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

Labib Rouhana [a,1], Norito Shibata [b], Osamu Nishimura [b], Kiyokazu Agata [a,*]

[a] Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan
[b] Laboratory for Biodiversity, Global COE Program, Division of Biological Science, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan

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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

* Corresponding author. Fax: +81 75 753 4203.
E-mail addresses: rouhana@life.illinois.edu (L. Rouhana), agata@mdb.biophys.kyoto-u.ac.jp (K. Agata).
[1] Current address: Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign (UIUC), C615 Chemical and Life Sciences Laboratory, 601 S. Goodwin, Urbana, IL 61801, USA.

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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).

Table 1
D. japonica homologues of RNP factors. Genes analyzed in this study and their mammalian homologues. Homology confidence and identity percentage (within amino acid length; parentheses) is shown. Reported association of to RNP granules in other organisms is noted (Component), as well as known functions (Function). Proteins whose RNP granule association has only been reported in non-somatic cells are highlighted (gray). Proteins known to localize to chromatoid bodies of planarian neoblasts are also indicated (bold font). Abbreviations: *D. japonica* (Dj); closest *Mus musculus* homolog (Mm homolog); RNA-Binding Protein (RBP); Nonsense Mediated Decay (NMD); mammalian chromatoid body (CB); planarian neoblast chromatoid body (nCB); neuronal granule (NG); P-body (PB); polar granule (PG); stress granule (SG); sponge body (SB). Asterisk (*) indicates *E*-value>1E-03. Superscript indicates reference for previously identified *D. japonica* gene or planarian ortholog. [1]Guo et al., 2006, [2]Yoshida-Kashikawa et al., 2007, [3]Salvetti et al., 1998, [4]Shibata et al., 1999, [5]Mochizuki et al., 2001, [6]Solana et al., 2009, [7]Solana and Romero, 2009, [8](*Smedwi-1*) Reddien et al., 2005a,b.

Dj Protein	Mm Homolog	Function	Component	Identity (# of AA)	E-value
[1]DjBruli	Bruno14	RBP	Nuage	30% (313)	1.00E-23
[2]BRUNOL-5	Brunol5	RBP	-	42% (395)	8.00E-68
[3]DjY1	Csda	RBP	SB	51% (95)	2.00E-22
[2]CSDA	Csda	RBP	SB	58% (89)	5.00E-21
ELAV	Elavl2	RBP	SG NG	65% (183)	5.00E-60
[2]FMRP-1	Fmr1	RBP	SG NG	32% (352)	5.00E-49
[2]G3BP	G3bp2	RBP	SG	32% (395)	4.00E-45
RBM-15	Rbm15	RBP	PB	52% (169)	3.00E-44
SMN	Smndc1	RBP	SG	30% (168)	1.00E-11
TDRD-1	Tdrd1	RBP	PG Nuage	21% (359)	2.00E-05
[2,6]DjTud-1	**Tdrkh**	**RBP**	**PG Nuage nCB**	**23% (471)**	**2.00E-25**
TIA-1	Tia1	RBP	PB SG	47% (216)	1.00E-56
TIAL-1	Tia1	RBP	PB SG	33% (281)	9.00E-33
[2]TIAL-2	Tia1	RBP	PB SG	27% (288)	3.00E-26
TIAL-3	Tia1	RBP	PB SG	36% (280)	5.00E-46
TIAL-4	Tia1	RBP	PB SG	38% (281)	2.00E-50
TIAL-5	Tia1	RBP	PB SG	37% (85)	7.00E-15
TNRC4	Tnrc4	RBP	-	43% (434)	1.00E-79
[2]PABPC-1	Pabpc1	poly(A) binding	SG	57% (342)	1.00E-139
PABPC-2	Pabpc4	poly(A) binding	SG	53% (167)	7.00E-35
[4,7]DjVlgA	D1Pas1	DEAD Helicase	PG Nuage CB	51% (604)	1.00E-159
[5]DjVAS-1	Ddx4 (Vasa)	DEAD Helicase	PG Nuage CB	41% (589)	1.00E-104
[2]DjCBC-1	**Ddx6 (p54)**	**DEAD Helicase**	**PB SG PG SB nCB**	**70% (411)**	**2.00E-178**
DICER-1	Dicer1	siRNA/miRNA pathway	CB	40% (244)	8.00E-45
[2,8]DjPiwi-A	Miwi	piRNA/retrotransposon silencing	CB	26% (805)	2.00E-69
AGO-2	Eif2c2(Ago2)	siRNA/miRNA pathway	PB CB	73% (838)	0.00E+00
XRN-1	Xrn1	Degradation- 5'-3' exoribonuclease	PB SG	33% (233)	1.00E-30
XRN-2	Xrn2	Degradation- 5'-3' exoribonuclease	-	67% (211)	1.00E-86
CNOT-6	Cnot6	Degradation- deadenylation	PB	42% (192)	3.00E-39
CNOT-7	Cnot7	Degradation- deadenylation	PB	40% (257)	6.00E-49
*DCP-1L	Dcp1a	Degradation- decapping	PB PG CB	31% (112)	0.14
DCPS	Dcps	Degradation- decapping	-	35% (178)	3.00E-25
*EDC-4	Edc4	Degradation- decapping	PB	33% (69)	0.006
*LSM-14	Lsm14b	Degradation- decapping co-activator	PB SG PG	25% (191)	0.07
LSM-6	Lsm6	Degradation- decapping co-activator	PB	83% (43)	8.00E-14
SMG-6	Smg6	NMD	PB	46% (203)	2.00E-44
*SMG-7	Smg7	NMD	PB	31% (182)	2.00E-03
UPF-1	Upf1	NMD/Translational regulator	PB	55% (274)	8.00E-79
UPF-2	Upf2	NMD	PB	31% (517)	5.00E-71
[2]eIF-4A3	Eif4a3	NMD/EJC- DEAD RNA helicase	NG PG	78% (390)	0.00E+00
eIF-2A	Eif2a	Translation initiation factor	SG NG	53% (275)	3.00E-74
eIF-3A	Eif3a	Translation initiation factor	SG	34% (773)	1.00E-128
[2]eIF-4A1	Eif4a1	Translation initiation factor	SG	69% (390)	1.00E-151
eIF-4G	Eif4g3	Translation initiation factor	SG	23% (649)	3.00E-29
eIF-5A	Eif5a	Translation initiation factor	PG	57% (147)	2.00E-41
eIF-4E	eIF4E	Translation initiation factor	PB PG NG	52% (132)	2.00E-37
GEMIN-5	Gemin-5	snRNP binding	PB	31% (123)	3.00E-08
DHX-8A	Dhx8	Nuclear export- DEAD helicase	splisosome	53% (265)	8.00E-76
DHX-8B	Dhx8	Nuclear export- DEAD helicase	splisosome	42% (278)	4.00E-86
DHX-8C	Dhx8	Nuclear export- DEAD helicase	splisosome	46% (310)	1.00E-109
TRAF-2A	Traf-2	TNF signal transduction	SG	30% (388)	7.00E-47
TRAF-2B	Traf-2	TNF signal transduction	SG	33% (296)	8.00E-54
KIF-19	KIF-19a	kinesin	-	50% (307)	2.00E-82
KIF-3A	KIF-3a	kinesin	CB	74% (67)	2.00E-21
KIF-3B	Kif-3b	kinesin	CB	76% (157)	5.00E-67

RNAi and regeneration experiments

Planarians were fed a mix of 4 µg *in vitro* synthesized dsRNA (synthesized using Ambion Megascript, Austin, TX) mixed with liquefied chicken liver and 0.55% Type IV agarose (Sigma) per group of 15 planarians. Feedings took place on day 1 (D1), D4 and D9 of the experiment, and amputations anterior and posterior to the pharynx on D10. Planarians were allowed to regenerate for 7 days, scored, and fixed. Groups observed to regenerate normally were amputated for a second time 10 days after the first amputation, allowed to regenerate for another 7 days, then scored and fixed. Most phenotypes in this study were observed after the second amputation.

For 20-day RNAi treatment for intact planarian experiments, planarians were fed *in vitro* synthesized RNA mixed with agarose and liver (as described above) once a week, for a total of three feedings in a 15-day period, and scored and fixed 20 days after the first feeding.

