The Retinoblastoma pathway regulates stem cell proliferation in freshwater planarians

Shu Jun Zhu \textsuperscript{a,c}, Bret J. Pearson \textsuperscript{a,b,c,*}

\textsuperscript{a} The Hospital for Sick Children, Program in Developmental and Stem Cell Biology, 101 College St., MaRS East Rm 11-704, Toronto, ON, Canada MSG1L7
\textsuperscript{b} University of Toronto, Department of Molecular Genetics, ON, Canada M5S1A1
\textsuperscript{c} Ontario Institute for Cancer Research, Toronto, ON, Canada MSG1L7

\textbf{Abstract}

Freshwater planarians are flatworms of the Lophotrochozoan superphylum and are well known for their regenerative abilities, which rely on a large population of pluripotent adult stem cells. However, the mechanisms by which planarians maintain a precise population of adult stem cells while balancing proliferation and cell death, remain to be elucidated. Here we have identified, characterized, and functionally tested the core Retinoblastoma (Rb) pathway components in planarian adult stem cell biology. The Rb pathway is an ancient and conserved mechanism of proliferation control from plants to animals and is composed of three core components: an Rb protein, and a transcription factor heterodimer of E2F and DP proteins. Although the planarian genome contains all components of the Rb pathway, we found that they have undergone gene loss from the ancestral state, similar to other species in their phylum. The single Rb homolog (\textit{Smed-Rb}) was highly expressed in planarian stem cells and was required for stem cell maintenance, similar to the Rb-homologs p107 and p130 in vertebrates. We show that planarians and their phylum have undergone the most severe reduction in E2F genes observed thus far, and the single remaining E2F was predicted to be a repressive-type E2F (\textit{Smed-E2F4-1}). Knockdown of either \textit{Smed-E2F4-1} or its dimerization partner \textit{Dp} (\textit{Smed-Dp}) by RNAi resulted in temporary hyper-proliferation. Finally, we showed that known Rb-interacting genes in other systems, \textit{histone deacetylase 1} and \textit{cyclin D} (\textit{Smed-HDAC1}; \textit{Smed-cyclD}), were similar to Rb in expression and phenotypes when knocked down by RNAi, suggesting that these established interactions with Rb may also be conserved in planarians. Together, these results showed that planarians use the conserved components of the Rb tumor suppressor pathway to control proliferation and cell survival.

* Corresponding author at: The Hospital for Sick Children, Program in Developmental and Stem Cell Biology, 101 College St., MaRS East Rm 11-704, Toronto, ON, Canada MSG1L7. Fax: +1 416 813 8456.

E-mail address: bret.pearson@sickkids.ca (B.J. Pearson.)

\textcopyright{} 2012 Elsevier Inc. All rights reserved.

\section*{Introduction}

The ability to regulate cell division is a fundamental property of all animals, and the Retinoblastoma pathway (Rb) is an ancient and critical component of this process. Originally discovered in humans, it is now clear that the Rb tumor suppressor pathway existed prior to multicellularity and prior to the split between humans, it is now clear that the Rb tumor suppressor pathway existed prior to multicellularity and prior to the split between humans, for example, when RB is mutated and activating-E2F complexes drive ectopic cell cycle entry at the G1/S transition (Burkhart and Sage, 2008; Classon and Harlow, 2002; Soleimani et al., 2012). RB binding to E2Fs, in turn, is negatively regulated through phosphorylation of RB by CDK/cyclin complexes (Dora et al., 1999). Cancer can result in humans, for example, when Rb is mutated and activating-E2F complexes drive ectopic cell cycle entry (Attwooll et al., 2004; Crosby and Almasan, 2004). RB proteins typically disrupt activating-E2F complexes and modify repressive E2F function, thus altering the activator/repressor balance to fine-tune cell cycle entry at the G1/S transition (Burkhart and Sage, 2008; Classon and Harlow, 2002; Soleimani et al., 2012). RB binding to E2Fs, in turn, is negatively regulated through phosphorylation of RB by CDK/cyclin complexes (Dora et al., 1999). Cancer can result in humans, for example, when Rb is mutated and activating-E2F complexes drive ectopic cell cycle entry (Attwooll et al., 2004).

