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Decreased neoblast progeny and increased cell death during starvation-induced planarian degrowth

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ABSTRACT The development of a complex multicellular organism requires a careful coordination of growth, cell division, cell differentiation and cell death. All these processes must be under intricate and coordinated control, as they have to be integrated across all tissues. Freshwater planarians are especially plastic, in that they constantly replace somatic tissues from a pool of adult somatic stem cells and continuously undergo growth and degrowth as adult animals in response to nutrient availability. During these processes they appear to maintain perfect scale of tissues and organs. These life history traits make them an ideal model system to study growth and degrowth. We have studied the unique planarian process of degrowth. When food is not available, planarians are able to degrow to a minimum size, without any signs of adverse physiological outcomes. For example they maintain full regenerative capacity. Our current knowledge of how this is regulated at the molecular and cellular level is very limited. Planarian degrowth has been reported to result from a decrease in cell number rather than a decrease in cell size. Thus one obvious explanation for degrowth would be a decrease in stem cell proliferation. However evidence in the literature suggests this is not the case. We show that planarians maintain normal basal mitotic rates during degrowth but that the number of stem cell progeny decreases during starvation and degrowth. These observations are reversed upon feeding, indicating that they are dependent on nutritional status. An increase in cell death is also observed during degrowth, which is not rapidly reversed upon feeding. We conclude that degrowth is a result of cell death decreasing cell numbers and that the dynamics of neoblast self-renewal and differentiation adapt to nutrient conditions to allow maintenance of the neoblast population during the period of starvation.

KEY WORDS: *planarian, starvation, regeneration, neoblast, stem cell*

Introduction

The planarian life history is highly plastic. Understanding their response to both injury and starvation has captivated the interest of researchers for more than 200 years (Morgan, 1901). However, we are still far from an understanding of the mechanics and regulation of this homeostatic plasticity. Several key traits that planarians posses demonstrate the incredible extent of this plasticity (reviewed in (Aboobaker, 2011; Gonzalez-Estevez, 2009; Reddien and Sanchez Alvarado, 2004; Salo, 2006). Most planarians are able to reproduce asexually by fission. For instance in *Schmidtea mediterranea* fission occurs at the post-pharyngeal level, whereby the tail of the animal attaches to the substrate while the head pulls away, resulting in two fragments that will regenerate two complete planarians. In addition, planarians are able to regenerate a whole functional organism from almost any part of their bodies. Even very tiny pieces are able to regenerate complete and functional planarians in about two weeks. Even more remarkable is the fact that during the regeneration process, these tiny pieces will remodel old tissue and organs to adapt it to their new size. Finally, planarians can survive long periods of starvation. During this time, they diminish in size from adult sexual planarian sizes of up to 2.5 centimetres to less than a millimetre. During this 25-fold decrease in length they maintain a full set of tissues and organs that all retain physiological function. This process is reversible since they can grow back

Abbreviations used in this paper: dS, days of starvation; hR, hours of regeneration; qPCR, quantitative PCR.

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to their full size when fed (reviewed in (Gonzalez-Estevez, 2009).

Planarian plasticity can be explained at least in part by the presence of pluripotent adult stem cells or neoblasts which are able to give rise to all differentiated planarian cell types and make up ~25% of all cells (Baguñà *et al.*, 1989; Wagner *et al.,* 2011). In the past 10 years significant advances have been made in the understanding of this particular cell type and the process of regeneration. However, the special process of degrowth during starvation has not been thoroughly researched with molecular tools. It is known that planarian degrowth during starvation is due to a decrease in cell number rather that to a decrease in cell size (Baguñà *et al.*, 1990). Supporting this observation there is a reported increase in apoptosis and cell death associated with autophagy during starvation (Gonzalez-Estevez *et al.*, 2007a; Gonzalez-Estevez *et al.*, 2007b; Pellettieri *et al.,* 2010). Although there have been contradictory reports (Bowen *et al.*, 1976), the prevailing view is that during planarian starvation the mitotic rate of neoblasts remains constant (Baguñà, 1976a). It has also been reported that planarians maintain a constant ratio of different cell types during changes in body size such as body remodelling during regeneration (Takeda *et al.*, 2009) or during degrowth due to starvation (Oviedo *et al.*, 2003). Other reports have shown that while this is true for some cell types, it is not for others (Baguñà and Romero, 1981; Romero, 1987; Romero and Baguñà, 1991). Recently advances have been achieved by studying the metabolic rate of planarians during their lifespan (Mouton *et al.,* 2011). It has been shown that juvenile animals show the highest metabolic rate and that later adult planarians keep a lower but constant metabolic rate. Thus, differences in metabolic age may not exist during planarian adulthood. Again, the influence of planarian size or starvation on the metabolic rate has not been systematically investigated yet.

It is fair to assume that some adjustment of the cell turnover and cell metabolic processes is necessary in order for planarians to be able to endure starvation without physiological impairment. By taking advantage of the molecular techniques and repertoire of neoblast and neoblast progeny markers now available in planarians we aim to give a novel overview of neoblast dynamics during starvation.

Results

Neoblast mitotic rate is constant during starvation

In order to better understand the dynamics of growth and degrowth in *S. mediterranea*, we selected a cohort of planarians of approximately the same size (~6 mm length; 5.12 mm^2 ± 0.79 s.d; n>130 planarians per experiment in 5 independent experiments). These planarians were kept under conditions of starvation at 20ºC and the area of their bodies was measured either while alive and in motion (Fig. 1A and B) or fixed (Fig. 1C) every 20 days until 60dS