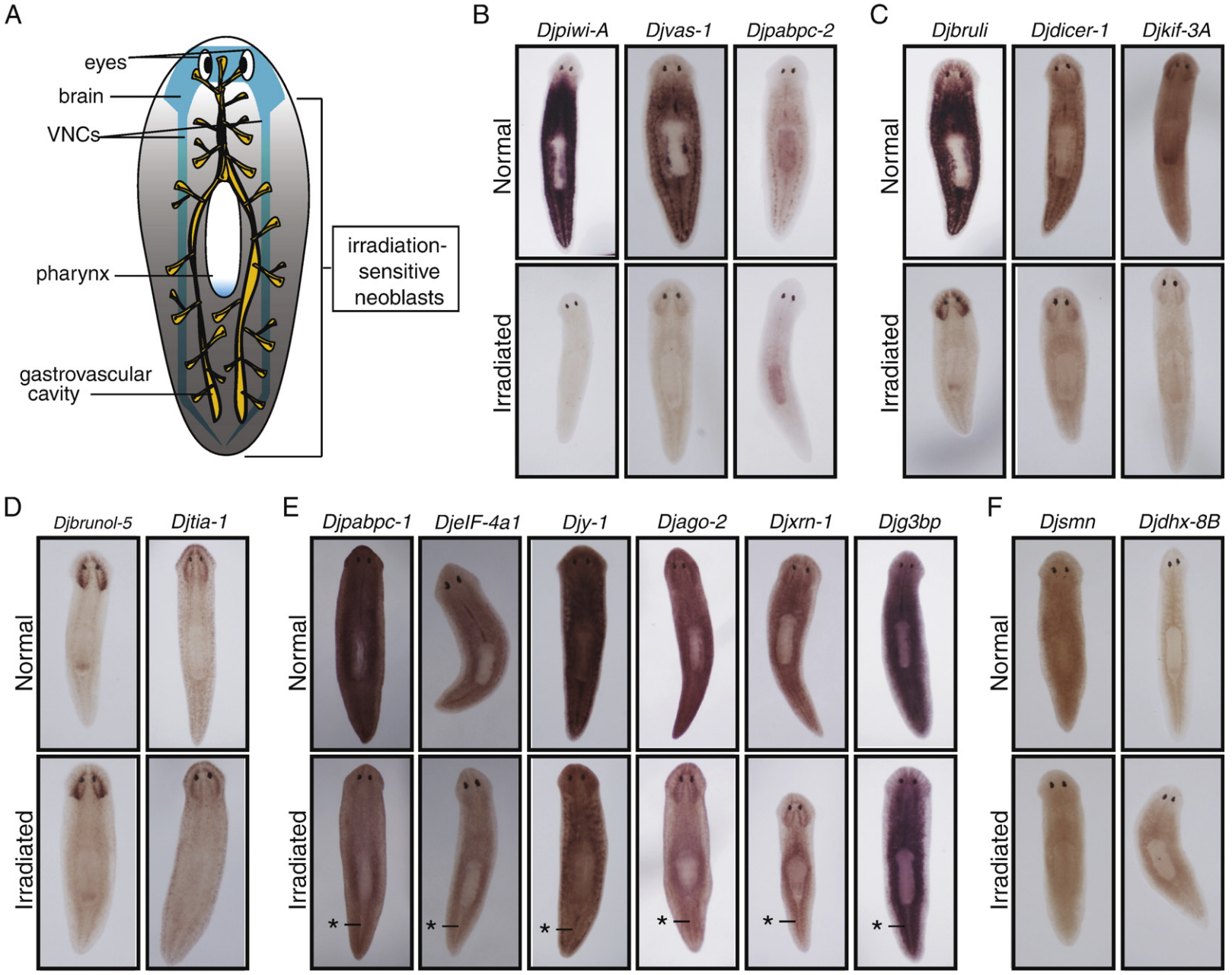
A
eyes
brain
VNCs
pharynx
gastrovascular cavity
irradiation-sensitive neoblasts
B
Djpiwi-A
Djvas-1
Djpabpc-2
Normal
Irradiated
C
Djbruli
Djdicer-1
Djkif-3A
Normal
Irradiated
D
Djbrunol-5
Djtia-1
Normal
Irradiated
E
Djpabpc-1
DjeIF-4a1
Djy-1
Djago-2
Djxrn-1
Djg3bp
Normal
Irradiated
F
Djsmn
Djdhx-8B
Normal
Irradiated

Table 2
Summary of observed phenotypes and expression distribution. List of genes from the RNAi study listed according to their regeneration phenotype and expression profile (A = Neoblasts, B = Neoblasts and CNS; and D = Neoblasts, CNS, and other). Abbreviations: Not determined (ND). Genes whose expression was restricted to the CNS (Type C: *brunol-5*, *elav*, *tia-1*, and *tnrc-4*), *piwi-A*, and *traf-2B*, were not included in our RNAi study. (*) An ortholog of *tud-1* (*SpolTud-1)* has been reported to be required for neoblast maintenance after RNAi treatment for longer periods than the ones used in this study (Solana et al., 2009).

Class	*Dj* Gene	Regeneration	Neoblasts	Mitosis	additional phenotype
A	*vas-1*	-	+	+	
	eIF-3a	-	+	-	
	eIF-2a	limited	+	-	
B	*bruli*	-	-	-	
	edc-4	-	+	+	
	dicer-1	-	ND	ND	
	cbc-1	limited	+	+	
	gemin-5	limited	+	+	
	eIF-4a3	lethal	ND	ND	
	kif-3a	+	+	ND	bloated
	kif-3b	+	+	ND	bloated
D	*ago-2*	-	-	-	
	upf-1	-	+	+	
	xrn-1	-	+	+	
	xrn-2	-	+	+	
	eIF-4a1	lethal	ND	ND	
	pabpc-1	lethal	ND	ND	
	g3bp	+	+	+	cone-head
	traf-2a	+	+	+	pleiotropic defects
	upf-2	+	ND	ND	pleiotropic defects

No phenotype observed				
A	**tud-1*	*eIF-4e*	*tial-2*	*lsm-6*
	tdrd-1	*eIF-4g*	*tial-3*	*csda*
	pabpc-2	*eIF-5a*	*tial-4*	*dcps*
	smg-6	*tial-1*	*tial-5*	*dhx-8a*
B	*DjvlgA*	*rbm-15*	*smg-7*	*dhx-8c*
	fmrp-1	*cnot-6*	*lsm-14*	*kif-19*
D	*dcp-1L*	*cnot-7*	*y-1*	
E	*dhx-8b*	*smn*		

Accession numbers for D. japonica sequences

Sequences of genes used in this study are accessible at the NCBI database from a previous submission (Yoshida-Kashikawa et al., 2007) or from accession numbers GU305857 to GU305878, GU305880 to GU305905, GU305908 and GU305909.

Results

Characterization of planarian homologues to cytoplasmic RNP factors reveals post-transcriptional regulation in planarian neoblasts

Fifty-one homologues of P body, stress granule, neuronal and germ line RNP granule components were identified by a Blastx search of a *D. japonica* EST database ($E \leq 2e^{-}05$; Table 1; Mineta et al., 2003). Less stringent search paradigms were required to identify homologues for SMG-7, EDC-4/Hedls, LSM-14 and DCP-1 (Table 1), which suggested weak conservation of some P body (Processing body) components in our database. In order to investigate this further, we searched the genomic sequence database of the closely related planarian species *Schmidtea mediterranea* (Robb et al., 2008) and failed to find clear homologues for DCP-1, EDC-3, GW182 and PATL-1 by primary sequence ($E = 1e^{-}03$ cutoff; Supplementary Table S1), which suggests an evolutionary loss in conservation of multiple P-body components in planarians.

Gene expression was assessed by comparing whole-mount *in situ* hybridization profiles in normal animals and animals depleted of neoblasts by X-ray irradiation (Fig. 1 and Supplementary Fig. S1;

Fig. 1. Enriched expression of post-transcriptional and translational regulators in planarian stem cells. Transcriptional expression analysis of genes involved in mRNA regulation by whole-mount *in situ* hybridization on normal and irradiated asexual planarians. (A) Simplified diagram of planarian anatomy. Neoblasts are located posterior of the photoreceptors (eyes), distributed throughout the mesenchyme (gray shading), and mostly absent from the pharynx. Neoblasts are not observed in X-ray irradiated planarians. The central nervous system is composed of a brain and ventral nerve cords (blue). A neuron-dense region is also found at the tip of the pharynx (blue in pharynx). The gastrovascular cavity (yellow), or intestine, is composed of one anterior and two posterior primary branches, which ramifies into secondary branches. (B) "Type A" genes are exclusively or preferentially expressed in neoblasts. These genes are expressed in a pattern reflective of neoblast distribution in normal planarians (top), but absent in irradiated animals (bottom). *DjPiwi-A* (first from left) is an abundant transcript exclusive to planarian neoblasts (C) "Type B" gene expression is enriched in both neoblasts and in cells of the central nervous system (CNS). Neoblasts detection is absent in irradiated animals (bottom), but detection of expression in the CNS remains intact. (D) "Type C" gene expression is exclusively detected in the CNS in normal (top) and irradiated (bottom) planarians. (E) "Type D" gene expression is readily detected in neoblasts, cells of the CNS, and other cells. Neoblast expression in "Type D" genes can be assessed by loss of detection in cells concentrated in the midline posterior to the pharynx of irradiated animals (asterisks). (F) Genes with no evident expression in neoblasts, classified as "Type E".

