent in regenerating planarian in anterior versus posterior fragments and suggests a link with differential β-catenin activity along the body-axis.

Abstract

Pluripotent stem cells hold great potential for regenerative medicine and as such understanding the fundamental mechanisms that effect their behaviour in adult tissue is important. Increased replication and division such is the case during regeneration, concomitantly increases the risk of adverse outcomes through the acquisition of mutations. Seeking for driving mechanisms of such outcomes, we challenged a pluripotent stem cell system during the tightly controlled process of regeneration in the planarian *Schmidtea mediterranea*. Exposure to the genotoxic compound methyl methanesulfonate revealed that despite a similar DNA-damaging effect along the anterior/posterior axis of intact animals, stem cell responses differed between anterior and posterior fragments after amputation. Stem cell proliferation and differentiation processes proceeded successfully in the amputated heads, leading to regeneration of missing tissues. Stem cells in the amputated tails however, showed decreased proliferation and differentiation capacity. As a result, tails were not able to regenerate. Interference with the body-axis-associated component *β-catenin-1* increased regenerative success in tail fragments, suggesting that differences in the Wnt signalling gradient along the body axis modulate different stem cell responses to MMS.

Introduction

Stem cells drive the development and tissue renewal of all multicellular organisms. Characterizing their key properties is crucial for advancing regenerative medicine, or for a better understanding of misregulation that underpins disease, such as tumorigenesis. Although stem cells are believed to have enhanced stress responses, ranging from upregulated DNA repair to rapid apoptosis (Dannenmann *et al.*, 2015; Liu *et al.*, 2013a; Maynard *et al.*, 2008; Vahidi Ferdousi *et al.*, 2014; Vitale *et al.*, 2017), their molecular behaviour also depends strongly on the tissue environment in which they reside (Blanpain *et al.*, 2004; Chacón-Martínez *et al.*, 2018; DeGregori, 2017; Rompolas *et al.*, 2013). Age-related changes in the tissue landscape, for example, provide a permissive environment for malignant growth, while in contrast embryonic niches or specific micro-environmental cues are able to remodel and exert control over teratomas to produce normal tissue (Bianchi-Frias *et al.*, 2019; Booth *et al.*, 2011; Hendrix *et al.*, 2007; Lan *et al.*, 2019; McCullough *et al.*, 1994; Mintz and Illmensee, 1975; Stoker *et al.*, 1990).
The molecular pathways controlling cell growth, morphogenesis and differentiation are key components of regeneration and tissue homeostasis but can also lead to tumorigenesis. During regeneration, strict control leads to an orchestrated tissue regrowth rather than malignant transformation. Albeit a contrasting outcome for both processes, gene expression patterns in for example renal regeneration versus renal carcinoma are regulated concordantly for 77% of the studied genes (Riss *et al.*, 2006). Another example of the commonality between both processes is the Wnt pathway, which is intimately linked with stem cell fate throughout development and regeneration and is mis-regulated in multiple cancer types (Beachy *et al.*, 2004; Clevers, 2006; Haegel *et al.*, 1995; Heasman *et al.*, 1994; Morin *et al.*, 1997). A deeper understanding of the underlying mechanisms that protect (cancer-) stem cells against various types of cellular stress and the parameters that influence the switch between a healthy (regeneration) and malignant (cancer) cell fate is crucial for a myriad of stem cell applications. Not coincidentally, tissues and species with a high regenerative ability, such as planarians,

seem to have a lower predisposition towards chemically-induced tumorigenesis (Enomoto and Farber, 1982; Farber, 1984; Okamoto, 1997; Oviedo and Beane, 2009; Pomerantz and Blau, 2013; Zilakos *et al.*, 1996). Planarians are known for their full-body regeneration, enabled by a large pool of adult pluripotent stem cells, called neoblasts. Neoblasts can provoke whole-body regeneration from a small fragment by altering proliferation, migration, differentiation and apoptosis patterns (Abnave *et al.*, 2017; Pellettieri *et al.*, 2010; Wenemoser and Reddien, 2010). Despite the high division rate of these stem cells, also during homeostasis, the incidence of malignancies is low and coincides with the absence of ageing (Oviedo and Beane, 2009; Plusquin *et al.*, 2012; Sahu *et al.*, 2017; Stevens *et al.*, 2017b; Tan *et al.*, 2012). Whether this can be solely attributed to the abundantly-present stem cells remains to be elucidated, as is the question how signal from the surrounding niche are involved. With proven conservation of many signalling pathways, planarians are well-suited to unravel stem cell responses within the complex multicellular entity of a regenerating tissue (Labbe *et al.*, 2012; Onal *et al.*, 2012; Resch *et al.*, 2012; Swapna *et al.*, 2018). They have an exceptionally high resilience against radiation or chemical exposure (Plusquin *et al.*, 2012; Salvetti *et al.*, 2009; Stevens *et al.*, 2017b; Zhang *et al.*, 2014), but several studies reported the formation of outgrowths or hyperproliferation by interfering with known mammalian tumour suppressors such as the damage transducer *smg1*, epigenetic regulator MLL3/4, tumour suppressors PTEN and p53 or the hippo pathway (de Sousa *et al.*, 2018; Gonzalez-Estevez *et al.*, 2012; Mihaylova *et al.*, 2018; Oviedo *et al.*, 2008; Pearson and Sanchez Alvarado, 2010). As in other systems, neoblasts receive guiding instructions from their surroundings, such as the musculature, extracellular matrix and the nervous system (Cebria and Newmark, 2007; Fraguas *et al.*, 2014; Isolani *et al.*, 2013; Oviedo *et al.*, 2010; Witchley *et al.*, 2013). Recent studies indicated that neoblasts respond differently to DNA damage depending on their location along the body axis (Peiris *et al.*, 2016) and that interfering with the extracellular matrix can influence the development of outgrowths (Van Roten *et al.*, 2018; Voura *et al.*, 2017).