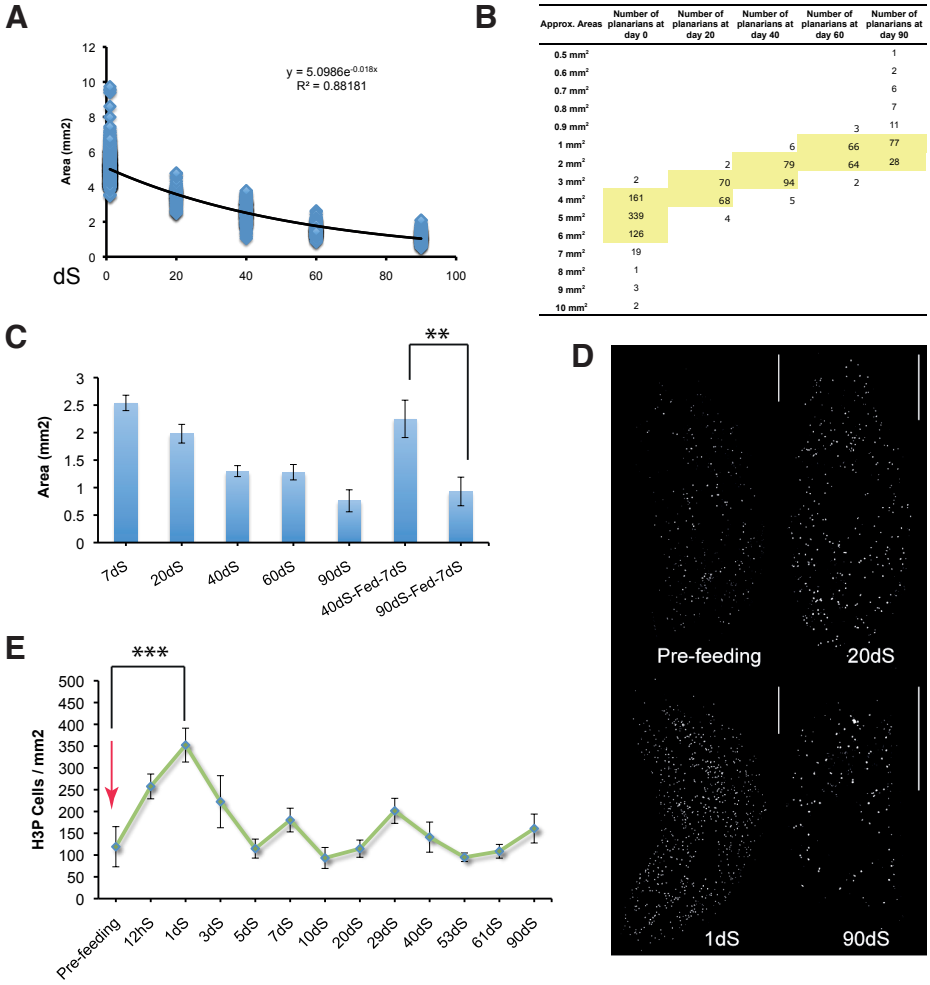

Fig. 1. Planarian size changes through growth/degrowth and the dynamics of mitotic neoblasts during starvation. (A) *Scatter plot that represents the change in area at different time points of starvation for all planarians used during this work. Areas are displayed in the ordinate and were measured in live planarians. Planarian degrowth processes follow a negative exponential pattern. n>130 planarians per experiment in 5 independent experiments; represented is the negative exponential distribution, R^2 indicates correlation coefficients. dS, days of starvation.* **(B)** *Table that shows the dispersion in area of the planarians for each time point of starvation represented in the scatter plot (A). In yellow is highlighted the most representative range in area for each time point of starvation.* **(C)** *The graph represents the change in area at different time points of starvation from a subset of planarians used in this work. Areas are displayed in the ordinate and were measured in fixed planarians. The graph also shows that the increase in area of planarians starved for 90d 7d after feeding is smaller than the one for planarians starved for 40d. n = 10.* **(D)** *Representative images of planarians stained for anti-H3P (mitotic neoblasts) at different days of starvation.* **(E)** *Planarian mitotic neoblasts during starvation. Values are normalised by total body area. n≥5 in each of 2 independent experiments. Red arrow indicates the time of feeding. Error bars in all the graphs are s.d from the mean and asterisks indicate p<0.01 (two asterisks) or p<0.001 (three asterisks) using two-tailed Student's test with equal sample variance. In (E), 7dS and 29dS show two peaks that are significant for p<0.01 respect the previous time point but not for p<0.001. However, they show no significant differences respect to the pre-feeding time point (p>0.01).*

(days of starvation) and at a final time point at 90dS. Under our culture conditions, the *S. mediterranea* BCN-10 clonal line degrowth process follows best a negative exponential distribution ($R^2 = 0.88$ versus $R^2 = 0.80$ if linear and $R^2 = 0.82$ if negative logarithmic). We could also observe that at 90 days of starvation, planarians diminished their body area by 76% (Fig. 1 A-C).

We also wished to assess if the nutritional status of the planarian affects the dynamics of growth once feeding is resumed. Thus we chose an intermediate time point and a late time point of starvation where we analyzed the dynamics of growth. We fed planarians that were previously starved for 40d and 90d and waited one week to measure their area increase as a measure of growth. Whereas 40dS planarians where able to grow on average 0.95 mm^2 (73.07% of the initial body area) in one week, 90dS planarians where only able to grow about 0.17 mm^2 (22.4 % of the initial body area) (Fig. 1A). This is quite remarkable since the square-cube law states that the surface of a small animal grows faster in relation to its volume than that of a bigger animal of the same shape. This suggests that nutritional status and not the size of the planarians before feeding that is the over-riding influence on the growth rate.

Planarians were collected at different time points, fixed and processed to observe neoblast dynamics during starvation using *in situ* hybridization and immunohistochemistry. We started by checking the pattern of mitotic neoblasts by using the Histone-3 phosphorylated at serine 10 (anti-H3P) antibody (Hendzel *et al.*, 1997) at different times within which a feeding proliferation response has been characterised (Baguñà, 1976a), and then every ~10 days until 61d of starvation and at a final time point at 90dS (Fig. 1 D,E). In accordance with previous reported work, we could confirm that feeding induces an increase in mitoses (Fig. 1E) (Baguñà, 1976a). We could observe that the number of neoblasts positive for anti-H3P increased rapidly after feeding showing a mitotic peak after 1d of feeding ($p<0.001$ with respect to the pre-feeding time point). Subsequently levels decreased until reaching baseline levels which were kept more or less constant through the different time points of starvation (Fig. 1E). Two extra mitotic peaks could be observed at 7dS and 29dS, however they did not show statistical differences respect to the pre-feeding time point ($p>0.01$) and only small differences ($p<0.01$) with respect to the previous time points. Together, our data confirms previous reports that planarian degrowth during starvation is not due to a decrease in the number of mitotic neoblasts (Baguñà and Romero, 1981).

