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Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance

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ABSTRACT

Planarian regeneration depends on the presence and precise regulation of pluripotent adult somatic stem cells named neoblasts, which differentiate to replace cells of any missing tissue. A characteristic feature of neoblasts is the presence of large perinuclear nonmembranous organelles named "chromatoid bodies", which are comparable to ribonucleoprotein structures found in germ cells of organisms across different phyla. In order to better understand regulation of gene expression in neoblasts, and potentially the function and composition of chromatoid bodies, we characterized homologues to known germ and soma ribonucleoprotein granule components from other organisms and analyzed their function during regeneration of the planarian *Dugesia japonica*. Expression in neoblasts was detected for 49 of 55 analyzed genes, highlighting the prevalence of post-transcriptional regulation in planarian stem cells. RNAi-mediated knockdown of two factors [*ago-2* and *bruli*] lead to loss of neoblasts, and consequently loss of regeneration, corroborating with results previously reported for a *bruli* ortholog in the planarian *Schmidtea mediterranea* (Guo et al., 2006). Conversely, depletion mRNA turnover factors [*edc-4* or *upf-1*], exoribonucleases [*xrn-1* or *xrn-2*], or DEAD box RNA helicases [*Djcbc-1* or *vas-1*] inhibited planarian regeneration, but did not reduce neoblast proliferation or abundance. We also found that depletion of cap-dependent translation initiation factors eIF-3A or eIF-2A interrupted cell cycle progression outside the M-phase of mitosis. Our results show that a set of post-transcriptional regulators is required to maintain the stem cell identity in neoblasts, while another facilitates proper differentiation. We propose that planarian neoblasts maintain pluripotency by employing mechanisms of post-transcriptional regulation exhibited in germ cells and early development of most metazoans.

Introduction

Regulation of eukaryotic gene expression at the level of messenger RNA translation, localization, and stability is vital for accurate early development, germ line formation, and neuronal plasticity (reviewed in Dever, 2002; Kimble and Crittenden, 2007; Martin et al., 2000; Wickens et al., 2000). Subsets of mRNA, and the post-transcriptional regulators that determine their fate, often aggregate in a number of different microscopically visible cytoplasmic foci (reviewed in Anderson and Kedersha, 2009a; Balagopal and Parker, 2009). Different outcomes are expected for mRNAs captured in different ribonucleoprotein aggregates (RNPs). In somatic cells, processing bodies (P bodies) are associated with repressed and decaying mRNAs, whereas translationally stalled mRNAs are captured in stress granules (reviewed in Anderson and Kedersha, 2009a,b; Balagopal and Parker, 2009; Eulalio et al., 2007; Ikenishi, 1998; Parker and Sheth, 2007). In the process of memory formation, mRNA-transporting neuronal granules mediate localized translation in response to synaptic stimuli (Kiebler and Bassell, 2006; Kwak et al., 2008; Martin et al., 2000; Richter, 2001). Whereas germ plasm, polar granules, germinal granules, and mammalian chromatoid bodies are RNP granules present in the germ line and early embryo that contain mRNA and small non-coding RNAs for localized processing and future specification (reviewed in Chuma et al., 2009; Ikenishi, 1998; Kotaja and Sassone-Corsi, 2007; Parvinen, 2005).

In order to analyze post-transcriptional regulation in somatic stem cells, we decided to study mRNA regulation in the planarian *Dugesia japonica*. Freshwater planarian flatworms are well-known models for understanding regeneration and stem cell regulation (reviewed in Agata, 2003; Agata and Umesono, 2008; Newmark and Sanchez Alvarado, 2002; Pearson and Sanchez Alvarado, 2008; Rossi et al., 2008). Almost any fragment of a planarian can regenerate into a complete organism including a well-organized brain, a pharynx, an

