Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Short Communication

The abrogation of condensin function provides independent evidence for defining the self-renewing population of pluripotent stem cells

Alvina G. Lai[1], Nobuyoshi Kosaka[1], Prasad Abnave, Sounak Sahu, A. Aziz Aboobaker*

Department of Zoology, University of Oxford, Tinbergen Building, South Parks Road, Oxford OX1 3PS, United Kingdom

ARTICLE INFO

Keywords:
Pluripotency
Condensins
Endoreplication
Endocycling
RNAi
Self-renewal

ABSTRACT

Heterogeneity of planarian stem cells has been categorised on the basis of single cell expression analyses and subsequent experiments to demonstrate lineage relationships. Some data suggest that despite heterogeneity in gene expression amongst cells in the cell cycle, in fact only one sub-population, known as sigma neoblasts, can self-renew. Without the tools to perform live in vivo lineage analysis, we instead took an alternative approach to provide independent evidence for defining the self-renewing stem cell population. We exploited the role of highly conserved condensin family genes to functionally assay neoblast self-renewal properties. Condensins are involved in forming properly condensed chromosomes to allow cell division to proceed during mitosis, and their abrogation inhibits mitosis and can lead to repeated endoreplication of the genome in cells that make repeated attempts to divide. We find that planarians possess only the condensin I complex, and that this is required for normal stem cell function. Abrogation of condensin function led to rapid stem cell depletion accompanied by the appearance of 'giant' cells with increased DNA content. Using previously discovered markers of heterogeneity we show that enlarged cells are always from the sigma-class of the neoblast population and we never observe evidence for endoreplication for the other neoblast subclasses. Overall, our data establish that condensins are essential for stem cell maintenance and provide independent evidence that only sigma-neoblasts are capable of multiple rounds of cell division and hence self-renewal.

1. Introduction

Throughout the lifespan of a multicellular organism with an adult lifespan of months or years, cells in many tissues will turn over, requiring mechanisms in place to control this tissue homeostasis. This requires stem cells of some kind that have the ability to self-renew, proliferate and differentiate into specialised cells for specialised tissue structures. Many animals appear to use germ layer and lineage specific tissue resident stem cells for these processes (Reddien and Alvarado, 2004; Tanaka and Reddien, 2011). Although the potency of such cells in animals varies greatly, most have limited potency. Planarians, however, have at least some individually pluripotent stem cells amongst the broader cycling population of adult stem cells (Wagner et al., 2011), collectively called neoblasts (NBs) (Aboobaker, 2011; Rink, 2013; Ross et al., 2017). NBs give planarians their ability to regenerate any missing tissue structure from small fragments and respond with startling homeostatic plasticity to changing nutritional status (Reddien and Alvarado, 2004; Saló, 2006; González-Estévez et al., 2012a). A current broad definition of NB is that they represent all cells that are actively cycling, and can be labelled with fluorescent in situ hybridization (FISH) markers such as *smedwi-1* (Reddien et al., 2005) and *histone2B (H2B)* (Guo et al., 2006; Solana et al., 2012), which act as pan-NB markers at the transcript level.

A number of studies have assayed the genes expressed in NBs and other planarian cells at the whole population level (Solana et al., 2012; Blythe et al., 2010; Labbé et al., 2012; Önal et al., 2012; Kao et al., 2013, 2017) and more recently at the single-cell level (van Wolfswinkel et al., 2014; Wurtzel et al., 2015, 2017; Issigonis and Newmark, 2015; Molinaro and Pearson, 2016; Scimone et al., 2016). This has led to the definition of NB subtypes based on gene expression profiles and revealed that planarians have at least three major subclasses of *smedwi-1*+ NBs. These *smedwi-1*+ subclasses were defined based on the expression of specific groups of transcription factors, with sigma-NBs expressing higher levels of *soxP-1* and *soxP-2*, zeta-NBs preferentially expressing *zfp-1 and soxP-3* and gamma-NBs expressing *gata4/5/6* and *hnf-4* (van Wolfswinkel et al., 2014). Zeta-NBs and gamma-NBs likely represent progenitors to restricted lineages that descend from sigma-NB, with zeta-NBs giving rise to the epidermal

* Corresponding author.
E-mail address: Aziz.Aboobaker@zoo.ox.ac.uk (A.A. Aboobaker).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.ydbio.2017.07.023
Received 28 May 2017; Received in revised form 18 July 2017; Accepted 27 July 2017

layer and gamma-NBs probably giving rise to intestinal cells (van Wolfswinkel et al., 2014). In the case of *zinc finger protein 1* (*zfp1*), as well as being a definitive FISH marker of zeta-NBs it is also reported, through *zfp1 (RNAi)* experiments, as being required for the transition from sigma-NBs to zeta-NBs (van Wolfswinkel et al., 2014). Evidence from this study, in particular for genes that define zeta-NB expression are co-expressed in newly minted post-mitotic epidermal progeny and that they increase in expression in NB over the course of S-phase, suggested that zeta-NBs may pass through mitosis once to give rise to post-mitotic daughter cells (van Wolfswinkel et al., 2014). This was also supported by the observation that changes in proliferation in response to amputation only impacted sigma-NBs, suggesting that only sigma-NBs, and not other classes of NBs, were self-renewing.

Here, we aimed to provide further independent evidence of sigma-NBs being the only NBs capable of multiple rounds of cell divisions and therefore self-renewal. Without the ability to perform transgenesis and in vivo lineage tracing to follow stem cell development in planarians, we employed a molecular genetics approach to target proteins that have pivotal roles in regulating cell division that might reveal which cells self-renew and which do not. This led us to study the role of planarian condensins.