Baguñà et al., 1989; Dubois, 1949; Ladurner and Rieger, 2000; Wolff and Dubois, 1948). The expression patterns of genes were categorized in five groups based on sensitivity to radiation and tissue type expression. The category with the largest number of genes was "Type-A" ($n=20$ genes), with genes exclusively or preferentially expressed in irradiation sensitive cells (Fig. 1B and Supplementary Fig. S1A). Type A genes included five Tia1-like RNA-binding protein homologues (*Djtial-1*, *Djtial-2*, *Djtial-3*, *Djtial-4* and *Djtial-5*), a poly(A)-binding protein homolog whose expression was only detected in a subset of neoblasts (*Djpabpc-2*); the *vasa* homolog *Djvas-1* (ortholog of *Dugesia dorotocephala PoVAS1*; Mochizuki et al., 2001), and homologues of a surprising number of translation initiation factor homologues (*eIF-2a*, *eIF-3A*, *eIF-4e*, *eIF-4g*, and *eIF-5a*) which are often considered as "house-keeping" genes (Supplementary Fig. S1A). Previously reported Type-A genes *Djpiwi-A* (*Smedwi-1* ortholog previously known as *DjPiwi-4*; Reddien et al., 2005b; Yoshida-Kashikawa et al., 2007) and *Djtud-1* (*Spoltud-1* ortholog; Yoshida-Kashikawa et al., 2007; Solana et al., 2009), served as controls for effective X-ray irradiation. The second largest category was "Type-B" ($n=16$ genes), composed of genes exclusively or preferentially expressed in both neoblasts and neurons (Fig. 1C and Supplementary Fig. S1B). Amongst category Type B are miRNA processing enzyme gene *Djdicer-1*, Tudor domain containing protein *Djdhx-8c*, and number of homologues of P body components (*cnot-6*, *edc-4*, *gemin-5*, *lsm-14*, *smg-7*, *rbm-15*). Type B category also includes the ortholog of *S. mediterranea bruli* (Guo et al., 2006), the RNA-binding protein *Djfmrp-1* (Yoshida-Kashikawa et al., 2007), RNA helicases *DjeIF-4a3* (an evolutionarily conserved eIF4A1 homolog associated with nonsense-mediated decay and the spliceosome; Gehring et al., 2009), *Djcbc-1* (Yoshida-Kashikawa et al., 2007), and Vasa-like PL-10 family member *DjvlgA* (previously shown; Mochizuki et al., 2001; Shibata et al., 1999). Three kinesin homologues (*Djkif-3A*, *-3B* and *-19*), which were analyzed because sequence similarity with a kinesins known interact with Piwi and chromatoid bodies in mammalian male germ cells (Kotaja et al., 2006b) and required for localization of *oskar* mRNA to the posterior pole in *Drosophila* (Brendza et al., 2000), were also categorized as Type B. "Type-D" genes ($n=13$) are expressed in neoblasts, neurons and additional differentiated cells (Fig. 1E and Supplementary Fig. S1D). *Djpabpc-1* and *DjeIF-4a1* are exemplary of genes with ubiquitous expression in both undifferentiated and differentiated cells, in planarians and other organisms. Not all "Type D" genes are necessarily expressed ubiquitously. *Djy1*, for example, is highly expressed in neoblasts, X-ray-resistant mesenchymal cells and the brain, whereas *Djago-2*, *Djxrn-1* and *Djg3bp* are readily detected in neoblasts, brain, and the intestine (Fig. 1E). A number of decay factor homologues (*ago-2*, *cnot-7*, *dcp-1L*, *upf-1* and *xrn-1*) belong to this category. Homologues of *elav*, *tia-1*, and *bruno*-like genes *tnrc-4* and *brunol-5* were found to only be expressed in neurons, and were categorized as "Type-C" ($n=4$; Fig. 1D and Supplementary Fig. S1C). Expression of "Type-E" genes ($n=2$) is neither exclusive to neurons, nor detected in neoblasts (Fig. 1F). In summary, expression analysis revealed evident representation of cytoplasmic RNP factor expression in neoblasts (89% of genes analyzed), which further supports the notion that planarian stem cells being under heavy post-transcriptional regulation.

Functional analysis of neoblast expressed genes during regeneration

Double-stranded RNA (dsRNA) mediated RNA interference (RNAi) is a well-established method to "knockdown" gene expression in planarians (Orii et al., 2003; Reddien et al., 2005a; Sanchez Alvarado and Newmark, 1999). We modified previously reported RNAi protocols, and instead of soaking into or injecting *in vitro* synthesized dsRNA or feeding bacteria expressing dsRNA, planarians were fed *in vitro* synthesized dsRNA mixed with chicken liver and agarose. After RNAi treatment, planarians were amputated transversely anterior and posterior to the pharynx, and the trunk fragment was allowed to regenerate. In cases where regeneration appeared normal, regenerate trunk fragments were amputated a second time, 10 days after the previous amputation, and allowed to regenerate for 7 days. Animals were scored for successful pigment cup and photoreceptor regeneration, as this is the most obvious characteristic of a regenerated head. Obvious phenotypes were seen for 20/49 genes subjected to RNAi (Table 2), which validates the effectiveness of our protocol. Due to our inability to monitor the protein levels of our knockdowns, we cannot rule out the possibility that some of the phenotypes observed here (or lack thereof) are reflective of insufficient aberration of gene expression by RNAi.

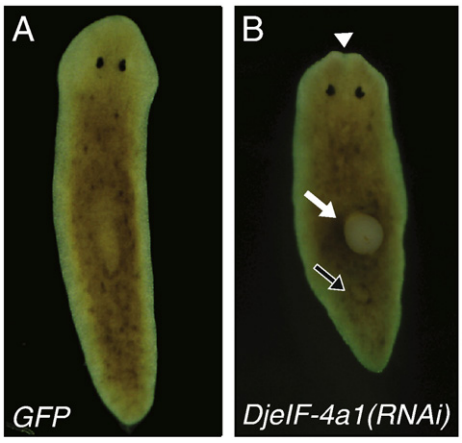

Fig. 2. Knockdown of factors required for general translation initiation and mRNA stability causes rapid somatic defects and lysis. Phenotype of intact planarians 10 days after first dsRNA feeding. (A) *GFP(RNAi)* control animals are characteristic of normal healthy planarians. (B) *DjeIF-4a3(RNAi)* planarians develop blisters (black arrow), deformation at the anterior tip (white arrowhead) and pharynx (white arrow).

Previous studies have shown that genes that disruption of expression of genes that encode for basal cell machinery factors, such as ribosomal proteins or tRNA synthetases, typically results in death by lysis prior to or soon after amputation without forming a blastema (Reddien et al., 2005a). Similar phenotypes were observed from experiments interfering with the expression of ubiquitously expressed factors known to promote general translation and mRNA stability (i.e. *DjeIF-4a1* and *Djpabpc-1*; Fig. 2), as well as with *DjeIF-4a3*. These animals failed to form a blastema if amputated, and underwent lysis within a couple of days of amputation. Knockdown of these three factors was classified as "lethal" (Table 2), concurrent with null mutations of their orthologues in *Saccharomyces cerevisiae* (Supplementary Table S2), and underwent no further analysis.

Failure to regenerate was observed in *Djago-2*, *Djbruli*, *Djdicer-1*, *DjeIF-3a*, *Djvas-1*, *Djxrn-1* and *Djxrn-2* RNAi treated planarians (Fig. 3B–E and G; Table 2). *Djdicer-1* knockdown planarians showed pleiotropic regeneration defects, including complete loss of regeneration (Fig. 3F). Given that DICER is a component of the RNA interference machinery (Bernstein et al., 2001), it is expected that the efficiency of RNAi will decrease as DICER protein levels decrease, and thus conclude that *Djdicer-1* is also required for regeneration. *Djedc-4(RNAi)* and *Djupf-1(RNAi)* animals not only failed to regenerate, but showed additional defects as well (Fig. 3H–I). *Djedc-4(RNAi)* trunk fragments developed an "hourglass" phenotype similar to that reported for *tropomyosin(RNAi)* in *S. mediterranea* (Fig. 3H; Reddien et al., 2005a), which suggests a role for DjEDC-4 in regulating transcripts of genes involved in maintaining actin filaments. *Djupf-1 (RNAi)* trunk fragments did not only fail to regenerate, but in the majority of the cases (60%) did not have a recognizable pharynx (Fig. 3I). Interestingly, knockdown of *Djupf-2*, another important component of the nonsense-mediated decay machinery (NMD), did not cause loss of regeneration (Fig. 3J). However, *Djupf-2(RNAi)*