We previously found that the planarian stem cell system alters its proliferation patterns during

genotoxic exposure and activates different defence mechanisms depending on the developmental stage of the animal (Stevens *et al.*, 2017a; Stevens *et al.*, 2017b). This work aims to characterize how regenerative tissues respond to a genotoxic insult and whether tissue-associated factors influence the outcome. Intact worms were exposed to a genotoxic agent, amputated and monitored during regeneration. Our data indicate that DNA-damage-induced stem cell responses and the regenerative success are tissue-dependent and can be altered by interfering with the body-axis-associated Wnt gradient.

Materials and methods

Experimental set-up

An asexual line of the planarian *Schmidtea mediterranea* was cultivated at 20°C in the dark in freshwater medium as described previously by Pirotte *et al.* (2015). Worms were fed once a week with veal liver, but were not fed during the experiments. As such, a fasting period of at least seven days prior measurements was taken into account to avoid food-related effects.

Worms were exposed to 50 µM methyl methanesulphonate (MMS, Sigma-Aldrich cat. number 129925) in variable experimental set-ups. In each case, medium was refreshed twice a week (every 2-3 days) with a freshly prepared MMS working solution, prepared in freshwater medium. Control animals were kept in freshwater medium and were refreshed concomitantly. Exposure was done in 6-well plates with 3 or 4 animals per well in 3 or 4 ml of medium respectively. Phenotypic follow-up was performed with individual worms in 1 ml of medium in a 24-well plate.

To examine how regenerative tissue recovers after a genotoxic insult, a set-up is used in which animals are exposed prior to the induction of regeneration (Fig. 1). Intact worms were exposed to 50 µM MMS for seven days. The genotoxic effect of MMS was established after this initial exposure period. Regeneration was then induced with a prepharyngeal cut to obtain a head and tail piece. The animals were kept in control medium during the regenerative phase; medium was refreshed twice a week (every 2-3 days). Samples for various measurements, as indicated below, were taken just before cutting the animals on day 0 and 2, 3, 7 and 14

days post amputation (dpa). Alternative cutting strategies, exposures, or combination experiments with RNA interference are indicated in the relevant figures and were exposed and refreshed as explained above.

Phenotypic follow-up and blastema size determination

Regenerative success was assessed by scoring the regrowth of missing body structures 14 dpa. The reappearance of a head with two photoreceptors, a centrally-located pharynx and the presence of a tail were visually inspected. Regenerative success was classified into three categories, scoring how well the missing body structures regenerated, i.e. complete, aberrant or absent. Within the considered time period heads have regenerated their missing body parts completely, i.e. a pharynx and tail (scored ‘complete’). In case a pharynx was present but the tail did not develop completely, samples were scored ‘aberrant’. In case the wound closure did not develop further into the missing body structures, this was scored as ‘absent’. For tails, the regeneration of a head structure with two visible eyes was scored as ‘complete’. In case a head with no eyes or only one eye developed, an ‘aberrant’ score was given. If wound closure did not lead to development of the missing body structures, this was considered as ‘absent’. For the experiments where animals were cut differently (anterior-trunk-posterior), the same categories scoring the presence of missing body structures were used per wound-type (anterior-facing or posterior-facing). Blastema areas were used as indication of early tissue regeneration and were measured at 4, 7 and 14 dpa. The blastema is an unpigmented region that is formed at the wound site, within which missing distal structures differentiate. The size of the blastema was measured relative to the size of the worm, determined as an average value based on three pictures per worm. Pictures were taken with a Nikon Ds-Ri2 digital camera mounted on a Nikon SMZ800 stereomicroscope (Nikon Instruments Inc.). The total amount of replicates and experiments is indicated in the respective figures.

Whole-mount (fluorescent) in situ hybridization

The expression of *smedwi-1* (general stem cell marker), *smed-NB.21.11e (*early progeny marker that is committed to the epidermal cell lineage) and *smed-notum* (anterior pole marker) and *smed-wnt-1* (posterior pole marker) were determined using whole-mount fluorescent or colorimetric in situ hybridization (ISH) (Adell *et al.*, 2009; Eisenhoffer *et al.*, 2008; Gurley *et al.*, 2010; Petersen and Reddien, 2011; Petersen and Reddien, 2009; Reddien *et al.*, 2005). The protocol was performed as described by King and Newmark (2013). For colorimetric ISH, development was performed using NBT/BCIP (Roche) as described by Pirotte *et al.* (2015). Probes were synthesized using the DIG RNA (SP6/T7) Labeling Kit (Roche) as indicated by the manufacturer, starting from a purified PCR product of the gene of interest. To obtain this, a general PCR reaction was performed with gene-specific primers: *smedwi-1* primers forward: 5' GTGACGCAGAGAAACGGAAG 3', reverse: 5' TTGGATTAGCCCCATCTTTG 3'. *NB.21.11e* primers forward: 5'GTGATTGCGTTCGCGTATATT 3', reverse: 5'ATTTATCCAGCGCGTCATATTC 3'. *notum* primers forward: 5'CGAGTGATTTGTGGTCTGG3', reverse: 5'CGTGGAGTCGTTGATTGTTG3'. *Smed-wnt-1* primers forward: 5'TGAAGGAATCAGAAAGGGTA3', reverse: 5'TTGTTAGGAAAGGTCGGTTG3' Samples were mounted with Immu-Mount (Thermofisher Scientific) and analyzed with a Nikon i80 fluorescence microscope with a Nikon Ds-Ri2 digital camera using the Nikon NIS-Br software (Nikon Instruments Inc.). Because of the dense signal for *smedwi-1*, the intensity of the signal was determined using the Nikon NIS-Br software. The average intensity of the entire worm or a specified region, as indicated in the figures, was used. For *NB.21.11e*, cells were counted in specified areas. In heads, one region covering the center of the animal was used. For tails, a prepharyngeal, postpharyngeal and the region at the right side of the pharynx were used to calculate an average value per animal. For intact animals, one region located either pre- or postpharyngeally was used as indicated in the figure. The total amount of replicates and independent experiments is indicated in the respective figures.