Neoblast progeny numbers decrease during starvation

We next assessed the ability of neoblasts to differentiate during starvation. A recent study has described a repertoire of markers

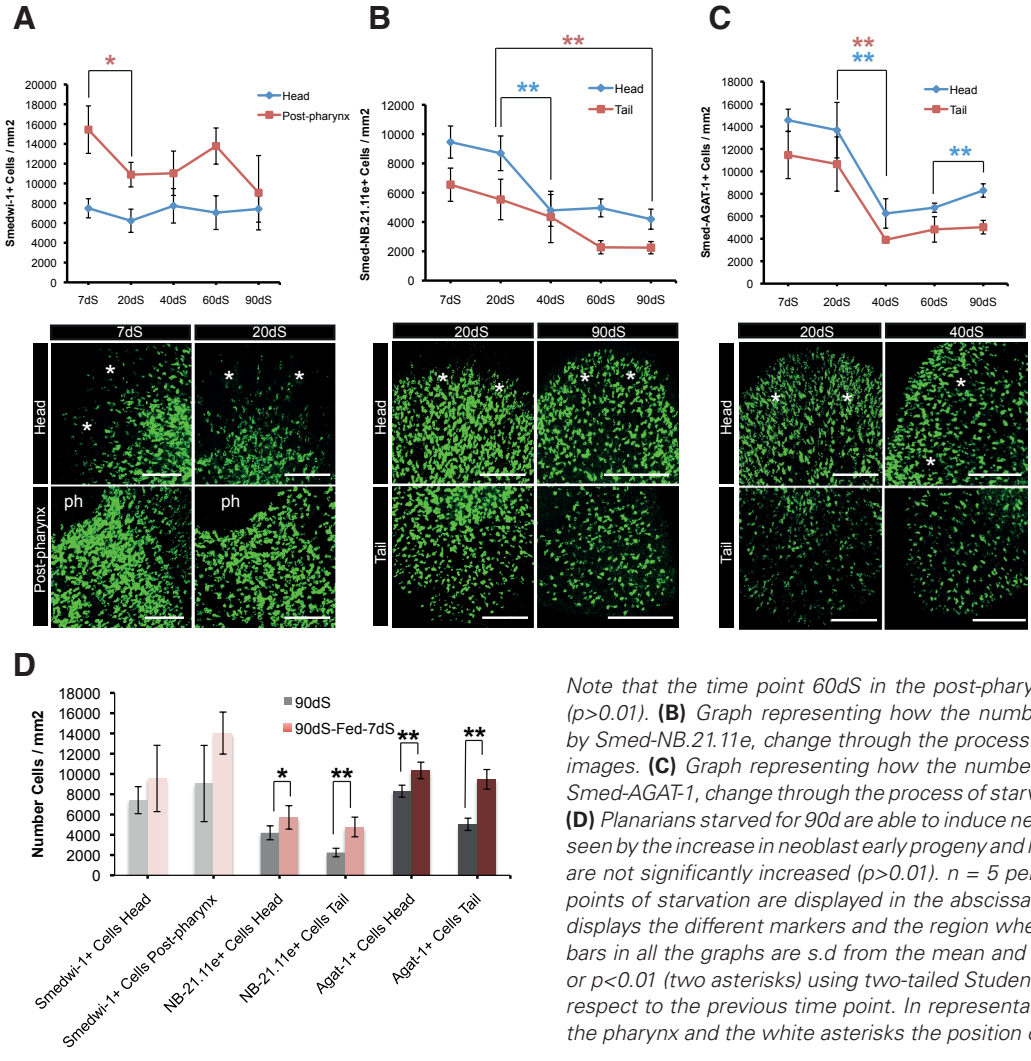

Fig. 2. Neoblast progeny dynamics during planarian starvation. *Results showed in this figure were obtained by counting positive cells for the described markers and normalised by the imaged area.* **(A)** *Graph representing how the number of cycling neoblasts, detected by the Smedwi-1 marker, change through the process of starvation. Below are representative images. Note that the time point 60dS in the post-pharynx region is not statistically significant ($p>0.01$).* **(B)** *Graph representing how the number of neoblast early progeny, detected by Smed-NB.21.11e, change through the process of starvation. Below are representative images.* **(C)** *Graph representing how the number of neoblast late progeny, detected by Smed-AGAT-1, change through the process of starvation. Below are representative images.* **(D)** *Planarians starved for 90d are able to induce neoblast differentiation 7d after feeding, as seen by the increase in neoblast early progeny and late progeny in heads and tails. Neoblasts are not significantly increased ($p>0.01$). n = 5 per time point in all graphs. Different time points of starvation are displayed in the abscissa for (A), (B) and (C). In (D), the abscissa displays the different markers and the region where these markers where counted. Error bars in all the graphs are s.d from the mean and asterisks indicate $p<0.05$ (one asterisk) or $p<0.01$ (two asterisks) using two-tailed Student's test with equal sample variance with respect to the previous time point. In representative images ph indicates the position of the pharynx and the white asterisks the position of the eyes. Scale bars indicate 100 μm.*

transiently expressed in neoblasts, early neoblast progeny and older neoblast progeny (Eisenhoffer *et al.*, 2008). We performed whole-mount *in situ* hybridization for *Smedwi-1* (neoblast marker), which is expressed deep within the body, *Smed-NB.21.11e* (early post mitotic progeny), which is expressed peripherally to *Smedwi-1* and *Smed-AGAT-1* (late post mitotic progeny), which is the most peripherally expressed of these genes (Fig. 2 A-C). We then quantified the number of positive cells for each marker in equivalent regions of the planarian body.

Smedwi-1 measurements were performed in the head region and the region posterior to the pharynx during different starvation time points. We could observe that the number of *Smedwi-1*$^+$ neoblasts in the post-pharynx area was significantly lower at 20dS compared to just 7dS (Fig. 2A). Interestingly, this is an agreement with the observation of a small mitotic peak ($p<0.01$) at 7dS (Fig. 1E). Later the proportion of *Smedwi-1*$^+$ cells does not change significantly. In the head region we observed constant values for *Smedwi-1*$^+$ neoblasts during the whole starvation process.

For early and late neoblast progeny we also performed two measurements of cell number per planarian, one in the head region and another in the tail region. We found that early and late progeny were significantly decreased during the process of starvation from 20dS. In the case of the early progeny marker *Smed-NB.21.11e* we observed a significant decrease in the number of positive cells between 20dS and 40dS in head regions (Fig. 2B), and between 20dS and 90dS in tail regions. The number of *Smed-AGAT-1*$^+$ cells significantly decreased between 20dS and 40dS and remained low through the rest of the starvation process in tails but displayed a significant increase in number between 60dS and 90dS in head regions (Fig. 2C).