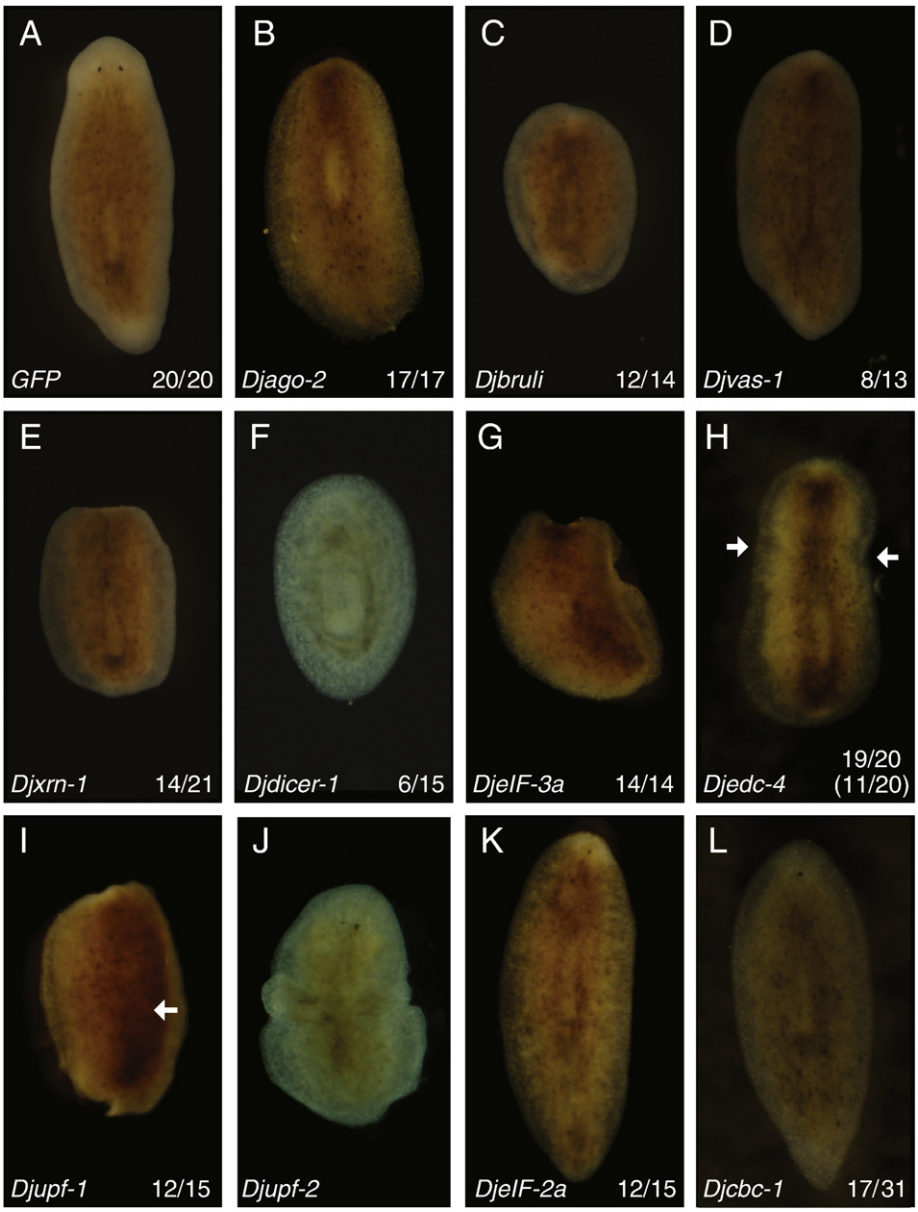

Fig. 3. Genes involved in post-transcriptional regulation are required for planarian regeneration. Representative phenotype of trunk fragments from planarians previously subjected to RNAi, 1 week after transverse amputation anterior and posterior of the pharynx. (A) Control *GFP* dsRNA fed planarians completely regenerated within a week of amputation. Planarians fed dsRNA for RNAi of (B) *Djago-2*; (C) *Djbruli*; (D) *Djvas-1*; (E) *Djxrn-1*; (F) *Djdicer*; (G) *DjeIF-3a*; (H) *Djedc-4*; and (I) *Djupf-1*; failed to regenerate. *Djedc-4* and *Djupf-1* RNAi-treated planarians showed additional phenotypes such as deformation into "hour-glass" shape (H; arrows), and loss of pharynx (I; arrow), respectively. (J) *Djupf-2* knockdowns were able to regenerate, but showed several pleiotropic abnormalities or died. (K) *DjeIF-2a*, and (L) *Djcbc-1* knockdowns could partially regenerate, but failed to form a complete set of photoreceptors. Number of planarians in RNAi test group scored pictured phenotype is shown in bottom right.

regenerates developed pleiotropic abnormalities and eventually lysis, which could be explained by a reduction in NMD surveillance (Cui et al., 1995; Leeds et al., 1992). *Djcbc-1*, *DjeIF-2a* and *Djgemin-5* knockdown planarians showed limited regeneration, as reflected by incomplete formation of photoreceptors or formation of a single photoreceptor (Fig. 3K–L; Table 2). In the case of *DjeIF-2a*, about half of the animals died before the regeneration period was completed.

Not all phenotypes observed were loss of regeneration phenotypes (Fig. 4). Planarians subjected to *Djg3bp*, *Djkif-3a* or *Djkif-3b* RNAi were able to regenerate to completion but developed distinctive phenotypes. *Djg3bp(RNAi)* developed an expansion of the head region during regeneration, a phenotype to which we assign the name "cone-head" (Fig. 4B). However, *Djg3bp(RNAi)* regenerates had normal neoblast distribution and complete photoreceptors and brain (not shown). *Djkif-3a* and *Djkif-3b* knockdown regenerates had well formed brain and photoreceptors, and a normal distribution of neoblasts (not shown), but developed transparent distended appearance and low mobility (Fig. 4C). Depletion of these kinesins causes the "bloated" phenotype previously reported for *β-tubulin* knockdowns (Reddien et al., 2005a), which involves water retention and is probably caused by deficiencies in cilia function as also reported for *Smed-iguana* knockdowns (Glazer et al., 2009; Rink et al., 2009). *Djtraf-2a(RNAi)* regenerates also showed pleiotropic defects, which included small head regeneration, abnormal pigment cups and photoreceptors, and no tail regeneration (Fig. 4D).

Cellular characteristics of regeneration-compromised RNAi-treated planarians

We speculated that planarians that failed to regenerate after RNAi, failed to do so because of a decrease in neoblasts, as previously reported for *DjPum*, *Smedbruli* and *SpolTud-1* (Guo et al., 2006; Salvetti

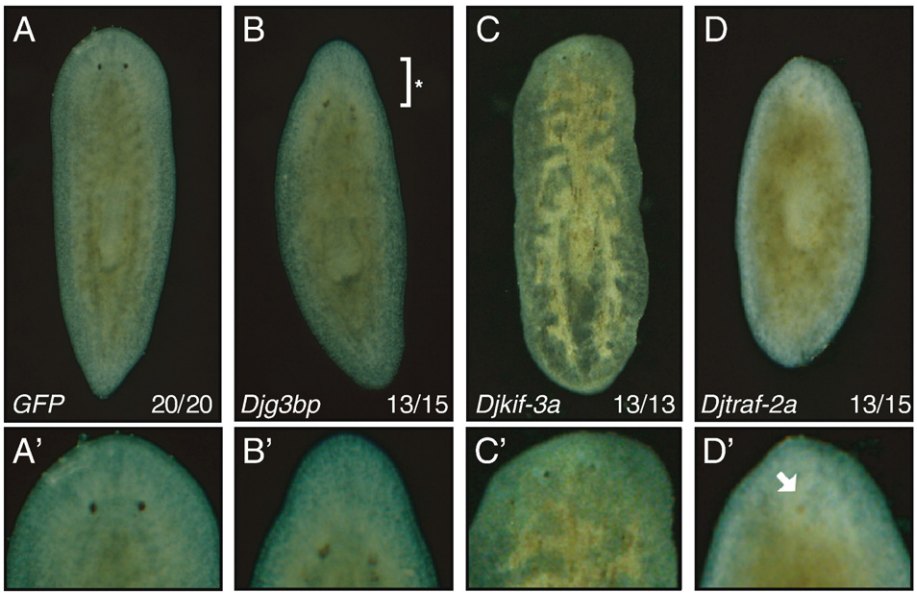

Fig. 4. Phenotypes observed in regeneration-capable knockdown planarians. Phenotype of regenerated trunk fragments from planarians previously subjected to RNAi, 1 week after transverse amputation anterior and posterior of the pharynx. (A) Control *GFP* dsRNA fed planarians completely regenerated within a week of amputation. (B) *Djg3bp* knockdown regenerates developed an abnormal extension of the area anterior to the photoreceptors (asterisks). (C) *Djkif-3a* knockdown regenerates developed transparence of tissue and swelling. (D) *Djtraf-2a* knockdowns developed smaller heads and abnormal pigmentation of photoreceptors (D′, arrow). (A′–D′) Magnified view of respective head region.

et al., 2005; Solana et al., 2009). In order to assess this, we analyzed the distribution of total and mitotically active neoblasts in regeneration compromised trunk fragments by performing anti-DjPiwi-A (Yoshida-Kashikawa et al., 2007) and anti-phosphohistone H3 Ser10 (anti-H3P) whole mount immunohistochemistry, respectively (Fig. 5 and Supplementary Fig. S2; Newmark and Sanchez Alvarado, 2000; Reddien et al., 2005a). Formation of differentiated tissue was analyzed by visualizing brain and photoreceptor regeneration with Hoechst and visual cell antibodies (anti-DjArrestin; Agata et al., 1998; Nishimura et al., 2007; Sakai et al., 2000), respectively (Fig. 5 and Supplementary Figs. S2 and S3).