Whole-mount immunohistochemistry

The mitotic activity of stem cells was determined by immunolabelling histone H3(Ser10) (Merck-Millipore, catalogue number 09-797, diluted 1:600), detected by a secondary anti-rabbit Alexa Fluor 568 antibody (Thermofisher Scientific, catalogue number A-11036, diluted 1:500) as described by Leynen *et al.* (2019). The total number of positive cells was normalized against the total body size of the animal (determined post-mounting). The total amount of replicates and independent experiments is indicated in the respective figures. The total amount of H3P positive cells in the entire animal was counted using the NIS-Br software (Nikon Instruments Inc.).

Neuroregeneration was determined by immunolabelling synapsin (mouse anti-SYNORF1 , C311, diluted 1:50, Developmental Studies Hybridoma Bank), detected by a secondary goat anti-mouse Alexa Fluor 488 antibody (Thermofisher Scientific, catalogue number A-1101, diluted 1:400) as described by Leynen *et al.* (2019). Samples were mounted with Immu-Mount (Thermofisher scientific) with the ventral side upwards. Pictures were captured with a Nikon Ds-Ri2 camera mounted on a Nikon eclipse i80 fluorescence microscope and analyzed using the Nikon NIS-Br software (Nikon Instruments Inc.). The presence of nerve cords and cephalic ganglia was scored visually. The relative brain size was measured as the width of the brain relative to the width of the entire head

Comet assay

The alkaline comet assay was used to analyse the DNA damaging effect of MMS in variable set-ups. After exposure, a stem-cell-containing fraction was obtained (from 1 worm per sample) using a dissociation protocol as described previously (Stevens *et al.*, 2017b), with some modifications that are mentioned below. After chemical and physical maceration, the samples were serially filtered with a 35 µm filter and a 10 µm filter (Pluriselect) to purify the fraction from larger cell types and debris. The obtained cell pellet was dissolved in 160 µl of 0.8% low-melting-point-agarose (Thermofisher) in phosphate buffered saline (PBS) and immediately gelled on an agar-coated slide (1% agar in ultrapure water, Invitrogen). Each sample was made in technical duplicates (70 µl per gel). The gels were lysed overnight in lysis

buffer (2.5M NaCl, 0.1M Na_2EDTA, 10mM Tris, 1% Triton X-100, 10% DMSO, pH 10) at 4°C. After washing twice with cold PBS, the samples were denatured for 20 minutes and electrophoresis was carried out during 20 min in electrophoresis buffer (300mM NaOH, 1mM Na_2EDTA, pH 13.0) at 0.95 V/cm over the platform. The buffer was continuously recirculated. Samples were neutralized first with PBS and subsequently ultrapure water and then stained with Sybr Gold Nuclear Acid Gel Stain (Invitrogen, catalogue number S11494) for 20 minutes. After two washing steps with water, samples were dried and scored with a Zeiss fluorescence microscope (AxioImager.Z2, equipped with SlideFeeder X80) using the 10x objective magnification. Images were automatically captured with Metafer 5 (Metasystems). Analysis of approx. 100 comets per sample (50 per technical duplicate) was performed with the comet assay IV software (Instem – perceptive instruments). Tail intensity, i.e. the percentage of intensity in the tail relative to the total intensity of the comet was used as output. Four biological replicates per condition with two technical replicates per sample were analysed. A mean value of the technical replicates was determined after which the average value of all biological samples was determined per condition. The total amount of samples and independent experiments is indicated in the respective figures.

RNA interference

To knockdown the expression of *smed-β-catenin-1*, worms were injected for three consecutive days with a gene-specific dsRNA probe. Injection was done prepharyngeally with three injections of 32.2 nl of a 1000 ng/µl dsRNA probe. The day after the final injection, the exposure period of intact worms began. The dsRNA probe was made based on a gene-specific amplified PCR product that was transcribed to dsRNA with the T7 Ribomax TM Express RNAi system kit (Promega) following the manufacturer's instructions. The control group was injected with ultrapure water following the same injection scheme. Primer sequences for *smed-β-catenin-1* : Forward 5'-GCTGGATTGTTGGTTGAGGT-3'; Reverse 5'-TGGTTGTGCATAATCGGAGA-3'. The total amount of samples and independent experiments is indicated in the respective figures.

Statistical analysis and figure information

Statistical analysis was performed in Open Source RStudio version 1.0143 (Team, 2015). Depending on the research question, a *t*-test or two-way ANOVA with a Tukey HSD *post-hoc* test was used. Normality of the data was analysed with the Shapiro-Wilk test, equal variance with the Bartlett test. If normality was not met, the dataset was transformed (log, sqrt, 1/x or e^x). If the normality criteria were still not met, a non-parametric Kruskal-Wallis test followed by a pairwise Wilcoxon Rank Sum Test were used. P-values < 0.05 were considered significant. Pictures were processed and assembled in Adobe Photoshop and Adobe Illustrator.