Next we wished to investigate if feeding modulates the progeny numbers during starvation. 90dS planarians were fed and 7 days later examined for progeny markers, a time when baseline levels of mitoses should have been reached. We observed that *Smedwi-1*$^+$ cell numbers already displayed basal levels similar to those before feeding (Fig. 2D). However, the number of early and late neoblast progeny was significantly increased in tails and heads (Fig. 2D).

Altogether, our data show that planarians significantly decrease the proportion of neoblasts in the post-pharyngeal region between 7dS and 20dS and this level is maintained thereafter. The proportion of neoblast progeny decreased significantly by 40dS. Thus, it appears that there is less differentiation of neoblasts from 20 days of starvation. Furthermore, we observed that feeding or growth is able to reverse the process and increase progeny cell numbers and the incidence of differentiation, and correlates with the fact that feeding induces proliferation (Baguñà, 1976a) (Fig. 1E).

We next wished to corroborate our observations by analyzing the levels of expression of neoblast and neoblast progeny markers by quantitative PCR (qPCR) at different time points of starvation. Since surface to volume ratio is smaller the larger an object is the relative level of expression of a gene that is expressed close to the epidermis should have a tendency to increase relatively with

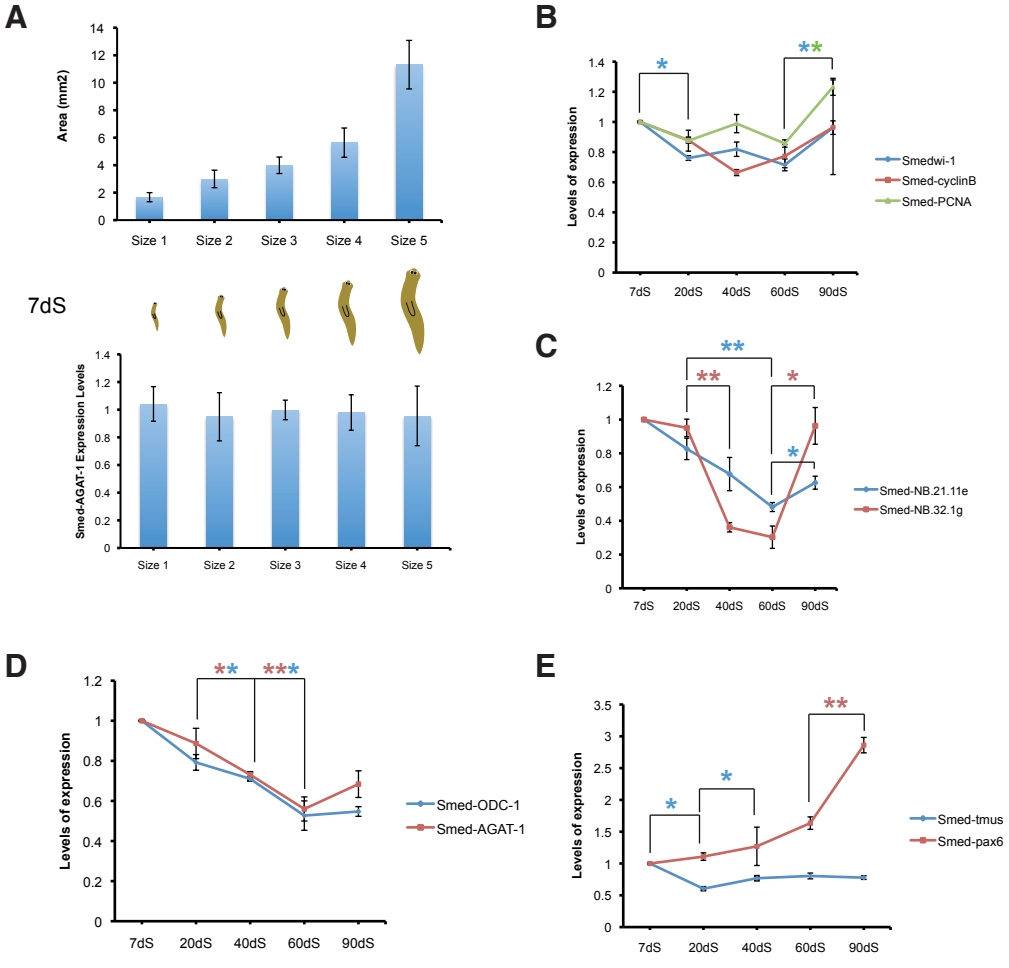

Fig. 3. Neoblast progeny dynamics during planarian starvation. *Results showed in this figure were obtained by qPCR for the described markers. All values are relative to 7dS* **(A)** *Planarians of different size but the same nutritional status (7dS) (graph on top) display the same values of Smed-AGAT-1 (graph below; no significant differences ($p>0.05$) were observed after running two-tailed Student's test with equal sample variance).* **(B)** *Level of expression of three neoblast markers through the process of starvation.* **(C)** *Level of expression of two neoblast early progeny markers through the process of starvation.* **(D)** *Level of expression of two neoblast late progeny markers through the process of starvation.* **(E)** *Level of expression of two markers for differentiated cells through the process of starvation. For details about number of worms per time point, see Materials and Methods. Error bars in all the graphs are s.d from the mean and asterisks indicate $p<0.05$ (one asterisk) or $p<0.01$ (two asterisks) using two-tailed Student's test with equal sample variance.*