GFP(RNAi) planarians, used as controls, showed characteristics representative wild-type regenerates (Fig. 5A, and Supplemental Figs. S2A and S3A). Neoblasts were distributed outside of the pharynx region and posterior to the photoreceptors, and mitotic cells distributed within DjPiwi-A expressing neoblasts (Fig. 5A′ and A″). Differentiated tissue had successfully regenerated, as a well-formed brain and two distinguishable photoreceptors connected by a chiasma were distinguishable (Fig. 4A and Supplementary Fig. S2A). Surprisingly, a number of knockdowns that failed to regenerate, such as *Djxrn-1(RNAi)*, *Djxrn-2(RNAi)*, *Djcbc-1(RNAi)*, *Djedc-4(RNAi)* and *Djvas-1(RNAi)* animals, contained populations of neoblasts and mitotic cells comparable to control animals (Fig. 5B and Supplementary Fig. S2B–E). Amongst these, *Djcbc-1(RNAi)* animals were the only ones capable of forming photoreceptors, although these were often abnormal (Supplementary Fig. S3B–E). Mitotic cells were also detected in *Djupf-1(RNAi)* and *Djupf-2(RNAi)* animals (Supplementary Fig. S2F; not shown), although photoreceptors failed to appear and brain formation was severely affected in *Djupf-1(RNAi)* regenerates (Supplementary Figs. S2F and S3F–G).

Another surprising observation came from analysis of knockdowns for translation initiation factors *DjeIF-2a* and *DjeIF-3a*. These animals displayed normal neoblast distribution but complete loss of mitotic cells, as characterize by absence of phospho-histone H3 Ser10 signal (Fig. 5C′ and C″; Supplementary Fig. S2H′ and S2H″). This was true even in regenerates whose mild phenotype exhibited partial brain regeneration (Fig. 5C and Supplementary Fig. S2H). These results indicate that neoblasts were capable of proliferating and replacing lost tissue until the decline in levels of DjeIF-2A or DjeIF-3A in these knockdowns caused mitotic arrest. The absence of phospho-Histone H3 (Ser10) signal indicates that such discontinuity occurred outside the M-phase of the cell cycle. It is expected that other translational initiation factors may also play a role in this regulation, but may have not shown a phenotype in our experiments due to incomplete aberration of expression by RNAi or by high genetic redundancy of multiple paralogs (see Supplementary Table S1). A decrease in expression of several genes and consequential defects is expected from reduction in translation initiation factors, but the fact that cells were specifically not detected in the M-phase was a clear molecular phenotype.

Other than the *Smed-bruli* ortholog *Djbruli*, only *Djago-2(RNAi)* animals showed a broad reduction of neoblasts (Fig. 5D′ and D″). As expected, no recognizable brain or photoreceptors were observed in these knockdowns (Fig. 4D and Supplementary Figs. S2G and S3H–I). To analyze whether loss of neoblasts is also true of intact *Djago-2 (RNAi)* animals, total and mitotic neoblasts were analyzed in planarians subjected to long-term RNAi without amputation after 20 days of RNAi treatment (Fig. 6). At this point, about one-third of *Djago-2(RNAi)* planarians had undergone lysis, and the remaining two-thirds had developed phenotypic abnormalities (Supplementary Fig. S4). As observed in regenerating animals, neoblasts were severely reduced in *Djago-2(RNAi)* intact planarians (Fig. 6B and E). *Djbruli* knockdown caused over 50% reduction in M-phase neoblasts in intact planarians, corroborating with results previously reported for *Smed-bruli* (Fig. 6E; Guo et al., 2006). Conversely, mitotic cell number remained unaffected, or even increased, after disrupting the expression of *Djvas*-1, *Djxrn-1*, *Djedc*-4, *Djcbc-1* or *Djupf-1* (Fig. 6C, D and E). These results reaffirm the trend observed in amputates of knockdown planarians (Fig. 5 and Supplementary Fig. S2). *Djago-2* and *Djbruli* are required for neoblast maintenance and consequentially regeneration, whereas *Djvas*-1, *Djxrn-1*, *Djedc*-4, *Djupf-1* and *Djcbc-1* are required for regeneration, but not for neoblast maintenance.

Heterogeneity and dynamics of RNP aggregates containing DjCBC-1

Three lines of evidence indicate that DjCBC-1-containing ribonucleoprotein aggregates are heterogonous. First, immuno-electron microscopy analysis of neoblasts revealed that DjCBC-1 aggregates

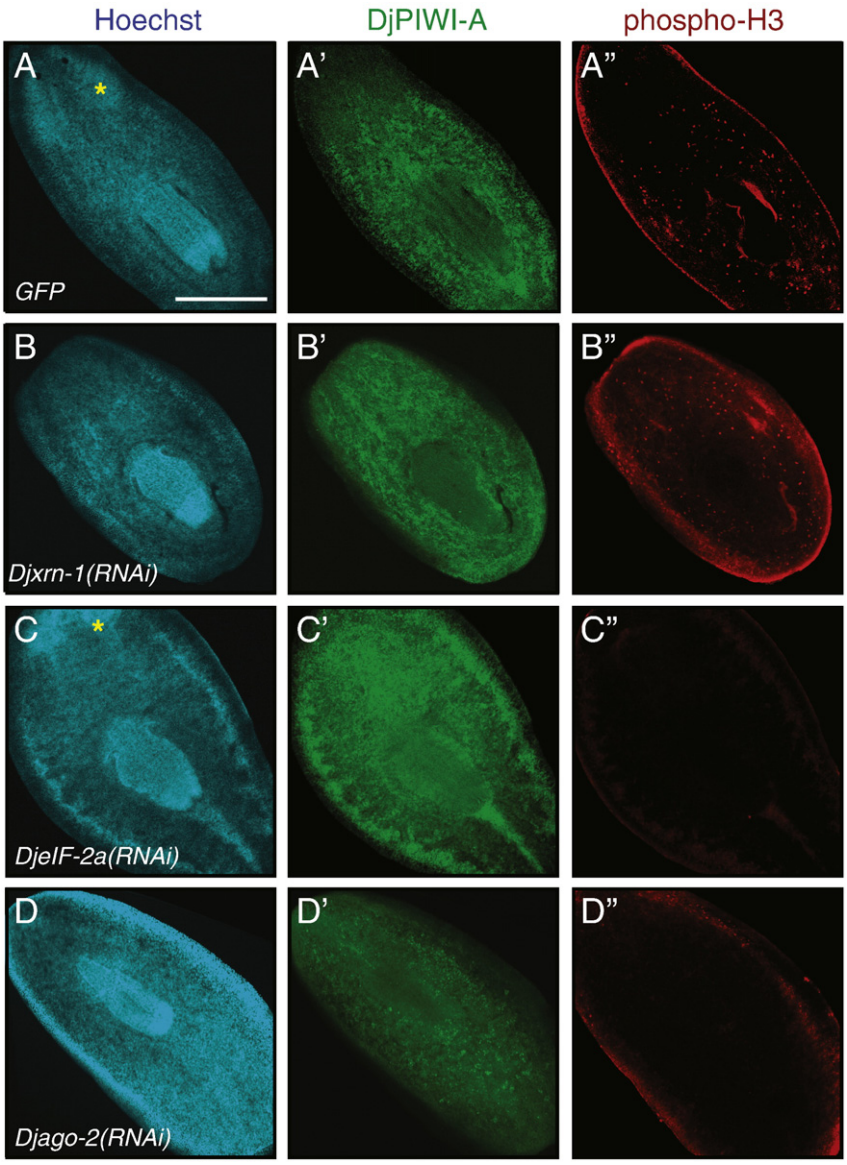

Fig. 5. Cellular analysis of regeneration-deficient knockdown planarians. Representative cellular outcomes of knockdown planarians 1 week post-amputation. Total (anti-DjPiwi-A; Green) and M-phase (anti-phosphohistone H3; Red) neoblasts, as well as differentiated tissue such as brain and pharynx (Hoechst; blue) were analyzed in regenerate trunk fragments 1 week after amputation. (A) *GFP(RNAi)* planarians, used as control, are able to regenerate a well-developed brain (asterisk), maintain normal distribution of neoblasts (A′), and mitotic activity is readily detected (A″). (B) *Djxrn-1(RNAi)* planarians fail to regenerate a head or brain, but preserve their neoblasts (B′) and mitotic cells (B″). (C) *DjeIF-2a(RNAi)* planarians partially regenerate a brain (asterisk), and preserve their neoblasts (C′), but mitotic cells are lost (C″). (D) *Djago-2(RNAi)* planarians do not regenerate a brain, and show a decrease neoblasts (D′), and mitotic neoblast population (D″). All panels on same scale. Scale bar = 500 μm.

are present in some (but not all) chromatoid bodies, and also away from chromatoid bodies, in neoblasts (Yoshida-Kashikawa et al., 2007). Second, DjCBC-1 foci are detectable in *bona fide* proliferating neoblasts, as well as in neighboring cells thought to be differentiating neoblasts (Fig. 7). These cells have neoblast-like morphology, but do not retain bromodeoxyuridine 48 h post-injection, nor do they express *Djpiwi-A* (Fig. 7; Yoshida-Kashikawa et al., 2007). Third, DjPiwi-A and DjCBC-1 sub-cellular distribution within the cytoplasm is different, since aggregates of these two proteins very rarely, but occasionally, partially overlap (Fig. 6; Yoshida-Kashikawa et al., 2007; reviewed in Shibata et al., 2010).