A large body of work has shown that the genes in the Rb pathway have a complex evolutionary history as well as diverse functions. For example, mammals have 3 RB homologs which are partially redundant, can cross regulate, and have specific preferences for which types of E2Fs they bind (van den Heuvel and Dyson, 2008). Furthermore, mammals have 8 E2F loci (E2F1-8); E2Fs 1–3 are primarily activators, E2Fs 4–5 are primarily repressors, and E2Fs 6–8 have lesser-known functions (van den Heuvel and Dyson, 2008). Finally, mammals have 3 DP homologs. In total, the high genetic redundancy in combination with the tissue or
developmental stage of the animal, has led to the discoveries that the Rb pathway functions in many diverse processes such as cell cycle control, DNA segregation, apoptosis, cellular senescence, and differentiation (Burkhart and Sage, 2008; Chicas et al., 2010; Classon and Harlow, 2002; Crosby and Almanan, 2004; Moon et al., 2008; Schwitzman et al., 2011; Wildwater et al., 2005). This complexity has made it difficult to ascribe functions to any single gene, and therefore, model organisms that have a simplified genetic context for the Rb pathway can shed light on both its evolution and function.

The Rb pathway has also undergone considerable evolution in different animal phyla. *Drosophila* has 2 RBs, 2 E2Fs (1 activator, 1 repressor), and 1 DP (Betz et al., 1998). *C. elegans* has 1 RB, 3 E2Fs (only one of which has a mutant phenotype), and 1 DP. Low genetic redundancy, typical of invertebrates, has allowed the elegant genetic dissection of Rb pathway functions (van den Heuvel and Dyson, 2008). These model systems have shown that RB can transcriptionally repress target loci through the recruitment of repressive chromatin remodelers such as histone deacetylase 1 (HDAC1) or complexes such as DREAM or NuRD (Cui et al., 2006; Dufourcq et al., 2002; Ferreira et al., 1998; Lu and Horvitz, 1998; Rossi et al., 1998). Interestingly, the *C. elegans* Rb pathway functions primarily to antagonize RAS-signalling in vulval differentiation, and has minimal roles in cell division control (Lu and Horvitz, 1998). In *Drosophila*, the Rb pathway controls cell cycle entry during development by balancing activator and repressor E2F function (Dimova et al., 2003; Frolov et al., 2001; Stevaux et al., 2002). Interestingly, mutating both fly E2Fs leads to relatively normal cell division and development until larval stages (Frolov et al., 2001). In summary, work from flies and *C. elegans* suggests that the Rb pathway does not function in tumor suppression per se, even though it has roles in aspects of cell division, cell death, and differentiation.

Despite the large body of work in vertebrates, flies, and *C. elegans*, it has been difficult to test Rb pathway function in adult animals or adult stem cells (ASCs), and there is virtually no functional data from animals outside of these clades. Adult freshwater planarians (flatworms) are ideal organisms to test the functional evolution of the Rb pathway because they have a large population of adult stem cells (ASCs), the ability to modulate cell division of their ASCs on a global scale, and their phylogenetic position in the Lophotrochozoan superphylum facilitates evolutionary comparisons. The asexual planarian, *Schmidtea mediterranea* (*S. med*), is a constitutive adult, with no known senescence or aging, and can regenerate from virtually any injury (Elliott and Sánchez Alvarado, 2012; Newmark and Sánchez Alvarado, 2002; Redden et al., 2005a; Redden and Sánchez Alvarado, 2004). Both their longevity and regenerative abilities depend on a large population of pluripotent ASCs. Planarians have already been shown to have conserved genetic mechanisms that control ASC proliferation, which can be drastically modulated in a given context (Wenemoser et al., 2012). For example, when planarians undergo injury, stem cells temporarily hyper-proliferate to replace missing structures through use of TOR signalling (Tu et al., 2012). In contrast, when planarians are starved, they decrease cell division and increase apoptosis (Kadosh and Struhl, 1998). Finally, non-injured planarians use the p53, PTEN, and EGFR pathways to limit proliferation in their stem cell lineages (Fragas et al., 2011; Oviedo, 2008; Pearson and Sánchez Alvarado, 2010). With a completely sequenced genome, low genetic redundancy, and the ability to test gene function in adult animals by RNAi, planarians are a powerful system to understand the mechanisms of proliferation control in ASCs.