decreasing volume of an animal. For that reason, we performed a control experiment that consisted of collecting planarians of different sizes all starved for 7d. We then performed quantitative PCR (qPCR) for *Smed-AGAT-1*, the marker for late progeny that showed the greatest decrease in cell number during starvation and that is the most peripherally expressed in the planarian body. We could not see any significant difference in expression among all the differently sized planarians (Fig. 3A). This suggests that planarians possess some kind of regulation for the level of expression of genes during degrowth. We therefore concluded that any changes in progeny expression that we could observe by qPCR are not likely due to size differences in the animal but rather due to the starvation process. To corroborate the results obtained by counting cells expressing the different markers we performed qPCR for *Smedwi-1*, *Smed-NB.21.11e*, *Smed-AGAT-1* and as well for other genes (Eisenhoffer *et al*., 2008; Fernandez-Taboada *et al.,* 2010) expressed in cycling neoblasts (*Smed-cyclinB*, *Smed-PCNA*), early progeny (*Smed-NB.32.1g*) and late progeny (*Smed-ODC-1*). For the cycling neoblast markers *Smedwi-1*, *Smed-PCNA* and *Smed-cyclinB* qPCR corroborated our data from counting *Smedwi-1*$^{+}$ cells or H3P^{+} cells (Fig. 2 A,B) that the expression of *Smedwi-1* decreases from 7dS to 20dS. For *Smed-NB.21.1e* and *Smed-NB.32.1g* we observed a decrease in the levels of expression from 20dS (Fig. 3C), as observed for the number of *Smed-NB.21.11e* expressing cells (Fig. 2B). Finally, the levels of expression of late progeny markers *Smed-AGAT-1* and *Smed-ODC-1*, two different markers which have been shown to have a minimal overlapping expression (Wagner *et al.,* 2011) also showed a decrease from 20dS (Fig. 3D), as we observed by counting the number of positive *Smed-AGAT-1* cells (Fig. 2C). We also noted that from 60dS the global level of expression of neoblast markers and early progeny markers significantly increased (Fig. 3 B-C). Thus, while neoblasts and early neoblast progeny keep constant numbers of positive cells from 60dS to 90dS (Fig. 2 A-B), the expression levels of these markers undergo a relative increase by 90dS (Fig. 3 B-C). The opposite pattern of behaviour was observed for neoblast late progeny markers, which increased in number but kept the same global expression level at this time point (Fig. 2C and Fig. 3D). Altogether qPCR with different neoblast markers has corroborated our observation that cycling neoblasts decrease from 7dS to 20dS and then are kept constant in number and expression until 60dS. Furthermore, qPCR on progeny markers confirms our previous observation that the levels of progeny are lower from 20ds to 60dS. The discordance in the number of positively expressing cells and global level of expression seen from 60dS may be interpreted as a regulation of the mRNA levels for these markers in the remaining fewer cells that express the markers.

Although it has been reported that planarians are able to maintain a constant ratio of different cell types during changes in body size (Takeda *et al*., 2009), there are other reports showing that some populations of cells, such as nerve cells proportionally increase during starvation (Baguñà and Romero, 1981). Hence, we examined the levels of expression of two genes, *Smed-tmus*, which is expressed in muscle cells and myocytes (Cebrià *et al*., 1997) and *Smed-pax6* (Pineda *et al*., 2002), which is highly expressed in the planarian central nervous system (Fig. 3E). Our results agreed with older reports (Baguñà and Romero, 1981), *Smed-tmus* was kept at constant levels, whereas, *Smed-pax-6* significantly and progressively increased during starvation. This suggests the response to starvation may vary between tissues.

Planarian degrowth correlates with an increase in cell death during the process of starvation

Degrowth is a process that involves high levels of tissue remodelling, since planarians are able to degrow in size while preserving their proportions. It has been suggested that degrowth results primarily from a reduction in cell number, rather than a reduction in cell size (Baguñà and Romero, 1981; Romero and Baguñà, 1991). As a result, processes of cell death may be important during degrowth due to starvation. In fact, TUNEL staining has already shown that during starvation there is a progressive increase in cell death from 5dS to 35dS (Pellettieri *et al*., 2010). It has been also recently shown that at least some planarian cell death, which is TUNEL negative but cleaved caspase-3 positive is related to autophagy and happens during starvation (Gonzalez-Estevez *et al*., 2007b). Caspase-3 activation plays a key role in the initiation of cellular events related to early cell death processes and thus the measure of its activity is a good assay for the detection of early cell death, both apoptosis- and autophagy-related. In planarians it has been already successfully used for the detection of cell death in the *Girardia tigrina* planarian species (Gonzalez-Estevez *et al*., 2007b).

We analyzed caspase-3 activity at different time points of starvation. We observed that cell death increased drastically by 20dS and that from 40dS to 60dS was maintained lower than the baseline levels found at 7dS ($p<0.01$). Interestingly, at 90dS it increased again, however never reaching the levels found at 20dS (Fig. 4A). These results show that cell death levels are higher at the beginning of the starvation process than much later. These

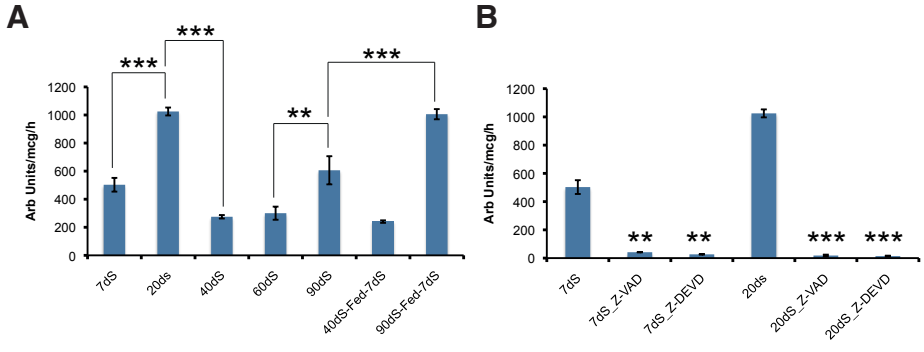

Fig. 4. Cell death dynamics during planarian starvation. (A) *The graph shows the levels of caspase-3 activity of cell lysates at different times of starvation. Caspase-3 activity is presented as arbitrary units per* μg *and hour of incubation (Arb Units/mcg/h). The abscissa displays the different nutritional conditions. Note that no significant differences were observed in 40dS compared to 40dS-Fed-7dS after two-tailed Student's test with equal sample variance ($p>0.05$). Also note that 40dS and 60dS are significantly lower respect to 7dS ($p<0.01$).* **(B)** *The graph shows a negative control of caspase-3 activity. After incubation of the cell lysates with Z-VAD-FMK and Z-DEVD-FMK, two caspase inhibitors, caspase-3 activity is almost completely abolished. Error bars in all the graphs are s.d from the mean and asterisks indicate $p<0.01$ (two asterisks) or $p<0.001$ (three asterisks) using two-tailed Student's test with equal sample variance.*

results correlate with some of our previous results that showed that degrowth follows a negative exponential distribution. The specificity of this assay was analyzed by incubating the cellular lysates with caspase inhibitors (Gonzalez-Estevez *et al.*, 2007b). Caspase inhibitors were able to inhibit caspase-3 and therefore no activity was observed (Fig. 4B).

