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Aurora kinase inhibitors delay regeneration in *Stentor coeruleus* at an intermediate step

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Abstract

The giant unicellular ciliate *Stentor coeruleus* can be cut into pieces and each piece will regenerate into a healthy, full-sized individual. The molecular mechanism for how *Stentor* regenerates is a complete mystery, however, the process of regeneration shows striking similarities to the process of cell division. On a morphological level, the process of creating a second mouth in a division or a new oral apparatus in regeneration have the same steps and occur in the same order. On the transcriptional level, genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during regeneration. This suggests that there may be some common regulatory mechanisms involved in both regeneration and cell division. If the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in *Stentor*. Here we show that two well-characterized Aurora kinase A+B inhibitors that affect the timing of oral apparatus regeneration. ZM447439 slows down the regeneration of the oral apparatus by at least one hour. PF03814735 completely suppresses the regeneration of the oral apparatus until the drug is removed. Here we provide the first direct experimental evidence that *Stentor* may harness the cell division machinery to regulate the sequential process of regeneration.

Introduction

The ability to heal wounds and regenerate damaged structures is essential for an organism's survival. Multicellular organisms mostly rely on cell division to patch wounds and regenerate lost structures with newly proliferated cells, but when a single cell is damaged, whether it be a free-living unicellular organism or a cell within a multicellular tissue, it must be able to recognize and repair that damage without being able to rely on other cells. Now here is this challenge more dramatic than in the giant unicellular ciliate *Stentor coeruleus,* for when cutting into pieces, each piece will fully regenerate into a healthy, full-sized individual [1]. *Stentor* cells are a millimeter long with a wine glass shape and have a complex and intricate ultrastructure. *Stentor* is binucleate ciliates with two morphologically distinct nuclei. The micronuclei is used for germline reproduction and the macronucleus is transcriptionally active throughout the cell cycle. *Stentor* has an oral pouch, a cilia-lined pore to intake food at its wide anterior and a holdfast, the structure by which the cell attaches to a surface, at its posterior. Connecting these two is a series of microtubule rows called cortical rows that resemble pinstripes. The oral pouch and the holdfast can each fully regenerate if removed, and a bisected cell can regenerate two normal-looking cells [1]. The molecular mechanism for how *Stentor* regenerates missing parts is a complete mystery. This study focuses on regeneration of the oral apparatus, which consists of a circular band of cilia-based structures known as the membranellar band, connected to an oral pouch located at a defined position. During feeding, the membranellar band creates a fluid flow to bring food to the anterior end of the cell, where it is engulfed through the oral pouch.

Regeneration in *Stentor coeruleus* can be induced by sucrose shock [1]. This leads to the shedding of the oral apparatus, which is comprised of the oral pouch and membranellar band (Fig. 1A). After sucrose shocking, *Stentor* look tear-drop shaped and stay stationary for approximately 3 h. After 3 or 4 h of regeneration, *Stentor* begins to form a

membranellar band in the middle of the cell body, initially oriented parallel to the body axis. The membranellar band grows simultaneously towards the top and bottom of the cell. At the top of the cell, the membranellar band will continue growing across the top. After 6 or 7 h of regeneration, the posterior end of the membranellar band will begin to curl to form the oral pouch and a physical indentation of the cell surface can be seen. Within the last 2 h of regeneration, the oral pouch will be moved to the top of the cell along with the membranellar band. *Stentor* usually completes regeneration within 8 h.

The process of regeneration shows striking similarities to the process of cell division. When a *Stentor* cell divides asymmetrically along its vertical axis, the anterior daughter cell retains the oral apparatus from the mother cell and the posterior daughter inherits a *de novo* oral apparatus that forms just prior to cytokinesis. This *de novo* creation of an oral apparatus during regeneration proceeds through a series of morphological steps virtually identical to those seen during the creation of a new oral pouch during division [1], namely, the formation of a membranellar band parallel to the body axis, curling of the band, and formation of the oral pouch. During division, the macronucleus undergoes a dramatic shape change from a moniliform string of small spherical nodes into a short tube, when then re-elongates just prior to mitosis. This same nuclear shape change also takes place during regeneration, further suggesting a similarity of the two processes [2].