In this study, we tested the function of the Rb pathway in proliferation control in planarians. We find that planarians (and their phylum) have undergone the most severe reduction in the Rb pathway described to date for any animal clade. Despite this, each component of the canonical Rb pathway is involved in regulating proliferation of adult stem cells. We find that Lophotrochozoa as a superphylum have lost an E2F7/8 homolog while the flatworms have additionally lost an activating E2F1/2/3 homolog, and have only retained a single, repressive E2F4/5-like molecule (Smed-E2F4-1). When Smed-E2F4-1 was knocked down by RNAi, animals showed briefly increased cell cycle entry, suggesting that this gene indeed functions as a repressor of cell cycle genes. Similar results were obtained for E2F2’s binding partner, Smed-Dp. Our analysis of Rb homologs showed that planarians have a single Rb (Smed-Rb) that was more similar to vertebrate RB-homologs, p107 and p130. This supported previous data that p107/p130 represent the ancestral protein state of the Rb family and that RB itself arose specifically at the base of the Deuterostomes via gene duplication (Cao et al., 2010; Wirt and Sage, 2010). When Smed-Rb was knocked down by RNAi, stem cell self-renewal was abolished. Finally, we investigated the roles of planarian orthologs of upstream (cyclins D and E) and downstream (HDAC1) regulators of RB in other systems. We found that both HDAC1 and cyclinD RNAi animals showed phenotypes consistent with function in the Rb pathway and stem cell self-renewal. Interestingly, planarians and other flatworms appear to have lost a cyclinE ortholog. From these data, we concluded that planarians have retained the ancestral function of the Rb pathway in proliferation control, however, they have done so without retaining cyclinE or an activating E2F-like molecule. From this, we hypothesize that planarians have evolved to transcribe cell cycle entry genes through the negative regulation of a repressive E2F complex.

**Materials and methods**

**Phylogenetics and cloning**

Rb pathway gene homologs were found in the sequenced and assembled planarian genome as previously described (Pearson and Sánchez Alvarado, 2010; Robb et al., 2008; Sánchez Alvarado et al., 2002, 2003). Primers were designed and full length genes were cloned by 3’ RACE. Cloned ORFs were then converted to predicted proteins and subjected to Bayesian phylogenetic analyses. Protein sequences used in phylogenies were obtained from the NCBI Entrez protein database or directly from the genome sequencing projects of included organisms. The program Geneious (www.geneious.) was used with the MUSCLE alignment plugin, and two tree building plugins for Geneious were used as independent analyses. Both Maximum Likelihood and Bayesian analyses were performed with the following settings: Maximum Likelihood – 10,000 bootstrap replicates, WAG substitution model, estimated distances; Bayesian – 1millon replicates, WAG substitution model, 4 heated chains, 25% burnin, subsample frequency of 1000. Consensus tree images were saved through Geneious, which were then manipulated in Adobe Photoshop. Alignments are provided as supplemental files A1-A2. Smed-Rb, Smed-E2F4-1, Smed-HDAC1, Smed-cyclinD, and Smed-Dp sequences were deposited in GenBank as accession numbers: JX967264-JX967268, respectively. Transcripts listed in Tables S2–S3 can be found in the transcriptome database associated with the NCBI GEO project: GSE37910.

**Animal husbandry and RNAi**

Asexual *S. mediterranea* CIW4 strain were reared as previously described (Sánchez Alvarado et al., 2002). RNAi experiments were performed using previously described expression constructs and HT115 bacteria (Newmark et al., 2003). Briefly, bacteria were grown to an O.D.600 of 0.8 and induced with
1 mM IPTG for 2 h. Bacteria were pelleted and mixed with liver paste at a ratio of 500 µl of liver per 100 ml of original culture volume. Bacterial pellets were thoroughly mixed into the liver paste and frozen as aliquots. The negative control, "control(RNAi)", was the unc22 sequence from C. elegans as previously described (Reddien et al., 2005a). All RNAi food was fed to 7-day starved experimental worms every 3rd day for 3 total feedings for Rb, HDAC1, and cyclinD, and 4 feedings for Dp and E2F4-1. All time points are given post-RNAi where day 0 was the day of the third feed for all conditions, and day 3 is the 4th feed for Dp and E2F4-1 and their respective controls. Amputations were performed 7 days after the final feeding unless noted otherwise. All animals used for immunostaining were 2–3 mm in length and size-matched between experimental and control worms.