It has also been shown that the changes in cell death observed in 35dS planarians are reversed if planarians are fed at day 28 (Pellettieri *et al.*, 2010). We performed a similar approach analyzing caspase-3 activity. We observed that 40dS planarians that were fed and analyzed 7 days later for caspase-3 activity showed levels of cell death similar to before being fed (Fig. 4A). However, 90dS planarians that were fed and then analyzed 7 days later for caspase-3 activity showed increased levels of cell death compared to before being fed (Fig. 4A). Altogether it seems that highly starved planarians have a larger cell death response 7d after feeding, compared to planarians starved for less time. This is in agreement with our previous results showing that at 90dS less growth is observed 7d after feeding than at 40dS. Our results together with the previous published results (Pellettieri *et al.*, 2010) suggest that although planarians may be able to reverse cell death levels upon feeding, this response is not rapid and may reflect the requirement for remodelling during growth.

Planarian response to amputation depends on the nutritional status

Older literature reports that starved planarians have a higher regenerative power or can regenerate more rapidly (Bardeen, 1901; Sivickis, 1933). However, again some contradictory results have been reported (Abeloos, 1930; Brøndsted, 1953; Child, 1911). To see if planarians respond differently to amputation depending on their nutritional status we checked the mitotic response of planarians at different time points of starvation. After amputation neoblasts undergo a broadly distributed increased mitotic response to injury in the first 4-10 h and a second more spatially restricted mitotic response at 48-72 h, which is a response to missing tissue (Baguñà, 1976b; Wenemoser and Reddien 2010). Neoblast progeny migrate and form an unpigmented tissue called the blastema. The blastema becomes progressively pigmented and neoblasts terminally differentiate to form missing structures.

We performed immunohistochemistry for anti-H3P at 6h, 20h and 48h after amputation on 7dS, 40ds, 90dS planarians as well as 40dS planarians that were fed and then processed 7 days after feeding (Fig. 5). We observed several interesting differences among the different nutritional situations. Firstly, starved planarians are able to respond to injury with same or even higher levels of mitoses. In fact, 90dS planarians were able to induce the largest proliferative response to amputation and to missing tissue compared to 40dS and 40dS fed and processed 7d later. In addition 90dS planarians had a higher proliferative response to amputation but not to missing tissue than 7dS planarians. Furthermore, 40dS planarians that were fed and processed 7d later had a similar response to injury to that observed for 7dS planarians. However, they did show a lower mitotic response to missing tissue than 7dS starving planarians. These results indicate that different nutritional statuses may translate into distinct responses to amputation and that, even 7d after feeding starved planarians, the response to missing tissue is not restored to that observed in animals of higher nutritional status. Finally, the intermediate time point 20h after amputation also showed some interesting features as it showed the same levels of mitosis as the first mitotic peak in 7dS and 40dS ($p>0.01$) but not at 90dS and fed 40dS ($p<0.01$). This may be explained by temporal differences in the mitotic peaks depending on the nutritional status. Further studies are necessary to better understand these differences. Furthermore, it will be interesting to elucidate if planarians that have been starved for a long time and show higher mitotic responses to injury display differences in the rate of differentiation, replacement of missing tissues and the proportions of new tissue derived from the blastema compared to old tissue.

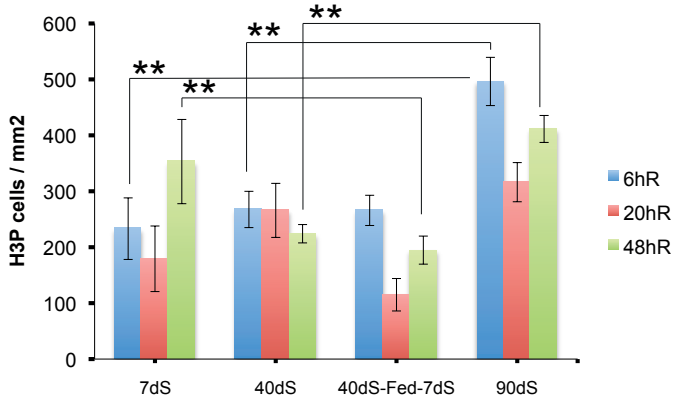

Fig. 5. Nutritional status influences the mitotic response to amputation. *The graph shows how the nutritional status can influence the first mitotic peak at 6 hours regeneration (hR) and the second mitotic peak at 48hR. The abscissa refers to the different nutritional status analyzed while the different colours indicate the different mitotic peaks after amputation and the intermediate time point of 20hR. The graph shows how 90dS planarians are able to respond stronger to amputation than 40dS. Error bars are s.d from the mean and asterisks indicate $p<0.01$ using two-tailed Student's test with equal sample variance.*

Discussion

It has been a matter of debate as to how planarians are able to degrow in size during starvation while keeping a perfectly scaled body and without suffering obvious physiological impairment. Although there have been some contradictory reports (Bowen *et al.*, 1976), the prevailing view to date has been that during planarian starvation, neoblast mitotic rate is kept constant (Baguñà, 1976a). Here we confirm that during planarian degrowth due to starvation there is not a decrease in the neoblast mitotic rate, and that during starvation planarians are able to keep a constant population of mitotic neoblasts (Fig. 6). This explains in part why starved planarians retain normal physiological performance over extended periods of time.

It has been suggested that cell death processes may play an important role during body remodelling and during degrowth due to starvation. Recently, reports have shown that apoptosis (Pellettieri *et al.,* 2010) and cell death related to autophagy (Gonzalez-Estevez *et al.*, 2007a; Gonzalez-Estevez *et al.*, 2007b) occur during starvation. However, a further description of these two processes through extended time points of starvation has not been previously studied. By measuring caspase-3 activity we have made an approximation of the distribution of cell death during an extended period of starvation. We have shown that cell death increases from 7dS

to 20dS and after that baseline levels are kept until 90dS when cell death increases again. These results suggest that much of degrowth process happens from 7dS to 20dS which can also be observed in the negative exponential distribution that planarian degrowth follows.