The similarity between regeneration and division has also been reported at the transcriptional level, based on studies of the RNA transcriptome during regeneration. Genes encoding elements of the cell division and cell cycle regulatory machinery, including Aurora kinases, are differentially expressed during the later stages of regeneration compared to the earlier stages of regeneration [3]. Such similarities suggest that there may be some common regulatory mechanisms involved in both regeneration and cell division. Since Aurora kinase signaling indicates that a spindle is properly assembled [4], a similar mechanism could be at work in *Stentor* to signal the correct assembly of one or more structures during regeneration. But it is also possible that the similarity has nothing to do with regeneration and instead plays some other role. For example, the micronuclei undergo mitosis during both cell division and regeneration [5], so perhaps the transcriptional changes in cell cycle-related genes have only to do with the micronuclear mitosis and not regeneration itself. However, if the cell cycle machinery really does play a role in regeneration, then inhibition of proteins that regulate the timing of cell division may also affect the timing of regeneration in *Stentor*. Here we show that two well-characterized Aurora kinase A+B inhibitors slow or stop regeneration in *Stentor*, providing the first direct experimental evidence that *Stentor* may harness the cell division machinery to regulate the sequential process of regeneration.

Objective

The general objective is to learn whether regeneration and division may harness conserved molecular mechanisms. The specific objective is to test whether inhibition of the Aurora kinases, well-known regulators of cell division, alters the process of oral apparatus regeneration in *Stentor*.

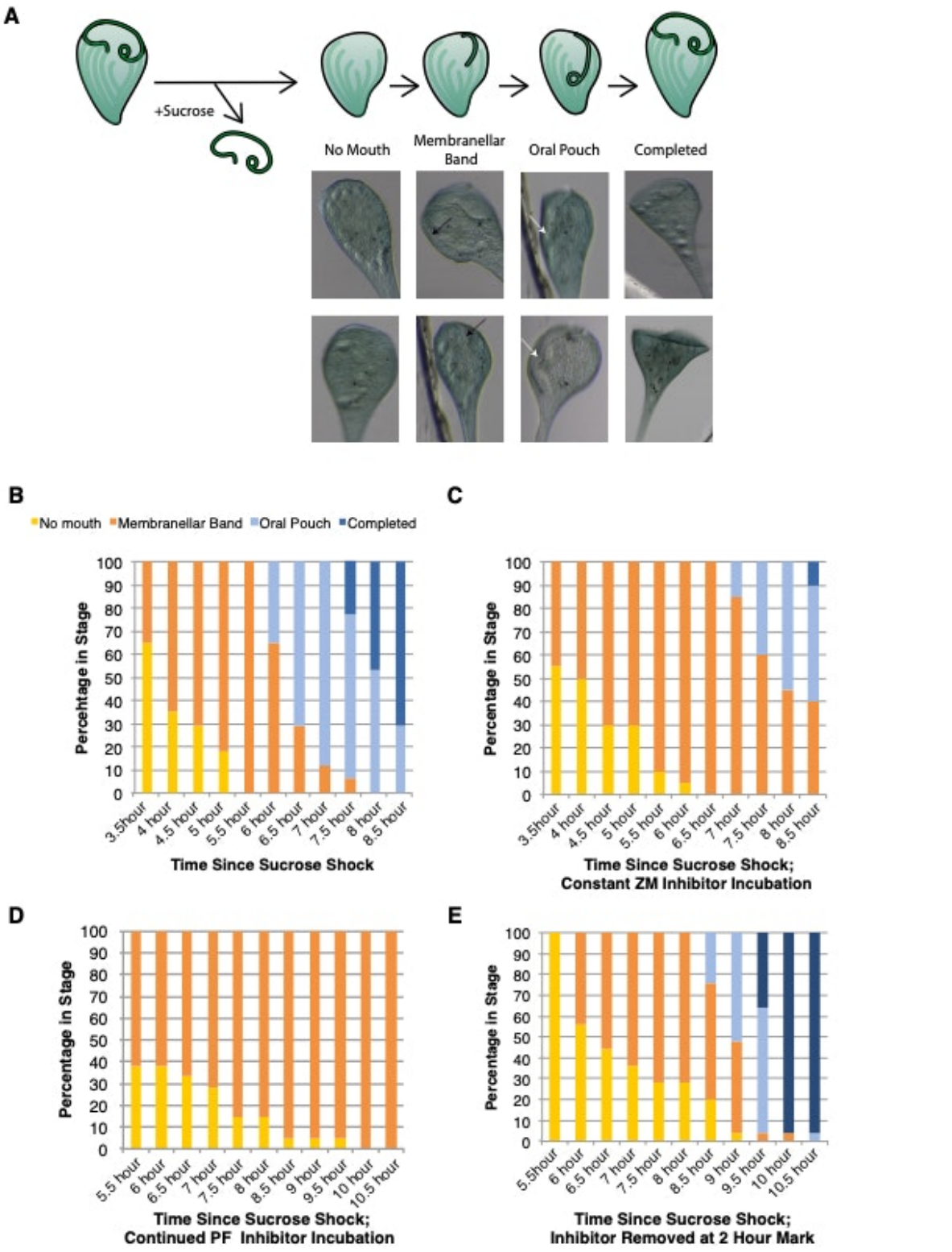

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Figure Legend

Figure 1. Aurora kinase A+B inhibitors slow or stop regeneration.