We analyzed the sub-cellular distribution pattern of DjCBC-1 in neoblasts of intact planarians subjected to RNAi of genes required for regeneration. *GFP(RNAi)* animals, which were used as control, showed normal distribution of chromatoid bodies in DjPiwi-A expressing neoblasts and differentiating neoblasts (Fig. 8A). *Djago-2* RNAi leads to abnormal morphology (Fig. 8B) and almost complete loss of DjPiwi-A-expressing neoblasts (as shown in Fig. 6B). Nevertheless, the lingering neoblasts of *Djago-2(RNAi)* planarians mostly displayed diffused DjCBC-1 protein and very few aggregates (Fig. 8B and F). This indicates a possible correlation between loss of DjCBC-1 aggregation (presumably chromatoid body stability) with neoblast decay. Although, at this point it is not clear whether the loss of DjCBC-1 aggregation precedes neoblast decay, or *vice versa*. Some changes in the distribution of DjCBC-1 aggregates were observed for two other knockdowns. *Djedc-4(RNAi)* animals showed an increase of DjCBC-1 aggregation in DjPiwi-A-expressing neoblasts, but not in differentiating neoblasts (Fig. 8C and F). DjCBC-1 signal noticeably variable in DjPiwi-A-expressing neoblasts of *Djxrn-1(RNAi)* animals (Fig. 8F). However, aggregates were readily detected in differentiating neoblasts (Fig. 8D). As expected, neoblasts of *Djcbc-1(RNAi)* animals showed a large decrease in overall DjCBC-1 signal, although some protein could still be detected in aggregation (Fig. 8E). A similar slow decay (>2 weeks) for SpolTud-1, another component of neoblast

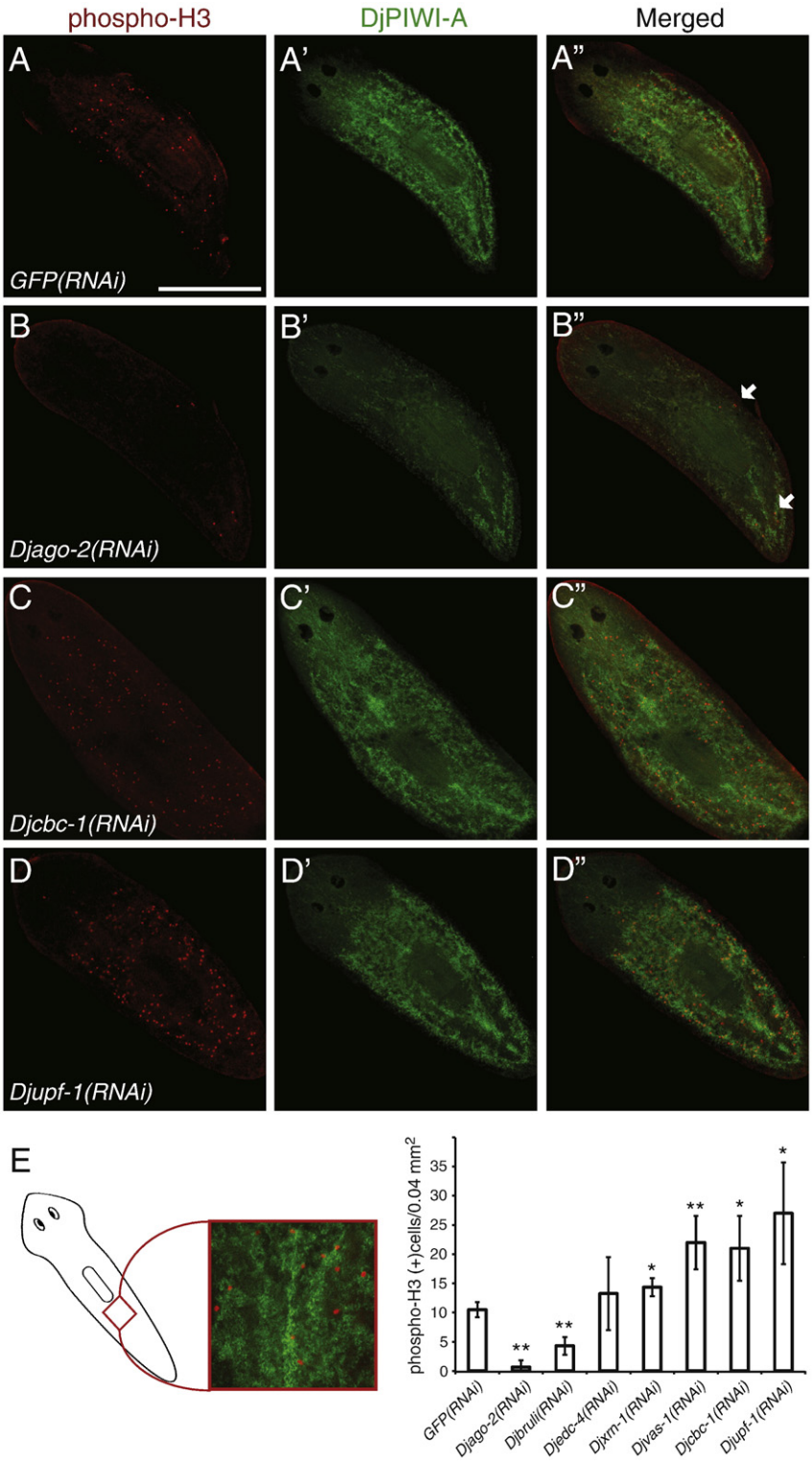

Fig. 6. Neoblast maintenance in knockdowns during homeostasis. Analysis of total and M-phase neoblasts (as in Fig. 4), in intact planarians submitted to a 20-day RNAi treatment. (A) *GFP(RNAi)* planarians, used as control, exhibit a normal population of mitotic (A) and total (A′) neoblasts posterior to the photoreceptors. (B) *Djago-2(RNAi)* planarians exhibit extensive loss of mitotic (B) and total (B′) neoblasts, but mitotic cells are detected amongst remaining neoblasts (B″, arrows). (C) *Djcbc-1(RNAi)* and (D) *Djupf-1(RNAi)* planarians exhibit a rich neoblast population (C′ and D′), with an increase in number of M-phase cells (C and D). Scale bar = 500 μm, panels A–D on same scale. (E) Average number of M-phase neoblasts (red) in a fixed area posterior to the pharynx (left), in intact animals submitted to a 20-day RNAi treatment. *GFP(RNAi)* planarians were used as controls. *Djago-2(RNAi)* and *Djbruli(RNAi)* planarians have a significant loss in M-phase neoblast number. Whereas *Djvas-1(RNAi)*, *Djcbc-1(RNAi)* and *Djupf-1(RNAi)* animals have about twice as many M-phase cells. *P*-values represented as * ≤0.02; and ** ≤0.005.

chromatoid bodies, has previously been reported for SpolTud-1 (Solana et al., 2009).

The increased aggregation of DjCBC-1 after disruption of *Djedc-4* expression and distribution *Djxrn-1* knockdowns indicates a possible association between chromatoid bodies and the mRNA decay machinery. It is not clear whether aggregation of DjCBC-1 in these knockdowns occurs in *bona fide* chromatoid bodies, or if neoblasts contain additional ribonucleoprotein granules involved in mRNA

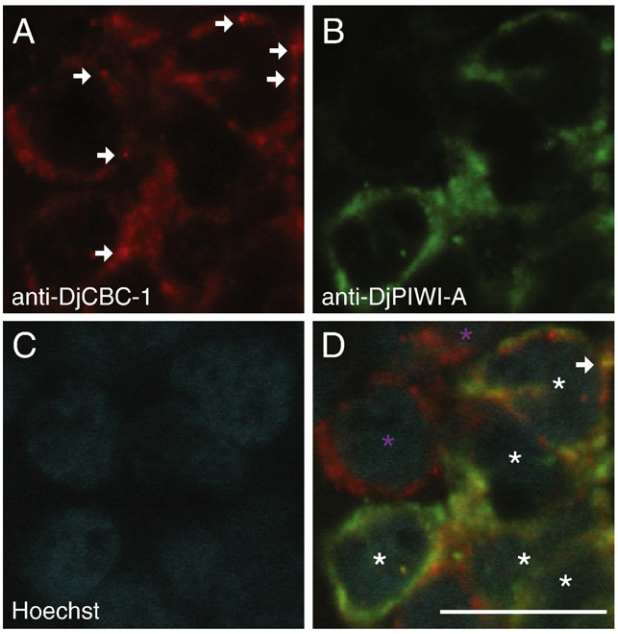

Fig. 7. DjCBC-1 granules are present in neoblast and presumed differentiating neoblasts. (A) DjCBC-1 protein aggregates (arrows) are visualized in whole intact animals using anti-DjCBC-1 antibodies (Red). (B) *Bona-fide* neoblasts are visualized with anti-DjPiwi-A antibodies (Green). (C) Hoechst staining of nuclei of all cells. (D) Merged images. Presumed differentiating cells (magenta asterisks) display neoblast morphology and DjCBC-1 aggregates, but lack DjPiwi-A expression. DjPiwi-A expressing neoblasts are considered *bona-fide* neoblasts, and also contain DjCBC-1 aggregates (white asterisks). DjCBC-1 and DjPiwi-A foci sometimes partially overlap (arrow). Scale bar = 10 μm.

turnover similar to P bodies. As previously indicated, DjCBC-1 also aggregates away from chromatoid bodies in neoblasts (Yoshida-Kashikawa et al., 2007), so development of reagents such as additional antibodies will be necessary to further characterize these granules and their dynamics during neoblast proliferation and differentiation.