Remarkably, we were also able to show by counting the number of positive cells for early and late neoblast progeny and by performing qPCR for these markers at different time points of starvation, that early and late neoblast progeny tend to decrease from 20dS to 60dS, whereas the cycling neoblast population only decreases from 7dS to 20dS and then is kept constant and even increases slightly later during the starvation process (Fig. 6). Thus we can say that from 20dS less post-mitotic progeny are produced and levels of cell differentiation decrease. At 40dS and 60dS we also observe that the levels of cell death are lower than at 7dS, taken together this suggests that tissue cell turnover as a whole decreases from 20dS to 60dS. Interestingly, the decrease in the expression of neoblast and neoblast progeny markers in postpharyngeal areas is higher than those from head structures, which match older results (Abeloos, 1930) that neoblast numbers at postpharyngeal regions decrease at higher rates than in head regions during starvation. More studies are needed to address how the number of neoblast progeny is modulated in response starvation and degrowth/scaling.

An outstanding point of contention is the discrepancy between transcript levels and cell counts at 90dS. One possibility is that regulation of transcription and/or mRNA stability is enhanced during late times of starvation. This is a further exciting clue as to the adaptations that might be happening during degrowth. It is interesting to note that this possible increase in neoblasts and neoblast progeny from 60dS is supported by the qPCR experiments on differentiated markers (i.e, *Smed-tmus* and *Smed-pax6*) where at least *Smed-pax6* shows also an increase from 60dS. Thus, from 60dS to 90dS it seems that there is more differentiation of some tissues such as the central nervous system where *Smed-pax6* is expressed. Alternatively this differentiated tissue may be favourably retained during later stages of starvation. Furthermore, this increase in the number of neoblasts, neoblast progeny and some differentiated tissues also coincides with an increase in cell death with respect to the starving baseline levels observed at 40dS and 60dS as shown in the caspase-3 activity experiments. Altogether these data suggest that from 60dS to 90dS cell death is increased in at least some differentiated cells and thus the proportion of stem cells increases with respect the total cell number. Tissues like the central nervous system (i.e *Smed-pax6*$^{+}$ cells) may have comparatively lower levels of cell death in order to maintain physiological function and the ability to find food. Alternatively, the central nervous system may show increased cell turnover. Our results agree with previous work reporting that planarians do not keep all tissues in proportion through starvation and degrowth (Baguñà and Romero, 1981; Romero, 1987; Romero and Baguñà, 1991). An observation which is further strengthened by the square-cube law, which predicts an increasing ratio of epidermis to parenchyma the smaller the planarian is. We also observed that 90dS planarians are able to grow less at 7d after feeding compared to 40dS planarians. This could be due to a decreased mitotic response to feeding. Therefore, it would be interesting to test if the mitotic values at 1dS are the same for planarians fed with differing starting nutritional status.

However, this also correlates with the higher levels of cell death observed at the same time points. This could mean that after 90dS planarian homeostatic mechanisms are focused on maintaining cell turnover of the remaining tissues, while at 40dS planarians are able to respond to being fed by growing. This could imply that a 90dS planarians have damaged or older differentiated tissues that urgently need to be replaced, compared to a 40dS planarian which have differentiated tissues that are physiologically uncompromised allowing resources to be focused on growth. This will be an exciting proposal to test in the future as it suggests that planarians might be a good model for understanding how aged differentiated tissues are replaced or maintained. At 90dS planarians are less that 1 mm in length and thus are probably close to the minimal size they can

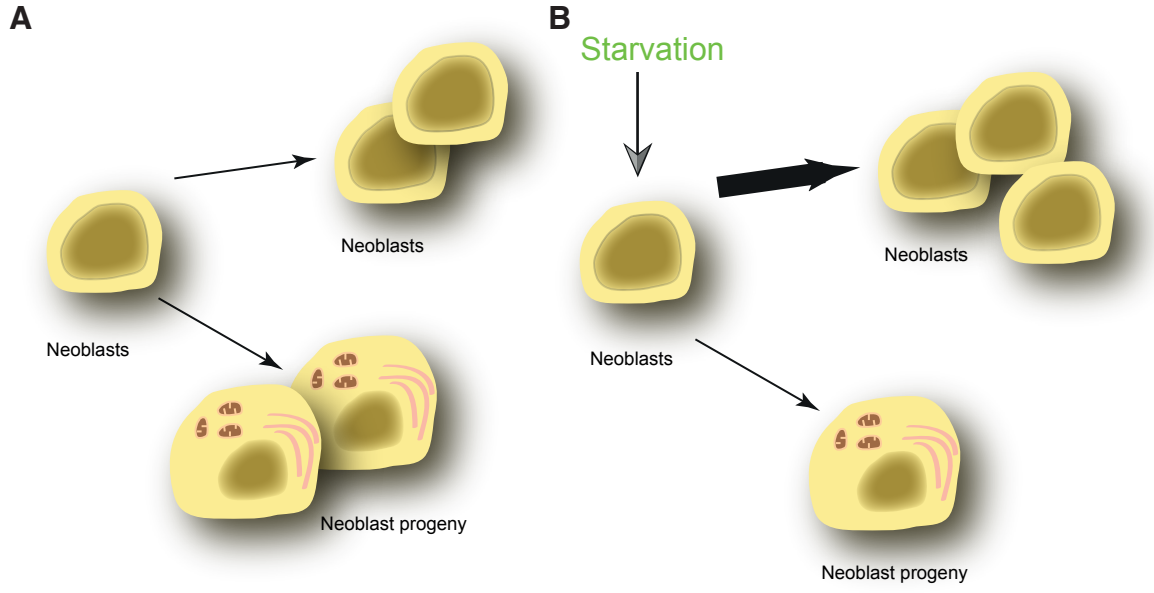

Fig. 6. Model of neoblast dynamics during starvation. *During starvation, neoblast progeny decrease in relation to neoblasts* **(B)** *and compared to high nutritional status* **(A)**.

reach before they die.

Our data also showed that 90dS planarians are able to respond stronger to amputation than 40dS or even 7dS planarians, displaying a higher first mitotic peak. This could be explained by the fact that it seems possible that from 60dS to 90dS there is an increase in neoblast and neoblast progeny and thus this would allow a stronger response to injury.