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intestine, ovaries, testis and copulatory organs (Agata, 2003; Agata and Watanabe, 1999; Newmark and Sanchez Alvarado, 2002; Newmark et al., 2008; Reddien and Sanchez Alvarado, 2004; Umesono and Agata, 2009). Their regenerative ability, which is exhibited during asexual reproduction and after lesion, is dependent on pluripotent adult stem cells, called neoblasts, which comprise roughly 25% of total cells in their body (Newmark and Sanchez Alvarado, 2000; Salo and Baguna, 1984). Neoblasts are also the only cells capable of proliferation in planarians and supply cells for replacement of older and damaged cells for tissue homeostasis (Newmark and Sanchez Alvarado, 2000; Orii et al., 2005). Recently, the abundance of neoblasts in planarians and their exclusive ability to proliferate was confirmed by fluorescence-activated cell sorting (FACS; Hayashi et al., 2006; Higuchi et al., 2007), a technique that has successfully been coupled with RT-PCR to analyze transcriptomes at the single-cell level (Hayashi et al., 2010).

An unambiguous feature of neoblasts is the presence of large perinuclear RNP granules known as "chromatoid bodies" (Coward, 1974; Hori, 1982; Le Moigne, 1967; Morita, 1967; reviewed in Shibata et al., 2010). These RNP granules received their name because of ultrastructural similarities to chromatoid bodies present in mammalian spermatocytes and spermatids. The precise function of chromatoid bodies in neoblasts is currently unknown, but some insight was recently obtained by identification of two components: DjCBC-1 (*D. japonica chromatoid body component-1*; Yoshida-Kashikawa et al., 2007) and SpolTud-1 (Solana et al., 2009). DjCBC-1 is a member of the highly conserved family of RCK/Xp54/Me31B DEAD box RNA helicases (Yoshida-Kashikawa et al., 2007), which are components of multiple RNP particles involved in diverse biological processes (reviewed in Eulalio et al., 2007; Weston and Sommerville, 2006). These helicases are thought to remodel RNP complexes to invoke nuclear export, decay, and entry into translation (reviewed in Weston and Sommerville, 2006), but are best known for their function in translational repression (Coller and Parker, 2005; Minshall et al., 2009; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001). SpolTud-1 is a Tudor domain-containing protein shown to be required for long-term maintenance of neoblasts in the related planarian species *Schmidtea polychroa* (Solana et al., 2009). Tudor domain containing proteins are important for germ plasm formation in flies (Boswell and Mahowald, 1985), as well as chromatoid body assembly, regulation of miRNA expression, retrotransposon silencing, and spermiogenesis in mice (Chuma et al., 2006; Shoji et al., 2009; Vasileva et al., 2009).

In addition to the presence of chromatoid bodies, several lines of evidence indicate the existence of heavy post-transcriptional regulation in neoblasts. Homologues of a few germ line post-transcriptional regulators have been shown to be indispensable for regeneration and adult somatic stem cell maintenance in planarians (Guo et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005a,b; Salvetti et al., 2005). *DjPum*, a member of the PUF-domain family of 3′UTR-binding proteins (Wickens et al., 2002); *Smedbruli*, a bruno-like family protein; and previously mentioned Tudor homologue *SpolTud-1*, are all required for planarian regeneration and neoblast maintenance (Guo et al., 2006; Salvetti et al., 2005). In addition, several members of the PIWI sub-family of Argonaute proteins are predominantly expressed in neoblasts and essential for precise cell differentiation and stem cell maintenance (*Smedwi-1*, *-2* and *-3*; Palakodeti et al., 2008; Reddien et al., 2005b). Finally, recurrent enrichment of RNA-binding proteins and other post-transcriptional and translational regulators in unbiased analyses of neoblast transcriptomes, once again suggest that planarian stem cells are under heavy post-transcriptional regulation (Eisenhoffer et al., 2008; Rossi et al., 2007; Shibata et al., 1999; Yoshida-Kashikawa et al., 2007).