(A) *Stentor* exhibits three, distinct, chronological, morphologies during regeneration of the oral apparatus. After sucrose shocking, they first adopt a tear-drop shape, then form a membranellar band (black arrow) parallel to their body axis. Next, they form an oral pouch (white arrow) at the posterior end of the membranellar band. Finally, they move the oral pouch to the top of the cell. At each stage, photos of two different individuals are shown in each column.

(B) Under normal conditions, *Stentor* needs approximately 8 h to regenerate. After about 3 h a membranellar band starts to appear, and after another 3 h the oral pouch becomes visible, after which 2 more hours are spent moving the membranellar band and the pouch to the correct position to complete regeneration. Data shown are from 27 control cells.

(C) Aurora kinase A+B inhibitor, ZM447439, has little effect on the first phase of regeneration, formation of the membranelle band, but dramatically slows down the second phase of regeneration, formation of the oral pouch. Data shown are from 25 cells treated with ZM447439.

(D) Aurora kinase A+B inhibitor, PF03814735, permits the formation of the membranellar band but completely blocks regeneration at the stage of oral pouch formation. Data shown are for 21 cells treated with PF03814735.

(E) Aurora kinase A+B inhibitor, PF03814735, can be removed and regeneration occurs subsequently within 8 h. Data shown are for 25 cells treated with PF03814735 followed by washout.

Results & Discussion

Compared to the timing of events in untreated cells (Fig. 1B), the addition of Aurora kinase A+B inhibitor ZM447439 [4] caused oral apparatus regeneration to be delayed by at least 1 h. 10% of treated cells did not form a membranellar band until 4 h into regeneration (Fig. 1C). Treated cells spent more time forming a membranellar band and the first oral pouch did not appear until 7 or 8 h after starting regeneration, compared to untreated cells where oral pouches appear in the 6.5 h time point. The first fully regenerated *Stentor* did not form until 8 and a 0.5 h later (Fig. 1C). We have observed the same pattern of delay 3 times in separate experiments (data not shown). The difference in the fraction of cells with a formed oral pouch at 6 h and completed regeneration at 8 h is highly significant (P=0.0018 and P=0.0001, respectively, by Fishers Exact Test).

Although ZM447439 is known to be a highly specific inhibitor of Aurora kinases in mammalian cells, any chemical inhibitor can show off-target effects, especially when applied in a different cell type. One type of off-target effect would be a fortuitous interaction of a drug to another target via a binding interaction that had no relation to the binding modality used with the intended target. This type of off-target interaction is less likely to be seen if one tests a second drug with a distinct chemical structure. To rule out this type of off-target effect and confirm our result that Aurora inhibition delays regeneration, we tested a second highly specific and reversible Aurora kinase A+B inhibitor, PF03814735 [6], which has a different chemical structure from ZM447439, and which also has the advantage of being reversible in many systems. We found that with this inhibitor, regeneration was suspended at the membranellar band stage (Fig. 1D). 38% of *Stentor* still had no oral pouch by 6 h, and none of the Stentor had regenerated by 10 and a 0.5 h. Regeneration was paused at the membranellar band stage for the duration of the experiment. As with ZM447439, in cells treated with PF03814735 the difference in the fraction of cells with a formed oral pouch at 6 h and completed regeneration at 8 h is statistically significant (P=0.003 and P=0.0004, respectively, by Fishers Exact Test). The fact that a second Aurora inhibitor whose chemical structure is different from ZM447439 shows delay in progression at similar stages of regeneration suggests that the delay of regeneration with ZM447439 may not have been an off-target effect, but our results cannot rule out an off-target effect that involves a binding site resembling the site present

on Aurora kinase itself.

PF03814735 is reversible in other systems, therefore we questioned whether the block on regeneration could be reversed after the inhibitor is removed. After a 2 h incubation and 3 subsequent washes, *Stentor* were able to regenerate in a timely fashion, forming membranellar bands after 5 h, oral pouches after 7 h and fully regenerating in 10 h (Fig. 1E).