Deep sequencing

RNA deep sequencing of the planarian stem cell, stem cell progeny, and differentiated tissue compartments was recently performed (Labbe et al., 2012). Briefly, we used flow cytometry to obtain a cell population highly enriched in stem cells as previously described (Hayashi et al., 2006). Approximately 1.3 million stem cells were isolated on a Becton–Dickinson FACSAria over 7 independent sorts. Two samples of RNA were subjected to next generation sequencing for comparison of differential expression enrichment in the stem cell compartment: (1) sorted stem cells; and (2) irradiated worms 7 days post-irradiation which have no enrichment in the stem cell compartment: (1) sorted stem cells; and (2) irradiated worms

Immuno-labeling, TUNEL, and in situ hybridization (ISH)

ISH, double-fluorescent ISH (dFISH), and immunostaining were performed as previously described (Pearson et al., 2009). Colorimetric ISH and fluorescent phospho-histone H3 (H3P) stains were imaged on a Leica M165 fluorescent dissecting microscope. The rabbit monoclonal antibody to H3ser10p from Millipore (04–817) was used for all cell division assays. TUNEL was performed as previously described (Pelletieri and Alvarado, 2007). Rabbit anti-PIWI-1 (gift of Dr. Peter Reddien (Wagner et al., 2011)) was used at 1:1500. TUNEL/PIWI-1 double stains were imaged on a Leica DMIRE2 inverted fluorescence microscope with a Hamamatsu Back-Thinned EM-CCD camera and spinning disc confocal scan head. H3ser10p and TUNEL were quantified using freely available ImageJ software (http://rsb.info.nih.gov/ij/).

Results

Platyhelminthes have single Rb and Dp homologs, and have undergone an evolutionary reduction in E2F genes

Based on previous studies, it is clear that the ancestral state of RB was closer in sequence and function to vertebrate p107/p130 proteins (Cao et al., 2010; Wirt and Sage, 2010). However, no study has examined sequences from the Lophotrochozoa, where several genome sequencing projects have been recently performed. Examining sequences in the Lophotrochozoa is critical because it is formally possible that RB and p107/p130 orthologs exist in this superphylum and were lost in the Ecdysozoa. Thus, the S. mediterranea genome and transcriptome were extensively searched (Labbe et al., 2012; Robb et al., 2008; Sánchez Alvarado et al., 2003), and similar to other non-chordates, a single homolog of Rb was found (Smed-Rb; Rb for the rest of this article). The full-length Rb was cloned and phylogenetic analyses were performed, which gave several interesting results (Fig. 1A). First, by using genomic sequences of four Lophotrochozoans, it was clear that the ancestral state of Rb homologs was a single p107/p130-like molecule. In addition, the two fly homologs of RB are a Drosophila-specific duplication as three other insects and a crustacean do not have more than a single RB homolog. Finally, in the Deuterostome lineage, RB and p107/p130 split from each other very early because two other non-vertebrate chordates have clear RB and p107/p130 orthologs. Together, phylogenetic analysis supported previous hypotheses that invertebrate RB homologs are structurally more similar to p107/p130 and also represent the ancestral sequence of the molecule (Cao et al., 2010; Wirt and Sage, 2010). However, despite the fact the SMED-RB had approximately 10% more amino acid identity to p107/p130, we were unable to identify domains that are present in vertebrate p107 and p130 and absent in vertebrate RB. These include an N-terminal CDK-inhibitory domain, and a cyclin-binding domain inside a split Rb-B pocket domain (Cao et al., 2010; Wirt and Sage, 2010). These domain rearrangements are thus likely to be vertebrate-specific alterations because fly and C. elegans Rb homologs also lack these features.

A search for a DP-like protein in the planarian genome uncovered the presence of a single Dp gene, which has been the case for all non-vertebrate organisms examined (Smad-Dp; Dp for the rest of this manuscript). The planarian E2F gene complement was more complex and interesting. A search of the planarian transcriptome returned a single E2F. However, a search of the planarian genome detected 4 other regions of E2F homology. Three of these other E2Fs appeared to be recent duplicates based on high nucleotide identity (i.e., 87–91% nucleotide identity) with the intact E2F transcript, which explained why transcript assembly performed, which explained why transcript assembly could not assemble multiple E2F genes (Labbe et al., 2012). In addition, two of the four genes had stop codons in key domains in their predicted amino acid sequences, which suggested that these may represent pseudogenes (we have named these sequences Smad-pE2Fa-d). We were unsuccessful in cloning any of these paralogous or pseudo-E2Fs, suggesting that they are either not transcribed in asexual planarians or are degraded quickly. Finally, it is noteworthy that even if the pseudo-E2Fs were transcribed, RNAi is predicted to knock them down due to the near identical nucleotide sequences.