Condensins are conserved multi-protein complexes essential for chromosome organization, assembly and segregation (Hirano, 2012, 2016). As the mode of action of condensins is specific to M-phase of mitosis or meiosis, perturbation of condensins should only affect cells passing through these cell cycle stages. Two condensin complexes, condensin I and II, have been identified in many animals. Both condensin I and II complexes share the structural maintenance of chromosomes (SMC) 2 and SMC4 subunits (Hirano, 2016) and each complex has its own unique non-SMC subunits: i.e., non-SMC condensin I complex subunit G (NCAPG), non-SMC condensin I complex subunit D2 (NCAPD2) and non-SMC condensin I complex subunit H (NCAPH) for condensin I and similarly NCAPG2, NCAPD3 and NCAPH2 for condensin II (Hirano, 2012).

Previous reports have shown that the perturbation of condensins leads to drastic defects in proliferating cells. In plants, abrogation of condensins resulted in enlargement of endosperm nuclei (Liu et al., 2002). Loss of condensin I in zebrafish causes chromatid segregation defects in neural retina progenitors and polyploidization (Seipold et al., 2009). In mammalian embryonic stem (ES) cells, simultaneous depletion of both condensin I and II resulted in the accumulation of enlarged interphase nuclei (Fazzio and Panning, 2010). Although complete elimination of condensin proteins appear to have lethal effects, reduction in condensin levels may be tolerated to allow functional studies on surviving cells (Gosling et al., 2007; Longworth et al., 2008; Murakami-Tonami et al., 2014; Nishide and Hirano, 2014; Frosi and Haering, 2015). The function of condensins in planarian NBs is not known. In light of this, we sought to confirm the contribution of condensins to NB proliferation. We reasoned that condensin depletion may lead to endoreplication of cells that remain in the cell cycle and self-renew. Cells that do not pass through mitosis but leave the cell cycle and differentiate would be depleted, as their own populations are not renewed by differentiation of daughter progeny from self-renewing stem cells.

We demonstrate that condensin I genes in the model planarian *Schmidtea mediterranea* have enriched expression in stem cells and are essential for tissue homeostasis and regeneration. RNA interference (RNAi)-mediated knockdown of all five condensin subunits resulted in a drastic decline in NBs. Remaining NBs positive for the stem cell marker *smedwi-1* or *H2B* in RNAi animals are often morphologically enlarged and have increased DNA content. These enlarged NBs are only ever positive for the sigma-class NB marker and never the zeta- or gamma-class markers. Enlarged sigma-NBs have increased DNA content but are non-mitotic, indicating that these cells may have undergone endocycling as a result of condensin depletion. Our results provide independent evidence that sigma-NBs are the only population of *smedwi-1*+ve stem cells capable of multiple rounds of cell division and hence self-renewal in *S. mediterranea*.

2. Materials and methods

2.1. Phylogenetic analyses

S. mediterranea condensin orthologs were identified by tBlastn against the planarian transcriptome and genome (Robb et al., 2008, 2015) using condensin protein sequences from *Drosophila melanogaster*, *Homo sapiens* and *Mus musculus* as queries. Condensins from other flatworm species (*Schmidtea polychroa, Planaria torva, Dendrocoelum lacteum, Polycelis nigra* and *Polycelis tenuis*) were obtained from Planmine (Brandl et al., 2016). Multiple sequence alignments of condensin proteins were performed using MAFFT (Katoh et al., 2009) followed by phylogenetic tree generation using RAxML (Stamatakis, 2014) using the gamma WAG model with 1000 bootstrap replicates. The best-scoring maximum likelihood tree was generated. The tree figure was made using Geneious (Kearse et al., 2012). Complete list of transcript sequences obtained are listed in Dataset S1.

2.2. Cloning of condensin family genes

S. mediterranea condensin family genes identified above were cloned into the double-stranded RNA expression vector (pT4P) as previously described (Rink et al., 2009). Colony PCR was performed using the M13 forward and reverse primers followed by Sanger sequencing using the AA18 or PR244 primer. Complete list of primer sequences used for PCR and cloning are listed in Table S1.

2.3. Animal culture

Asexual *S. mediterranea* animals were cultured at 20 °C in 1X Montjuic salts (Cebrià and Newmark, 2005). The 1X Montjuic salt solution was prepared using milliQ ddH2O with the following composition: 1.6 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 mM $MgCl_2$, 0.1 mM KCl, 1.2 mM $NaHCO_3$. The worms were fed with organic beef liver once a week and were starved for 1 week prior to any experimental procedures to minimize non-specific background from gut contents. Animals were kept in the dark at all times apart from during feeding and water changing.

2.4. RNA interference (RNAi) by injection

All RNAi experiments were performed on 3–4 mm worms, according to procedures previously described (Felix and Aboobaker, 2010). Control RNAi was performed by injecting worms with double stranded (ds) RNA encoding for green fluorescent protein (GFP) that is not present in *S. mediterranea*. Condensin dsRNA was prepared by in vitro transcription of the gene amplified from the pT4P vector mentioned above with primers listed in Table S1. The GFP gene was amplified from the pGEMT-GFP plasmid for use as control during RNAi (Solana et al., 2012). PCR products were purified using the Wizard SV Gel and PCR clean-up kit (Promega). This was then followed by in vitro transcription using T7 RNA polymerase (Roche). The in vitro transcribed dsRNAs were treated with Turbo DNAse (Ambion), lithium chloride precipitated in the presence of glycogen and re-suspended in molecular grade water to a final concentration of 2 mg/mL. 1 week starved animals were injected using the Nanoject II microinjector (Drummond Scientific, Oxford, UK) for three consecutive days in the first week and another three consecutive days in the second week. Worms were monitored throughout the procedure and day 1 post RNAi in all experiments is considered to be the first day after the sixth dsRNA injection. All experiments were performed in triplicates and at least 10 worms were used for each time point.