Results

In the current study, we aimed to determine stem cell capacity and associated regenerative success of a developing tissue after inducing DNA damage. Intact (i.e. uninjured) planarians were exposed to the DNA-alkylating compound MMS to induce DNA damage. Cellular damage and subsequent responses were evaluated after seven days of treatment. Subsequently, a regenerative response was provoked by amputation. Within this regenerative tissue, the progression of DNA damage, stem cell responses and regeneration efficiency were monitored over time (Fig. 1). By comparing responses in anterior and posterior body parts, and by interfering with the anterior/posterior Wnt gradient, the impact of tissue-related factors on DNA damage responses was assessed.

MMS exposure induces genotoxic effects throughout the entire body of intact animals

Intact worms were exposed for seven days to 50 µM MMS, a sublethal concentration that was previously found to evoke DNA damage in our model organism (Stevens *et al.*, 2017a; Stevens *et al.*, 2017b). An overall significant increase ($p < 0.001$) in DNA damage was observed together with an overall significantly increased amount of apoptotic cells ($p < 0.001$), a significantly decreased amount of proliferating stem cells ($H3P^+$ cells, $p < 0.001$), a decrease in the overall amount of stem cells (*smedwi* intensity, $p < 0.001$) and a decrease in the presence of the early-progeny marker committed to the epidermal lineage *NB.21.11e* ($p < 0.01$) (Figs. 2A-D, S1), confirming previous measurements (Stevens *et al.*, 2017b). All parameters were compared between the anterior (prepharyngeal region) and posterior (postpharyngeal region) part of intact animals to screen for tissue-dependent effects, but no significant differences were observed ($p > 0.05$).

Regenerative success varies along the body axis

The regenerative capacity of the exposed animals was screened by monitoring early tissue formation (blastema size) and regenerative success up to 14 dpa (Fig. 3B,C). After cutting the animal in front of the pharynx, creating an anterior (head) and posterior (tail) piece, blastema

growth was most affected in the tail (Fig. 3B). While both fragment types had a significantly smaller blastema at 4 dpa ($p < 0.001$), tail fragments failed to regenerate and a protruding blastema was never observed. The blastemas of the head fragments kept growing, yet remained proportionally smaller as compared to the non-exposed animals at each time point ($p < 0.001$). The difference in blastema development between head and tail fragments was reflected in their final regenerative success at 14 dpa (Fig. 3C). This was scored as complete, aberrant or absent, based on the reappearance of missing body structures. The majority of the heads was able to fully regenerate the missing pharynx and tail (63%, 26/41). Manifested aberrations were the reappearance of a pharynx but not a tail (7/41) or a 'notched' tail (7/41). One animal did not regenerate. In contrast, seven percent of the tails developed the missing head-tissue aberrantly (a head with 1 cyclopic eye (2/43) or no eyes (1/43)) or did not develop at all (74%, 32/43). The body edges of the latter were smoothly aligned, indicating that wound closure took place but did not evolve further to reconstruct missing body parts. In accordance with the phenotypes, labelling the nervous system showed either connected nerve cords in the tail, a notched tail, or no further development of nerve cords behind the pharynx (Fig. 3D). In tail fragments, nerve cords looped around the pharynx without the formation of cephalic ganglia (18/20 animals). The expression of the anterior marker *smed-notum* (Petersen and Reddien, 2011), was not present at 3 dpa in recovering tail fragments (6/7 animals) (Fig. 3E). The posterior marker *smed-wnt-1* (Adell *et al.*, 2009; Gurley *et al.*, 2010; Petersen and Reddien, 2009) was present in the regenerating head fragments, but the signal was diminished as compared to control animals (Fig. 3E). An additional experiment suggested that the location of the cut along the A/P axis influences the final outcome, both for anterior and posterior fragments. The ability to regenerate a head increased when the cut was made more anteriorly. Likewise, the ability to regenerate a tail also increased the more posteriorly the cut was made (Fig. S2)

Anterior/posterior differences were again confirmed when excising small fragments of anterior, central (trunk), or posterior parts of the planarian body, with a decreasing regenerative success rate towards the posterior part of the animal (Fig. 3F). Secondly, anterior-facing

wounds had a lower success rate in all fragment types as compared with posterior-facing wounds. The highest regenerative success was observed in anterior fragments, which regenerated a complete head in 46% of the samples and a complete tail in 69% of the samples. The remaining samples regenerated aberrantly at both the anterior wound side (a head with one cyclopic eye for 4/13 animals or an eyeless head; 1/13), and at the posterior-facing wound (notched-tail, 2/13). The posterior-facing wound of the trunks either regenerated completely (73%, 19/26 animals) or with a notched-tail (23%, 6/26). Anterior regeneration was drastically lowered in trunks (8% complete success, 2/26 animals) and mostly did not develop at all (85%, 22/26). The overall success-rate decreased in the posteriorly-located fragments where anterior regeneration was only successful in 1/12 animals. Fifty percent of these animals (6/12) was still able to regenerate a tail. The mortality rate was higher in the smaller fragments (2/13 anterior fragments; 3/12 posterior), which is possibly related to their higher fragility during handling.

Stem cell dynamics in regenerating tissue varies along the body axis

We next sought to characterize underlying stem cell responses leading up to the observed regenerative success or failure. We assessed how the induced genotoxic effects in intact animals (Fig. 2) affected regenerative stem cell dynamics (Figs. 4-5). During normal regeneration, an initial proliferation peak near the wound site is followed by a body-wide increase in mitosis at 2-3 dpa (Wenemoser and Reddien, 2010). The latter was chosen as a first time point to assess stem cell dynamics (Fig. 4). In both heads and tails, the induced DNA damage was still present 3 dpa ($p < 0.001$ and < 0.1 respectively) and was concomitant with a significant decrease in the total amount of stem cells (*smedwi* intensity) 2 dpa as compared to control animals, which was stronger in tails than heads ($p < 0.001$ versus $p < 0.05$, Fig. 4A-B). The amount of proliferative cells in head fragments was similar as in controls (NS), while their early progeny was significantly lower ($p < 0.001$). In tail fragments, both proliferation ($p < 0.001$) and early differentiation ($p < 0.001$) were significantly decreased as compared with the

control. Both proliferation and differentiation were significantly higher in recovering heads than tails ($p < 0.01$) (Fig. 4C,D).