The evolution of the E2F family has never been completely understood, primarily because Lophotrochozoan genomes have not been accessible until recently. To understand the evolution of this gene family as a whole and in the flatworms, phylogenetic analysis was performed using 6 Lophotrochozoans, which included 4 flatworm species (Fig. 1B). This complement of species yielded several interesting results. First, the ancestral E2F gene complement for all animals was a single member of each: E2F1/2/3, E2F4/5, and E2F7/8. This conclusion was reached because we observed that C. elegans, the insect Nasonia vitripennis, and the cnidarian Nematostella vectensis, have representative molecules of all three E2F types (as do vertebrates). Second, it is clear that E2Fs 1, 2, 3, and 6 arose specifically in the vertebrates through duplication of a single gene. Third, Lophotrochozoans appear to have lost an E2F7/8 homolog, which could not be found in members of molluscs, annelids, or platyhelminthes. In terms of the evolution of E2F genes in the Platyhelminthes, we observed that an activating E2F1/2/3-like molecule was additionally lost in this phylum. Finally, the observed S. mediterranea pseudo-E2F
Fig. 1. Evolution of the Rb pathway and analysis of Smed-Rb expression. (A) A Bayesian phylogeny of RB homologs. The S. mediterranea homolog used in this study clearly groups with p107/p130. Non-vertebrate chordates have clear RB orthologs, and thus the split of RB from p107/p130 occurred at the base of the Deuterostomes. Only Drosophila melanogaster was found to have more than a single RB homolog in the Protostomes used. (B) A Bayesian phylogeny of the E2F family. Nematodes, insects, and vertebrates have representative members of each E2F sub-type. No E2F7/8 homolog could be found in the Lophotrochozoans, and no activating E2F1/2/3/6 could be found in the flatworms. The S. mediterranea homolog used in this study clearly groups with E2F4/5. (C) WISH for the stem cell marker piwi-1, and Rb. Rb showed a stem cell specific pattern, which was ablated by 24 h post-irradiation, similar to piwi-1. Rb expression in the brain remains following irradiation (blue arrowheads). White asterisks mark the pharynx. (D) Double FISH for piwi-1 and Rb. White cells indicate double-positive cells which make up 90.5% of the piwi-1+ cell population. Examples of the remaining 9.5% of piwi-1 single-positive cells are marked by yellow arrowheads. The blue arrowheads mark the Rb brain-specific expression. For phylogenies: only bootstrap values over 50 or key nodes are shown; arrows indicate the S. mediterranea homolog used in this study. Species used: Mb = Monosiga brevicollis; Nv = Nematostella vectensis; Crei = Chlamydomonas reinhardtii; Ce = C. elegans; As = Ascaris suum; Bm = Brugia malayi; Dm = Drosophila melanogaster; Tc = Tribolium castaneum; Ag = Anopheles gambiae; Nvit = Nasonia vitripennis; Dpul = Daphnia pulex; Lg = Lottia gigantea; Hr = Helobdella robusta; Cc = Capitella capitata; Mlig = Macrostomum lignano; Sman = Schistosoma mansoni; Em = Echinococcus multilocularis; Smed = Schmidtea mediterranea; Sp = Strongylocentrotus purpuratus; Bf = Branchiostoma floridae; Dr = Danio rerio; Tn = Tetraodon nigroviridis; Gg = Gallus gallus; Mm = Mus musculus; Hs = Homo sapiens.
duplications do not exist in other sequenced flatworms, nor do these molecules represent other E2F types (i.e., all are predicted to be E2F4/5 homologs). Whether these duplicates are specific to S. mediterranea or are representative of other free-living flatworms of the triclads or polyclads remains to be determined upon further genome sequencing. In conclusion, while Rb and Dp genes are represented by the ancestral single homolog in planarians, the E2F family had an interesting evolutionary history and has been reduced from 3 ancestral genes to 1 of the repressive-type E2F4/5 in flatworms. Thus, we have named this gene Smed-E2F4-1 (E2F4-1 for the rest of this study).