Discussion

We set out to test the requirement of factors involved in post-transcriptional regulation in planarian regeneration. From an initial list of 55 factors identified by primary sequence conservation, expression of 89% was readily detected in neoblasts. Phenotypes ranging from physical abnormalities to lethality were observed from RNAi-mediated functional analysis, with loss of regeneration being the most prevalent phenotype (Table 2). Homologues of germ granule components, namely DjBruli, DjAgo-2, DjDicer, and DEAD Box RNA chaperones DjCBC-1 (RCK/p54/Me31b) and DjVAS-1, were all required for planarian regeneration. Concurrent studies have shown the requirement of members of the PUF, Bruno, Piwi, and Tudor families of post-transcriptional regulators for planarian regeneration and stem cell maintenance/function (Guo et al., 2006; Palakodeti et al., 2008; Reddien et al., 2005b; Salvetti et al., 2005; Solana et al., 2009). Together, these studies show that planarian adult somatic stem cells utilize mechanisms of post-transcriptional regulation historically uncovered by studies of germ line development.

Different post-transcriptional events during the life of a neoblast

Interestingly, most of the factors found to be required for regeneration in this study were not required for neoblast maintenance (Fig. 9; summarized in Table 2), as is the case for RNA-binding proteins previously reported to be required for planarian regeneration (Guo et al., 2006; Salvetti et al., 2005; Solana et al., 2009). Regeneration was compromised after knockdown of P body component homologues such as DjXRN-1 5′ to 3′ exoribonuclease, DjCBC-1 (RCK/Dhh1p homolog), DjUPF-1, and the enhancer of decapping factor DjEDC-4/Hedls, but neoblast maintenance was unaffected. Given the known function of these factors in mRNA turnover (reviewed in Eulalio et al., 2007; Franks and Lykke-Andersen, 2008), and their observed phenotype (summarized in Table 2), we predict that these are required for a wave of decay of "stemness" promoting mRNAs during the differentiation process. On the other hand, DEAD box helicases such as DjVAS-1 and DjCBC-1, which displayed similar phenotypes, may interact with a number of factors to ensure timely translation of a subset of stored mRNAs required for lineage specification during the process of differentiation, as is the case in other organisms (reviewed by Abdelhaleem, 2005; Raz, 2000; Weston and Sommerville, 2006). There were two factors which were actually required for stem-cell maintenance in our study: DjBruli and DjAGO-2/EIF2C2 (Fig. 6). These factors, along with previously shown Pumilio, are likely repressing neoblasts transcripts in order to maintain differentiation potential. While Piwi and Tudor homologues silence transposons and maintain neoblast genome stability (Reuter et al., 2009; Shoji et al., 2009).

We demonstrate that DjAGO-2 is required for maintaining stem cell identity in regenerating and intact planarians (Figs. 5D and 6B), which suggests that associated miRNAs, and perhaps endo-siRNAs (reviewed in Kim and Rossi, 2009), are required for neoblast identity. Studies in mouse spermatids have shown that not only Ago-2, but also Dicer and a number of miRNAs, localize to chromatoid bodies (Kotaja et al., 2006a; reviewed in Chuma et al., 2009). So far, different groups have identified dozens of miRNAs in planarians, but their role in planarian neoblast function or regeneration is still elusive (Friedlander et al., 2009; Gonzalez-Estevez et al., 2009; Lu et al., 2009; Palakodeti et al., 2006). Given the notion that neoblasts give rise to the germ line and soma, it would be interesting to compare the population of DjAGO-2 associated miRNA in neoblasts to that of human embryonic stem cells and neural precursors (Goff et al., 2009) or germ cells. Further study of DjAGO-2 and associated mRNAs will shed light on the importance post-transcriptional regulation by small RNAs, and the function of their targeted transcripts in planarian stem cells.

What about the germ line?

Given the germ-like nature of post-transcriptional regulation in neoblasts, one speculates as to what events give rise to the germ line in planarians. This question was partially answered by the identification of the Nanos planarian homolog (Handberg-Thorsager and Salo, 2007; Sato et al., 2006; Wang et al., 2007). Germ cell specification in planarians is not predetermined by cytoplasmic factors during embryogenesis, but apparently arises from neoblasts by inductive interactions between cells (reviewed by Newmark et al., 2008) and requires Nanos (Wang et al., 2007). In asexual planarians, nanos is only expressed in presumptive germ line stem cells located dorso-laterally and posterior to the head (Sato et al., 2006; Wang et al., 2007). The mechanism by which nanos expression is driven in these cells is unknown. Nevertheless, one can imagine that with the presence of Pumilio (Salvetti et al., 2005) and Vasa (Fig. 1) already in neoblasts, the addition of Nanos could direct germ line specification.

Relationship of different RNP granules

Although germ granules and P bodies are classified as different types of RNPs, they are known to partially overlap in germ cells of flies and mammals (Chuma et al., 2003; Chuma et al., 2009; Kotaja et al., 2006a; Lim et al., 2009; Seydoux and Braun, 2006; Zabolotskaya et al., 2008). The phenotypes found in this study suggest that a similar interaction between homologues of germ granule and P body components occurs in planarian neoblasts. Thus, it seems likely that planarian chromatoid bodies are somatic analogues of germ granules. Cytoplasmic RNPs granules, such as P-bodies and stress granules, can

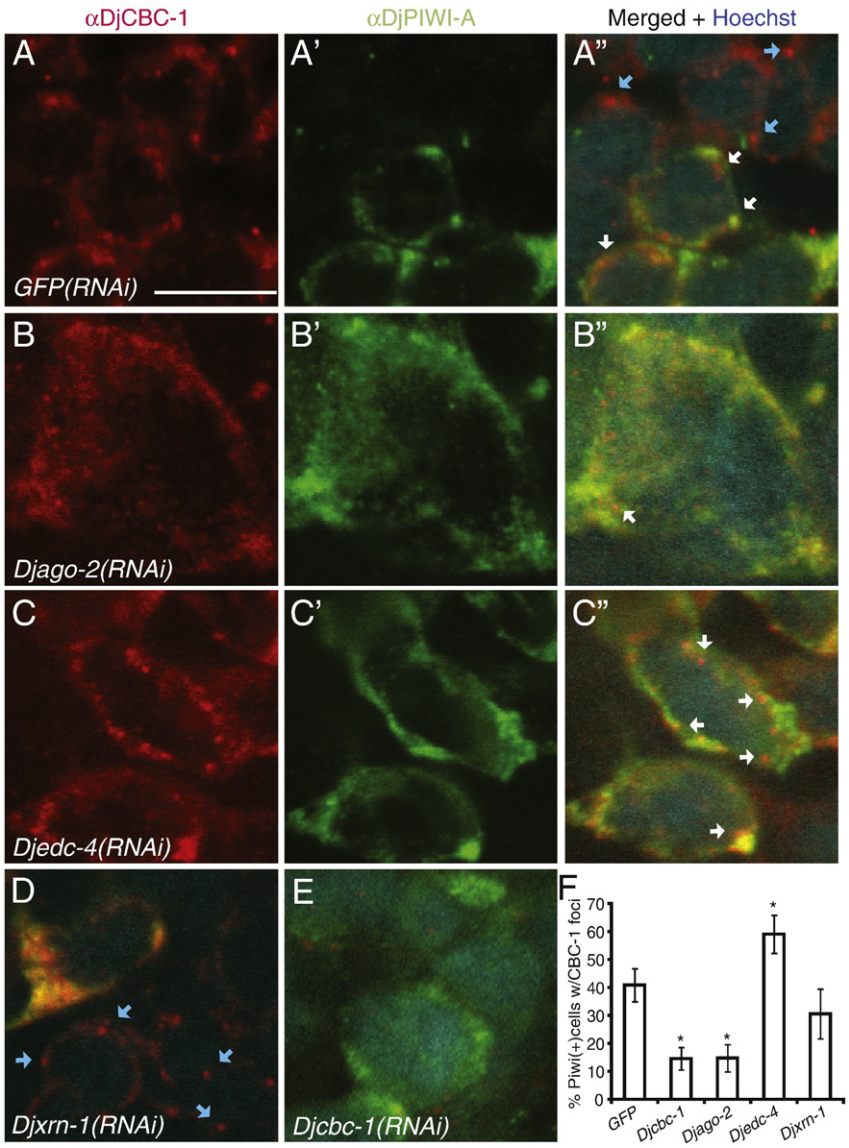

Fig. 8. Distribution of DjCBC-1 granules in neoblasts of intact planarians subjected to RNAi. Representative distribution of DjCBC-1 granules (as analyzed in Fig. 7) in intact planarians subjected to RNAi. (A) *GFP(RNAi)* planarians, used as control, contain DjCBC-1 granules in DjPiwi-A expressing neoblasts (white arrows) and differentiating neoblasts (blue arrows). (B) *Djago-2(RNAi)* exhibit neoblasts morphological deformation and significant diffusion of DjCBC-1 protein. (C) *Djedc-4(RNAi)* planarians show substantial DjCBC-1 aggregation in DjPiwi-A expressing neoblasts (white arrows). (D) *Djxrn-1(RNAi)* planarians show substantial DjCBC-1 aggregation in presumptive differentiating neoblasts (blue arrows). (E) *Djcbc-1(RNAi)* planarians show substantial decrease of diffused DjCBC-1, but some protein remains in aggregates. (F) Average number of Piwi-A-expressing neoblasts with detectable DjCBC-1 aggregates taken from confocal sections of the anterior midline neoblast population of 20-day knockdown planarians, 120× magnification. Asterisks represent unpaired *t*-test two-tailed *P*-values ≤ 0.02. All panels are on same scale. Scale bar = 10 μm.