By 7 dpa, no significant differences in the measured parameters were observed for regenerating heads compared to their control (Fig. 5A-D). In recovering tails, only the amount of early progeny cells was significantly decreased ($p < 0.05$).

Similar responses are observed in continuously-exposed tissue over time

When animals were continuously exposed to MMS, i.e. before and after amputation, regenerative success again decreased in tails but not in heads (Fig. S3C). Stem cell proliferation and differentiation in continuously-exposed heads remained significantly higher than in continuously-exposed tails both on the short term (2 dpa, $p < 0.05$) as well as on the long term (7 dpa, $p < 0.01$). Both continuously-exposed heads and tails had a significantly lower amount of proliferation and differentiation compared to their respective controls 2 dpa ($p < 0.001$, Fig. S3D). While differentiation remained significantly decreased in both fragment types at 7 dpa ($p < 0.001$ and < 0.01), only continuously-exposed heads showed a similar proliferation level as controls at that time point (NS).

Independent of the treatment, head and tail fragments showed similar proliferative and differentiation patterns over time, but their kinetics differed (Fig. S3F). Both parameters increased stronger in heads than tails. While proliferative responses in treated animals eventually equal that in controls in all recovering fragments, this is obtained more quickly in heads. Similarly, recovering fragments increase their proliferation faster than continuously-exposed animals. Control levels of early differentiation on the other hand, are never achieved in tail fragments, independently of the exposure treatment.

A long-term follow-up experiment (up to 21 dpa) was performed to estimate the occurrence of malignant development. Abnormalities were only observed in animals that were continuously exposed to MMS before and after amputation (3% for all conditions combined n=156) (Fig. S3B). Their incidence in regenerating anterior and posterior fragments was equal. Aberrancies manifested themselves as blister-like structures on the dorsal side of the animal but were not

further characterized because of their low occurrence and fragility.

Interfering with the Wnt gradient rescues otherwise impaired tail fragments

Since proliferative and differentiation responses were activated to a lesser extent in impaired tail fragments, we reasoned that the inhibitory effect could have disturbed the normal response to injury. Recutting impaired tail fragments 7 dpa rescued regeneration completely, but only in recovering animals and not in continuously-exposed ones (Fig. S4). In search for factors responsible for blocking or activating regeneration specifically in posterior fragments, we interfered with the Wnt-gradient, which is known to determine the anterior-posterior fate decision during homeostasis and regeneration (Gurley *et al.*, 2010; Petersen and Reddien, 2011; Petersen and Reddien, 2009). An increasing gradient of *β-catenin-1* activity towards the posterior end activates posterior fate determination, while knockdown of *β-catenin-1* induces the formation of a head structure, also in posteriorly-facing wounds (Gurley *et al.*, 2008; Iglesias *et al.*, 2008; Petersen and Reddien, 2008). The failure to regenerate a head structure in recovering tails was rescued by *smed-β-catenin-1* knockdown (KD), both phenotypically (54% developed completely, 37% aberrantly) and at the level of brain development (16/17 animals developed cephalic ganglia) (Fig. 6A,B). Eyes were nonetheless under developed as compared with non-exposed animals, and their regenerated brains were significantly smaller ($p < 0.001$). Stem cell proliferation patterns at 2 dpa remained significantly lower than in control animals in both recovering water-injected and recovering *smed-β-catenin-1* KD animals (Fig. 6C, $p < 0.001$ and < 0.01 respectively). Also the amount of early progeny cells of the epidermal lineage remained significantly lower in both recovering water-injected and *β-catenin-1* KD animals (Fig. 6D, $p < 0.001$). Also under continuous exposure, tails of *β-catenin-1* KD animals had an increased regenerative success (Fig. S5A). *R*ecovering *β-catenin-1* KD heads regenerated worse than without KD, ranging from multi-lobal structures without visible eyes, the absence of regeneration or bloated animals and even lysis (Fig. S5B).

Discussion

The inherent plasticity of stem cells confers a tissue with regenerative potential. However, an uncontrolled execution of this plasticity can be the onset of diseases, failed wound healing or regenerative impairment (Beachy *et al.*, 2004; Knoepfler, 2009; Ma *et al.*, 2010; Sundaram *et al.*, 2018). Increased lifetime, frequent replication and micro-environmental signals make stem cells vulnerable to both exogenously and endogenously-induced DNA damage (Espada and Ermolaeva, 2016; Tao *et al.*, 2015; Tomasetti and Vogelstein, 2015). Regenerative tissues and tissues in early development have the ability to restrict and tightly control cellular functioning, or on the opposite, could pave the way for malignancies to arise because of the highly active tissue-context (Enomoto and Farber, 1982; Hendrix *et al.*, 2007; Li and Ye, 2017; Ma *et al.*, 2010; Oviedo and Beane, 2009; Pomerantz and Blau, 2013). In order to use stem cells at their full potential, it is crucial to gain more insight into the way how newly-regenerating tissues respond to inflicted damages, preserve genomic integrity and guard their cellular and physiological functions. Studying these processes in a regenerative model can reveal key determinants that tip the scale towards successful tissue restoration or lead to abnormal growth. Planarians have the advantage of linking underlying molecular mechanisms and stem cell behaviour to outcomes at the tissue level such as successful development or impaired regeneration. We used the genotoxic compound MMS to challenge the planarian stem cell system and characterized (1) the regeneration potential upon induced DNA damage, (2) the underlying stem cell dynamics, (3) whether these responses led to adverse outcomes and (4) which factors influenced these outcomes. To achieve this, the regeneration process was induced via amputation after MMS exposure; the induced responses were monitored in the regenerating fragments (Fig. 1).