In an attempt to learn more about the regulatory events that mediate pluripotency and stability of neoblasts, we systematically analyzed 55 *D. japonica* homologues of genes implicated in the function or stability of RNP granule mediated post-transcriptional regulation. We found that the genes analyzed are predominantly expressed in neoblasts, indicative of prevalent post-transcriptional regulation in adult planarian stem cells. Analysis of regeneration and stem cell viability in asexual animals revealed unexpected outcomes in response to depletion of different post-transcriptional regulators. Disrupting expression of *Djago-2*, or *Djbruli*, caused loss of neoblasts and consequently regeneration, whereas knockdown of mRNA turnover factors DjEDC-4/Hedls, DjUPF-1 or DjXRN-1, as well as homologues of DEAD box RNA helicases RCK/Me31b/Xp54 and VASA, inhibited planarian regeneration without reducing neoblast maintenance. Disruption of "house-keeping" factors, such as canonical cytoplasmic poly(A)-binding protein and translation initiation factor eIF4A, which are ubiquitously expressed and required for viability in other organisms, lead to lesions and rapid lysis in intact planarians. Conversely, knockdown of translation initiation factors DjeIF2A or DjeIF3A homologues whose expression was primarily in neoblasts, caused cessation of mitosis but no noticeable defects in the soma or loss of neoblast identity. Our results demonstrate that planarian neoblasts maintain pluripotency by employing mechanisms of post-transcriptional regulation manifested in germ cells and early development of most metazoans (Ewen-Campen et al., 2010), and supports the notion that different translation initiation mechanisms are employed at different stages of the cell cycle (reviewed by Sivan and Elroy-Stein, 2008).

Methods

Animals

Planarians (*D. japonica*) of asexual SSP3 strain were used in all experiments (Ito et al., 2001). Planarians of 8–10 mm in length were used for regeneration experiments. Planarians of 5–6 mm in length were used for *in situ* hybridization and 20-day RNAi experiments of intact planarians. Planarians were maintained in autoclaved tap water at 23 °C, and starved for at least 7 days prior to any procedure.

X-ray irradiation

Groups of 30 planarians were placed on three sheets of wet filter paper on ice and irradiated with 100 R of X-rays, using an X-ray generator (SOFTEX B-4; SOFTEX, Tokyo, Japan).

Whole-mount in situ hybridization and immunofluorescence

Samples were killed with 4 °C 2% HCl in 5/8 Holtfreter's solution, vigorously agitated for 5 min, and fixed in 4% paraformaldehyde containing 5% methanol for 2 h in motion at 4 °C. Hybridization was performed using digoxigenin-labeled complementary RNA probes, to cDNA clones in their entirety, as previously described (Agata et al., 1998; Umesono et al., 1997). For immunofluorescence, fixed samples were washed with cold methanol, bleached overnight in methanol with 6% H_2O_2 under fluorescent light, treated in 1:1 methanol/xylene for 30 min at 4 °C, rehydrated and incubated with 10% goat serum in 0.1% Triton in PBS (TPBS) for 2 h, and then with primary antibodies (1:1000 dilution in 10% goat serum TPBS) overnight at 4 °C. Samples were then washed extensively in TPBS and incubated overnight with secondary antibody Alexa Fluor 488 and/or Alexa Fluor 594 (2 μg/ml final concentration, Molecular Probes, Eugene, OR) and Hoechst 33342 (Sigma, St. Louis, MO) at 4 °C, washed four times, mounted in mounting medium (DakoCytomation, Carpinteria, CA), and analyzed in an Olympus FluoView FV10i (Olympus, Japan) confocal microscope. The following antibodies were used: anti-DjCBC-1 and anti-DjPiwi-A (rabbit and mouse, respectively; Yoshida-Kashikawa et al., 2007), anti-phospho-histone H3 (Ser10) (rabbit; Millipore, Billerica, MA), anti-Djarrestin antibody (rabbit; Nishimura et al., 2007; Sakai et al., 2000).