be stabilized or destabilized after depletion or over-expression of components important for their function (reviewed in Balagopal and Parker, 2009; Eulalio et al., 2007; Parker and Sheth, 2007). Here we see a similar trend, with inhibition of *Djxrn-1* expression leading to an increase in aggregation of DjCBC-1 in differentiating cells (Fig. 8D), and in proliferative neoblasts after disruption of *Djedc-4* expression (Fig. 8C). Further characterization of planarian chromatoid bodies, as well as other DjCBC-1 containing RNP granules, will require identification and production of antibodies of additional components, as well as identification of RNA substrates, and their fate in these structures. A trend that seems clear is the conservation of post-transcriptional regulation in RNP aggregates in stem cells, neurons and germ line, which is reflected by the expression of many homologues of germ line RNA-binding proteins of other organisms in planarian neoblasts and brain (Fig. 1; Yoshida-Kashikawa et al., 2007; reviewed by Shibata et al., 2010).

Regulation of translation initiation and the cell cycle

Not surprisingly, inhibition of some translation initiation factors leads to lethality or compromised regeneration. We find it noteworthy, however, that many translational initiation factors (i.e. *DjeIF-2a*, *DjeIF-3a*, *DjeIF-4e*, *DjeIF-4g* and *DjeIF-5a*) are preferentially expressed in neoblasts (Fig. 1). In accordance with this, expression of several other translation initiation factors and ribosomal protein genes have been shown to be severely down-regulated in planarians 1 day after irradiation, which further suggests heavy translational regulation in neoblasts (Cormier et al., 2003; Eisenhoffer et al., 2008). This might be due to the fact that neoblasts are the only proliferating and differentiating cells in planarians, and thus require significant changes in their proteome during these processes. Translation initiation factor over-expression is a characteristic of cancerous cells (reviewed in Cormier et al., 2003), and studies in yeast and cultured mammalian

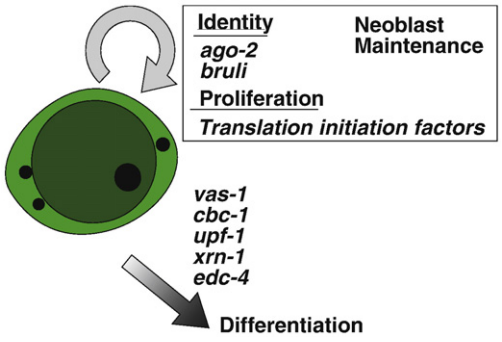

Fig. 9. Model. Post-transcriptional and translational regulators required for regeneration affect neoblasts by different modes. *Djago-2* and *Djbruli* are required to maintain identity of neoblasts. Neoblast proliferation is sensitive to levels of translation initiation factors, probably by a cell-cycle checkpoint mechanism. *Djvas-1*, *Djcbc-1*, *Djxrn-1*, *Djupf-1* and *Djedc-4* are not required for neoblast maintenance, but their requirement during regeneration indicates that they have a role in neoblast differentiation processes.

cells have shown that robust cap-dependent translation is required for G_1/S transition, but suppressed during the M-phase of the cell cycle (Bonneau and Sonenberg, 1987; Pyronnet et al., 2001; Scharff and Robbins, 1966; reviewed in Cormier et al., 2003; Pyronnet and Sonenberg, 2001). *DjeIF-2a(RNAi)* and *DjeIF-3a(RNAi)* planarians, which fail to regenerate but are alive and motile, completely lose M-phase neoblasts (Fig. 5C and Supplementary Fig. S2H). This suggests that neoblasts are able to progress through M-phase regardless of reduced levels of DjeIF-2A or DjeIF-3A, but fail to progress through the cell cycle. IRES-dependent translation initiation has been reported to prevail over cap-dependent translation initiation during mitosis in mammalian cells (Bonneau and Sonenberg, 1987; Cornelis et al., 2000; Pyronnet et al., 2000; reviewed in Sachs, 2000). The ability of neoblasts to progress through mitosis despite low levels of DjeIF-2A and DjeIF-3A suggests that a similar mechanism may occur in neoblasts. Another possibility is that neoblasts possess a response mechanism that negates entry into M-phase if cap-dependent translation is compromised. We wonder how differentiated cells can function with minimal or no expression of translation initiation factors, as compared to neoblasts. Perhaps, spliced-leaders found in planarian mRNAs (Zayas et al., 2005) are involved with a form of non-canonical translation initiation in differentiated cells. Another possibility is that during the constant replacement of differentiated cells by neoblasts, translation initiation factors are supplied during differentiation and linger for the life of the differentiated cell, although their transcription is only detected in neoblasts.

Different approaches aimed at the identification of genes involved in planarian regeneration

Planarian regeneration is a fascinating phenomenon to which recent molecular dissection has been made possible by an array of technological advances (Agata and Watanabe, 1999; Hayashi et al., 2010; Sanchez Alvarado, 2004, 2006). Candidate gene approaches have been fruitful in identifying and analyzing components of entire pathways in planarians, as shown for members of the Hedgehog and wnt/β-catenin signaling pathways involved in determination of A/P polarity (Gurley et al., 2008; Rink et al., 2009; Yazawa et al., 2009). High throughput non-bias efforts in *S. mediterranea*, as the one carried out by Reddien et al. (2005a), successfully identified the requirement of Smedwi-2 (HB.14.06D; Reddien et al., 2005b) and 139 other gene perturbations that "blocked, limited or reduced regeneration" from a total of 1065 unique knockdowns. These include a homolog of Sam68, a post-transcriptional regulator known to localize to stress granules (Henao-Mejia and He, 2009; Henao-Mejia et al., 2009); as well as a homolog of eIF-6, a protein involved in RISC-mediated miRNA silencing (Chendrimada et al., 2007); and a homolog of Tropomyosin 2, a factor required for *oskar* RNA localization to the posterior pole in *Drosophila* (Erdelyi et al., 1995; Tetzlaff et al., 1996; Trucco et al., 2009; Zimyanin et al., 2008). However, given the generality of their approach, much effort went into characterizing that factors required for global gene expression, such as ribosomal proteins L3, L8, L13a, L15, L17, L7a, L18, L21, L22, L26, L35, L36, P0, P2, S2, S4, S5, S8, S13, S15a, S18, S19 and S27; seryl, glutaminyl, lysyl, phenylalanyl and tyrosyl tRNA sythetases; and eukaryotic translation termination factor 1 (Reddien et al., 2005a). Another unbiased method for identification of genes involved in planarian regeneration has been the use of microarrays to identify transcripts highly enriched in neoblasts of *S. mediterranea* (Eisenhoffer et al., 2008) and *D. japonica* (Rossi et al., 2007). From these, three different Piwi homologues were identified as enriched in the transcriptome of *D. japonica* neoblasts (Rossi et al., 2007), and expression of *Smedwi-1*, *Vasa/DDX4*, *Sam-68*, *eIF-6*, and the Plasmonigen activator inhibitor 1 RNA-binding protein in neoblasts of *S. mediterranea* (Eisenhoffer et al., 2008). Here we show that analysis of conserved macromolecular machinery components is a successful approach for the identification of genes involved in a certain process. In this specific case, analysis of 55 putative cytoplasmic RNP components lead to the identification of 49 genes expressed in neoblasts, 15 of which (27.3%) are involved in the process of regeneration or viability.

Summary and outlook

At this moment, we do not know enough about post-transcriptional regulation in mammalian adult stem cells, or induced pluripotent stem cells, in order to compare with what is seen in planarian neoblasts. However, it appears that the mechanisms of post-transcriptional regulation present in neoblasts parallels regulatory pathways seen in early development and gametogenesis in other metazoans. Neoblasts are under heavy regulation by mRNA silencing factors such as miRNAs, AGO-2, Pumilio, and Bruno-like proteins, which maintain the stem cell identity by regulating specific mRNAs. Additionally, Piwi-family proteins, Tudor and piRNA secure genome stability by repressing transposable elements, while degradation of specific mRNAs and/or miRNAs by exoribonucleases and the decapping machinery seem to be required for progression of differentiation. The abundance of neoblasts in planarians makes them a valuable model for understanding regulation of gene expression in stem cells. Whether similar mechanisms regulate gene expression in adult stem cells of vertebrates, or in induced pluripotent stem cells (iPS) remains to be investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.037.

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