The alkylating agent MMS irreversibly binds to DNA strands, stalls the replication fork and disturbs normal DNA replication (Lundin *et al.*, 2005). With stem cells being the only dividing cells in planarians, stem cell cycling was affected by the increased amount of DNA damage in

our set-up (Fig. 2A), probably by halting them in the S-phase. As a consequence, the amount of proliferating and descending progeny cells decreased (Fig. 2C, D). The overall decrease in the total amount of stem cells (*smedwi*) can be due to damage-induced cell death, although the detected increases in apoptosis can also take place in differentiated cells (Figs. 2B, S1). The observed alterations in stem cell dynamics did not result in severe phenotypic effects in intact animals. We hypothesize that in non-regenerating conditions, the pool of undamaged stem cells is large enough to restore the loss of damaged cells (Sahu *et al.*, 2017).

As far as could be determined, the amount of DNA damage was equal in both anterior and posterior regions of the animal, as was previously also observed after *rad51* and *ubc9* knockdown (Fig. 2A) (Peiris *et al.*, 2016; Thiruvalluvan *et al.*, 2017). Different than what was observed by Peiris *et al.* (2016) after *rad51* KD, we did not find regional differences in proliferation or differentiation responses upon DNA damage in intact animals (Fig. 2). However, when challenged by tissue loss, the regenerative outcome differed along the body axis as only heads were able to regenerate (Fig. 3C). In tails, the increasing amounts of proliferating and differentiating stem cells during regeneration were insufficient to induce the formation of a blastema (Figs. 3B,C, 4C,D, 5C,D S3F). The absence of the anterior marker *notum* (Petersen and Reddien, 2011) indicates that the anterior/posterior decision does not take place in impaired tails and regenerative responses are already affected at an early time point (Fig. 3E). In general, amounts of proliferating and differentiating cells were significantly higher in heads than tails, at early and later stages of regeneration. These discrepancies were again observed when exposure was continued after amputation (Figs. 4C,D, 5C,D, S3D,E). This corresponds to the better regenerative capacity of heads in all conditions and indicates that, although amputation-associated stem cell responses remain active following genotoxic stress in both fragment types, their outcome depends on the tissue location (Figs. 3C, S3F). We did notice a delay in the regenerative responses of the heads, exemplified by the restricted blastema size, decreased *smed-wnt-1* expression and an initially decreased amount of early progeny cells 2 dpa (Figs. 3B,E, 4D). The latter quickly increased by 7 dpa, while proliferation was already compensated at 2 dpa (Figs. 4C, 5D). Following amputation, the amount of DNA

breaks decreased in both heads and tails after three days and DNA damage was completely absent at 7 dpa (Figs. 2A,4A,5A). This corresponds with a previous study in *Dugesia schubarti* describing a similar decrease in, but not the absence of DNA breaks 24 hours after an acute MMS exposure in intact animals (Guecheva *et al.*, 2001). Also after sublethal irradiation, *smedwi*-positive cells repopulate and regain their mitotic activity within a similar timespan (Lei *et al.*, 2016; Salvetti *et al.*, 2009; Wagner *et al.*, 2012; Wagner *et al.*, 2011).

Anterior-located amputation sites have a faster rate of head regeneration in a number of planarian species and differences in mitotic activity between body-regions have been described previously (Baguñà, 1976; Oviedo and Levin, 2007; Reddien and Sanchez Alvarado, 2004). Accordingly, in *S. mediterranea,* higher proliferative capacities and a higher resistance to cell death in the anterior have been found following *rad51* KD-induced DNA damage, which is in line with our observed differences between head and tail fragments, specifically affecting anterior regeneration of posterior body-parts (Figs. 3-5,S3) (Peiris *et al.*, 2016). We did on the contrary not observe regional differences in stem cell responses in intact animals (Fig. 2), suggesting that regeneration-associated signals can influence stem cell fate following MMS exposure differently (Stevens *et al.*, 2017b). To date, several (positional) cues that influence stem cell fate have been identified in planarians (Fraguas *et al.*, 2014; Miller and Newmark, 2012; Pirotte *et al.*, 2015; Rossi *et al.*, 2012; Scimone *et al.*, 2017; Seebeck *et al.*, 2017; Witchley *et al.*, 2013). Neuronal signalling and the presence of the brain are often associated with a stimulating effect on stem cells (Cebria and Newmark, 2007; Peiris *et al.*, 2016; Rossi *et al.*, 2012), but we found no significant differences in regenerative success rates in smaller anterior fragments without a brain (Fig. 3F).