Our results indicate that Aurora kinase function may normally be required to drive a specific step of oral apparatus regeneration that takes place after the membranellar band has formed but before it moves to the anterior of the cell and forms an oral pouch. Such a temporal requirement is reminiscent of the requirement of Aurora kinases for specific stages of mitotic progression. As with the cell cycle, the ability to reversibly arrest regeneration and then analyze the timing of events after the arrest is alleviated may, in the future, provide a way to determine whether regeneration is timed by a series of domino-like events, each triggering the next, or a master clock like that used in the cell cycle. Actinomycin D [7], puromycin [7], concanavalin A [8] and DNA synthesis inhibitors [9] have previously been shown to affect cell growth or regeneration in *Stentor*, but the Aurora inhibitors reported here have the advantage that they target a very specific signaling pathway.

Conclusions

Our results indicate that regeneration of the oral apparatus in Stentor takes place in two separately regulated steps, with Aurora kinase possibly regulating the second step. These results support the idea that regeneration of the oral apparatus in *Stentor* is regulated by components of the cell division machinery, suggesting that the similarity between the two processes is more than just a superficial coincidence. These small molecule inhibitors provide new tools to perturb the process and study its effects.

Limitations

Bioinformatic analysis of the Aurora kinase family in *Stentor* indicates that there are 44 different Aurora kinases [10], and that these cannot be clearly mapped onto the Aurora classes A, B, and C in mammals. Consequently, it is not currently clear which of the Aurora kinases in *Stentor* is actually being affected by the inhibitors during regeneration. We also note that because both Aurora kinase inhibitors were developed to target mammalian kinases, and have not to our knowledge been previously tested in ciliates, hence the possibility that the drugs have off-target effects in *Stentor* cannot be excluded.

Alternative Explanations

Conjectures

Both mitosis and regeneration proceed through a series of distinct steps that must take place in the correct order, and each step must not start until the preceding steps are completed. We conjecture that the cell cycle machinery, which has evolved to regulate the sequential steps of division, may provide the necessary timing and order of events that allows proper regeneration. For example, regeneration might require a series of checkpoints, one of which is mediated by Aurora signaling. Early observations of washing out the competitive inhibitor suggested that subsequent events took place more synchronously. However, measurements of regeneration timing will be needed to confirm this impression.

Additional Information

Methods

Sucrose shock

Sucrose shock was performed according to [1]. Cells were gathered by pipette individually and washed with pasteurized spring water (PSW; Carolina Biological Supply). An equal volume of 25% (w/v) sucrose was added to cells in PSW to give a final concentration of 12.5% sucrose. Cells were incubated for approximately 3 min or until the membranellar band was shed. Sucrose was then diluted 50x by the addition of PSW. After 20–30 min, cells that have rounded up (indicating imminent death) or that still had membranellar bands present were discarded.

Identification of stages

Cells were examined at 30 min or 1 h- intervals, using a Zeiss Stemi 2000 at 5X to identify cells that retained a non-spherical shape. 3 h after sucrose shock, the presence of a membranellar band was assessed by looking for a faint band of randomly beating cilia in the middle of the cell. Since these may be facing away from the camera lens, it was important to look at the other side of *Stentor*. If the cells had been starved, the membranellar band was more likely to be visible through the cell. To locate the oral pouch, the most posterior part of the membranellar band was examined for the presence of an indentation that represents the oral pouch. It was observed that immediately before the oral pouch first appeared, the membranellar band began curling. Cells were considered to have completed regeneration if the oral pouch was present and the membranellar band had migrated to the anterior end of the cell. Bar graphs in figure 1B–E depict the distribution of regeneration stages as a function of time. The number of cells analyzed in the 4 panels was 27 (control), 25 (ZM447439), 21 (PF03814735), and 25 (ZM447439 with washout). Percentages were obtained by dividing the number of cells at each stage of regeneration by the total number of cells in each experiment. Statistical analysis was performed using Fishers Exact test. Experiments were done at 21℃ in room light.

Inhibitor treatment

The inhibitors ZM447439 and PF03814735, purchased from Selleck Chemicals, were dissolved in DMSO at concentrations of 5.0 mM and 2.1 mM respectively. These stock solutions were then diluted to final concentrations of 0.1 nM and 42 pM in wells containing *Stentor* cells in PSW.

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Ethics Statement

Not Applicable.

Citations

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