The outstanding question is why planarians are able to maintain a stable population of neoblasts and show a decrease in neoblast progeny from 20dS to 60dS? One attractive and simple explanation is that the stem cell population as a whole switches to stem cell maintenance rather than the production of progeny cells (Fig. 6). Conversely the higher numbers of progeny in well-fed worms could result from higher levels of neoblast mitoses, with the mitoses above baseline levels giving rise to more neoblasts but also more neoblast progeny. Planarians would channel resources that are internally available into efficiently maintaining the essential neoblast population in preparation for a response to a more favourable nutritional environment or in response to injury. In agreement with this, we could observe that starving planarians are able to raise a normal or even higher response to injury. We observed that from 20dS, there is a clear decrease in neoblast progeny, however the neoblast population is kept constant. Thus 20dS planarians could effectively be entering into a stand-by state that is optimal for their survival through lean nutritional times. From 20dS there would be reduced cell turnover and cell survival pathways for the differentiated tissues could play an important role. This agrees with our data that cell death from 20dS is at the minimum. If this model was correct a corollary would predict that differentiated tissues would age during starvation as the rate of homeostatic cell replacement is reduced. One exciting possibility is that essential cells within differentiated tissues would initially activate survival pathways in order to lengthen their life span however later would become aged cells and would become the main population of cells being replaced. This agrees with our observation that some cells, like nerve cells seem to proportionally increase through the starvation process. In the future it will be important to test this model by understanding whether the differentiated cells are randomly or selectively removed with respect to their age during degrowth.

To understand which signalling pathways are regulating the degrowth and growth during planarian starvation is going to be very informative not only for a better understanding of planarian biology but also for the understanding on how body and organ proportions are regulated in all animals.

Materials and Methods

Animals

Planarians used in this work belong to the species *Schmidtea mediterranea* asexual strain clonal line BCN-10. Planarians were maintained at 20ºC in tap water treated with activated charcoal and buffered with 0.5 ml/L 1 M $NaHCO_3$. All planarians for all experiments were fed organic veal liver (Veritas, Barcelona, Spain). Experiments involving feeding were done with observation of feeding behaviour. All animals were observed by eye to make sure they ate. This was monitored by watching them attract to liver and by observing typical distension and red liver coloration of the gut. Starvation experiments were done in 5 independent experiments or pools (n>130) that were started at different times of the year. Planarians needed for each set of experiments were taken from at least two of the pools.

in situ *hybridization (ISH) experiments*

Digoxigenin-labelled RNA probes and/or fluorescein-labelled RNA probes were prepared by using an *in vitro* labelling kit (Roche, Basel, Switzerland). Fluorescent whole mount ISH was carried out as described previously (Cebria *et al.*, 2007; Gonzalez-Estevez *et al.*, 2009; Pearson *et al.*, 2009; Umesono *et al.*, 1999).

Immunohistochemistry

Whole-mount immunohistochemistry was carried out as described elsewhere (Cebria and Newmark, 2005). Anti-Histone H3 phosphorylated at serine 10 (Hendzel *et al.*, 1997) was used to detect mitotic neoblasts (diluted 1/500, Millipore). Alexa488-conjugated or Alexa568-conjugated goat anti-rabbit were used as secondary antibodies (Molecular Probes; diluted 1/1000).

Real Time PCR

RNA was extracted using Trizol reagent (Invitrogen). cDNA was obtained from 2 μg of total RNA by using MMLV Reverse Transcriptase (Promega). Elongation Factor 2 (EF2) was used as an internal control. Two qPCRs per starvation time point were performed for *Smedwi-1*, *Smed-NB.21.11e*, *Smed-NB.32.1g*, *Smed-AGAT-1* and *Smed-ODC-1*. Each qPCR were performed with two biological replicates per starvation time point. Five animals were used per replicate, and each sample was replicated three times in each real-time PCR experiment. PCR reactions were performed using the SensiMix SYBR No-ROX Kit (Bioline). Reactions were aliquoted using a Corbett Robot (Corbett Robotics) and analysed with a Rotor Gene 6000 (Corbett, Qiagen). qPCR oligos were used as already described (Eisenhoffer *et al.*, 2008; Fernandez-Taboada *et al.,* 2010), except for:

ODC-1F:	5'-AGTGAAGTGCAATCAGTTGG-3'
ODC-1R:	5'-GAATGTTGAAAGAGCCATAGAC-3'
NB.32.1gF:	5'-AGAAACGAGAAATGAGTGCC-3'
NB32.1gR:	5'- CTCAAATTTGCCATGTAAGCTG-3'

Imaging and quantifications

Measurements on live planarians were done in photographs taken with a Zeiss Discovery V8 (Carl Zeiss) and recorded on an AxioCam MRC (Carl Zeiss). Confocal laser scanning microscopy was performed for fluorescent samples with a Leica SP2 confocal microscope (CLSM) (Leica). Confocal stacks were processed using ImageJ 1.43l or Adobe Photoshop CS5 software and compositions done on Adobe Illustrator CS5. *Smedwi-1*$^+$, *Smed-NB.21.11e*$^+$ or *Smed-AGAT-1*$^+$ cells were quantified by Volocity (Perkin Elmer) after performing 2 μm Z confocal stacks from dorsal to ventral and normalised by the area measured. Two different regions of the planarian body were quantified for each marker. Head and tail regions were quantified for *Smed-NB-21.11e* and *Smed-AGAT-1* positive cells at 40x. The area quantified was measured by Image J and used to normalise the values over shape. Head and post-pharynx regions were used for *Smedwi-1*. The head region was measured for positive cells at 40x and the area quantified was measured by Image J to normalise the values over shape. The postpharyngeal region (from the end of the pharynx until half the length to the tail) were used to quantify the number of cells. The area quantified was measured by Image J and used to normalise the values. Position of the eyes in Fig. 2 was determined by the background of Hoechst in the eyes. H3P quantification was performed on whole planarian confocal stacks and using the Object Counter 3D plugin from ImageJ 1.43l and normalised by the total body area. For all graphs, error bars represent standard deviation of the mean (sd) and asterisks indicate $p<0.05$ (one asterisk), $p<0.01$ (two asterisks) or $p<0.001$ (three asterisks) using two-tailed Student's t-tests with equal sample variance.

Analysis of caspase-3 activity

Fluorimetric analysis of caspase-3 activity was performed as described previously for *Girardia tigrina* (Gonzalez-Estevez *et al.*, 2007b) with 20 μg protein extract and incubated for 2 hours at 37ºC with 20 μM caspase-3

substrate Ac-DEVD-AMC or 2 μl from a stock of 1mg/ml for a final volume of 150 μl. For a detailed protocol please refer to (Gonzalez-Estevez, 2008). Fluorescence was measured in a Microplate Fluorescence Reader Fluostar Optima (BMG Labtech) (1 excitation, 380 nm; 1 emission 440 nm). Protein concentration of the cell lysates was measured using the BioRad protein reagent.

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