The Wnt pathway is one of the most important positional cues and is present in a gradient along the planarian anterior/posterior axis, decreasing its activity from tail to head and determining posterior identity (Adell *et al.*, 2009; Gurley *et al.*, 2008; Iglesias *et al.*, 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009; Sureda-Gómez *et al.*, 2016). The dual role of *β-catenin* is segregated in two distinct genes in planarians and *β-catenin-1* is functioning solely in the Wnt pathway as a positive mediator of Wnt-signalling (Chai *et al.*,

2010; Iglesias *et al.*, 2008). By downregulating *smed-β-catenin-1*, we were able to induce regeneration in impaired tail fragments (Fig. 6A,B). This is in line with the previously reported rescue of head regeneration by *β-catenin-1* KD in *Dendrocoelum lacteum, Procotyla fluviatilis* and *Phagocata kawakatsui,* all species that are normally unable to regenerate anteriorly in posterior body fragments (Liu *et al.*, 2013b; Sikes and Newmark, 2013; Umesono *et al.*, 2013). Similarly to what was found in *D. lacteum* and *P. fluviatilis*, we did not observe the presence of the anterior marker *smed-notum* in recovering (non-KD) tail fragments (Fig. 3E). In contrast to a normal proliferative response in impaired *D. lacteum* and *P. fluviatilis* wild-type tails, we did observed a diminished proliferative response and the absence of blastema growth, indicating that the genotoxic exposure also affects other processes than establishing A/P fates (Figs. 3B, 4C). Wnt signalling is correlated with stem cell control in general, as well as with the DNA damage response pathway (Clevers, 2006; Karimaian *et al.*, 2017; Reya *et al.*, 2003; Zhang *et al.*, 2011; Zhao *et al.*, 2018). A transcriptomic study in *S. mediterranea* found stem cell gene expression to be affected by *β*-catenin knockdown, indicating that stem cells respond to differential *β*-catenin signalling (Reuter *et al.*, 2015). However, we did not observe significant differences in stem cell proliferation or early differentiation associated with *β-catenin-1* KD. Differentiation did show a visual increasing trend and was also the most affected parameter during recovery without KD (Figs. 6C,D, S3F). A link between Wnt-signalling and differentiation was also reported by Reuter and colleagues (2015), where *β*-catenin-responsive genes were found to be co-expressed with an early-progeny marker in *S. mediterranea*. In murine embryonic stem cells, Wnt signalling was upregulated following genotoxic stress in a p53-dependent manner, thereby inhibiting differentiation (Lee *et al.*, 2010). Also murine intestinal stem cells are more sensitive to DNA damage when localized in a region with high Wnt activity and as a result increased apoptotic responses (Tao *et al.*, 2015). Taken together, we hypothesize that the high presence of *β-catenin-1* in tail fragments induces an exaggerated response to DNA damage that impairs regeneration completely, possibly by affecting polarity signalling (observed as the inhibition of the anterior organizer *notum*), blocking differentiation and/or apoptosis. While Wnt signalling is also active during

homeostasis (Iglesias *et al.*, 2008), we did not observe differential effects in intact (non-KD) animals between anterior and posterior regions (Fig. 2) . As such, *β*-catenin might also function differently in a regenerative versus adult tissue state, in accordance with the variable role of Wnt signalling in different cell types or tissues (Clevers, 2006).

In conclusion, genotoxic exposure provokes equal stem cell responses in adult animals, while the recovery potential of stem cells in a regenerative context depends on their location along the body axis. Anteriorly-localized fragments quickly regain normal stem cell processes and are capable of restoring missing tissue. Posterior fragments activate stem cell proliferation and differentiation processes to a lesser extent, and fail to regenerate. The latter can be improved by *β-catenin-1* KD, which activates regeneration in otherwise impaired tail fragments. These results designate *β-catenin-1* as a regulator of tissue-specific stem cell responses to DNA damage, although future research will have to elucidate its role in DNA-damage responses at the molecular level.

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Author contributions

Conceptualization and methodology: A.W., K.S., A.A. Formal analysis: A.W., K.S.

Investigation: A.W., J-P. P. Resources: T.A., K.S., S.A.S.L., A.A Writing original draft: A.W., K.S. Writing review and editing: A.W. J-P.P., S.A.S.L., T.A., A.A., K.S. Supervision and project administration: K.S. Funding acquisition: A.W., T.A., K.S., S.A.S.L., A.A.

Figure legends

Fig. 1. Overview of the general experimental set-up. Intact (i.e. non-regenerating) animals are exposed to 50 µM MMS during seven days and then amputated prepharyngeally to create an anterior (head) and posterior (tail) part. Regenerating pieces are further incubated in control medium. Initial genotoxic effects are assessed prior the induction of regeneration (day 0). The following stem cell responses are determined at an early (2 dpa) and later (7 dpa) time point; the phenotypic outcome at 14 dpa. Responses are always compared between different fragment types to screen for differences along the body axis. Alternative cutting strategies, exposure or combination experiments with RNA interference are indicated in the relevant figures.

Fig. 2. Effects of MMS exposure on intact animals. An overview of the experimental procedures is summarized on top. (A) The average median % tail DNA of 4 (3 for MMS tail) replicates per condition is depicted (1 independent experiment). Animals were cut prepharyngeally right before processing the sample. (B) The average *smedwi* intensity/mm². Combination of 3 independent experiments (total replicates ≥ 15 per group). (C) The average number of $H3P^+$ cells/mm². Four independent experiments (total replicates ≥ 22 per group). (D) The average number of *NB.21.1e*$^+$ cells/mm². Combination of 3 independent experiments (total replicates ≥ 15 per group). All parameters were compared between anteriorly-located and posteriorly-located regions, indicated by the red squares. Per parameter, a representative image is shown on the right side. The values represent mean±s.e.m. (2-way ANOVA with Tukey HSD multiple comparison for A,D; Kruskal-Wallis with Pairwise Wilcoxon rank sum-test for B,C, ***P<0.001, **P< 0.01; *P<0.05). Non-significant differences are indicated by 'NS'. Scale bars: 500 µm.

Fig. 3. Regenerative success of recovering fragments. (A) Overview of the experimental procedures, referring to the corresponding figure. (B) The average relative blastema size was determined at 4, 7 and 14 dpa. Combination of 2 (14 dpa) or 3 (4 and 7 dpa) experiments with

a total of respectively 13 or 19 biological replicates per group. The values represent mean±s.e.m (2-way ANOVA with Tukey HSD multiple comparison per timepoint; Pairwise Wilcoxon rank sum-test at 14 dpa, ***P<0.001). Significant differences between recovering head and tail fragments are positioned in between. Significant differences between control and recovering fragments are positioned on top. (C) Regenerative success in recovering head and tail fragments. The regenerative success is represented percentually and is divided into 3 categories, based on the reappearance of missing body structures, i.e. complete (green colour), aberrant (orange colour) or absent (red colour). For heads, the reappearance of a pharynx and a tail was assessed and was scored aberrant in case the tail was absent or notched. For tails, the reappearance of a head with 2 photoreceptors was assessed and was scored aberrant in case a head with no or only one eye developed. The number of biological replicates per category is depicted in a representative example. Control animals all manifested the 'complete' phenotype and are not depicted. Combination of 7 independent experiments (number of biological replicates ≥ 41 per group). The pharynx is indicated by an asterisk, photoreceptors by an arrow. (D) Immunostaining against SYNORF1, a panneuronal marker, in head and tail fragments. For heads, the reappearance of nerve cords branching together in the tail tip is depicted. For tails, the reappearance of a brain ganglion is depicted. The number of replicates manifesting the shown level of nerve development is shown. Combination of 2 independent experiments. (E) WISH of *Wnt-1* in head fragments and *notum* in tail fragments from 1 independent experiment (F) Regenerative success in smaller fragments, located anteriorly (A), centrally (Trunk, Tr) or posteriorly (P). For all fragment types, a separate analysis was done for the anterior-facing wound (A) versus posterior-facing wound (P). For both wound types, the same categories were applied as for the head and tail pieces, scoring the reappearance of missing body structures as explained above. The number of replicates showing the depicted replicates is shown in the figure and is the combination of 2 (anterior and posterior pieces) or 3 (trunks) independent experiments with a total of minimal 11 (anterior and posterior pieces) or 23 (trunks) replicates per group. Scale bars: 100 µm.

Fig. 4. DNA damage and stem cell dynamics in recovering fragments 2 dpa. An overview of the experimental procedures is summarized on top. (A) The average median % tail DNA is depicted. Two independent experiments (amount of biological replicates n ≥ 7 per group). (B) The average *smedwi* intensity/mm². Combination of 3 independent experiments (amount of biological replicates n ≥ 14 per group). (C) The average number of H3P+ cells/mm². Three independent experiments (amount of biological replicates ≥ 16 per group). (D) The average number of *NB.21.1e*+ cells/mm². An average value per sample was determined based on counting 2 regions (one in front of the pharynx and one next to the pharynx). Combination of 3 independent experiments (number of biological replicates ≥ 15 per group). The values represent mean±s.e.m (2-way ANOVA with Tukey HSD multiple comparison for A-C,Kruskal-wallis with pairwise Wilcoxon-rank sum test for D, ***P<0.001, **P< 0.01; *P<0.05). Non-significant differences are indicated by 'NS'. Significant differences between recovering head and tail fragments are depicted with a two-headed arrow. Scale bars: 100 µm.

Fig. 5. DNA damage and stem cell dynamics in recovering fragments 7 dpa. An overview of the experimental procedures is summarized on top. (A) The average median % tail of 4 biological replicates per group is depicted. One independent experiments. (B) The average *smedwi* intensity/mm². Combination of 3 independent experiments (number of biological replicates ≥ 14 per group). (C) The average number of H3P+ cells/mm². Three independent experiments (number of biological replicates ≥ 15 per group). (D) The average number of *NB.21.11e*+ cells/mm². An average value per sample was determined based on counting 3 regions (in front of the pharynx, next to the pharynx and behind the pharynx).Combination of 3 independent experiments (number of biological replicates ≥ 13 per group). The values represent mean±s.e.m (2-way ANOVA with Tukey HSD multiple-comparison, ***P<0.001, **P< 0.01; *P<0.05). Non-significant differences are indicated by 'NS'. Significant differences between recovering head and tail fragments are depicted with a two-headed arrow. Scale bars: 500 µm.

Fig. 6. Effects of *β-catenin-1* knockdown in recovering tail fragments. An overview of the experimental procedures is summarized on top. (A) Regenerative success 11 to 13 dpa. Regenerative success was scored by assessing the reappearance of a head structure with two eyes, i.e. 'complete (green color, head with 2 eyes), 'aberrant' (orange color, head with 1 or no eyes), 'absent' (red color, no outgrown blastema). The number of replicates per group is indicated in the figure and is a combination of 3 independent experiments at 11 or 13 dpa. (B) Immunostaining against SYNORF1. The reappearance of a brain ganglion is depicted. The relative brain size of *β-catenin-1* KD animals was measured as the width of the brain relative to the width of the entire head and is a combination of 2 independent experiments (number of biological replicates per group ≥ 11). (C) The average number of $H3P_+$ cells/mm². Three independent experiments (number of replicates ≥ 16 per group). (D) The average number of *NB.21.11e*$_+$ cells/mm², measured 3 dpa. An average value per sample was determined based on counting 3 regions (in front of the pharynx, next to the pharynx and behind the pharynx). Combination of 2 independent experiments (number of replicates ≥ 8 per group). The values represent mean±s.e.m (2-way ANOVA with Tukey HSD multiple comparison for (C-D) Student *t*-test for (B), ***P<0.001, **P< 0.01; *P<0.05). Non-significant differences are indicated by 'NS'. Significant differences between recovering head and tail fragments are depicted with a two-headed arrow. Scale bars: 100 µm (A-B) or 500 µm (C-D).

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