Planarian Rb, E2F4-1, and Dp are all required for stem cell function during normal tissue homeostasis and during regeneration

To investigate the effects of Rb, E2F4-1, or Dp on stem cell function, we knocked down expression of these genes using RNAi, then examined animals for defects in tissue homeostasis in uninjured animals. Stem cell loss in planarians by irradiation or RNAi against stem cell-specific genes results in characteristic stem cell deficient phenotypes including head regression, dorsal lesions, and ventral curling (Pearson and Sanchez Alvarado, 2010; Reddien et al., 2005a). Administration of RNAi against either Rb, E2F4-1, or Dp led to the development of these stem cell deficient phenotypes in 100% of the worms by 14–16 days post-RNAi (Fig. 2A). It should be noted that a single lesion, directly dorsal to the pharynx, was observed in E2F4-1 and Dp RNAi animals, while multiple dorsal lesions were observed in Rb(RNAi) animals. Although dorsal lesioning is commonly observed in the course of stem cell deficient phenotypes, it is not a required step in the progression, and it is largely unknown why it is observed. Together, however, these phenotypes indicated that stem cell output during tissue homeostasis requires the activity of the Rb pathway.

The ability of planarians to regenerate is another assay to examine proper stem cell functions in response to injury, which involves temporary hyper-proliferation to make the missing tissue, and production of the correct differentiated progeny. RNAi-treated worms were assayed using the same feeding schedule as the homeostasis experiments combined with amputation of the worms into 3 fragments 7 days after the final feedings (the very beginning of any observable phenotypes). In all 3 RNAi treatments, regeneration ability was markedly reduced at 3 days of regeneration and severely compromised by 7 days of regeneration (Fig. 2B). This suggested that perturbation of the Rb pathway impairs stem cell function, concordant with observations during tissue homeostasis. Despite clear stem cell defective phenotypes for both intact homeostasis and regeneration, these

---

**Fig. 2.** Live images representing the RNAi phenotypes from knockdown of Rb, E2F4-1 and Dp genes. (A) Intact phenotypes. Rb typically presented multiple dorsal lesions approximately 9 days following RNAi feeding (yellow arrows). E2F4-1 and Dp showed a single dorsal lesion directly over the pharynx. Despite this, all RNAi treatments ultimately resulted in a stereotypical stem cell deficient phenotype of ventral curling by 14 days post RNAi. (B) Time course of head regeneration. Each image shows a tail regenerating a new head at either day 3 or 7 post amputation. White arrowheads mark the edges of the amputation, and any newly regenerated tissue is unpigmented. Rb(RNAi) animals showed virtually no regeneration by day 7. E2F4-1 and Dp RNAi animals showed slightly more regeneration than Rb, yet were deficient for regenerative ability. These results demonstrated a requirement for the Rb pathway in both tissue homeostasis and regeneration.

---

gross phenotypes do not predict a specific molecular mechanism. Therefore, we next measured proliferation, survival, and differentiation following Rb pathway knockdown.

The Rb pathway regulates stem cell division in planarians

Because the core Rb pathway is well established in other model systems to have key roles in cell division, we first examined how numbers of dividing cells may be changing during the time course of the Rb, E2F4-1, or Dp RNAi phenotypes. To achieve this, we measured the number of cells undergoing the G2/M transition of the cell cycle using the antibody to Histone-H3 phosphorylated on serine 10 (H3P). We quantified the staining every 3 days for 15 days following RNAi, where day 0 in our time course is the day of the third RNAi feeding. When knocked down, Rb(RNAi) animals showed a slight, yet significant increase in cell proliferation at day 0 compared to controls, while days 3 and 6 showed no difference with controls. After day 6, however, Rb(RNAi) animals showed significant hypo-proliferation, which suggested that Rb is either required for mitotic progression, stem cell self-renewal, or for stem cell survival.

Due to the sequence similarity of E2F4-1 to the repressive E2Fs 4 and 5, we predicted that SMED-E2F4-1 may function as a repressor. If the function of E2F in controlling cell cycle genes has been conserved in planarians, then knockdown of a repressive E2F may show de-repression of cell cycle genes and increased cell cycle entry. In addition, because planarians only have a single Dp gene, we predicted that all E2F function requires DP, and both should have closely matched RNAi phenotypes. When we knocked down either E2F4-1 or Dp by RNAi, significant increases in cell division were observed for both conditions during the first 3–6 days following RNAi (Fig. 3). However, similar to Rb(RNAi) animals, E2F4-1 and Dp RNAi animals ultimately resulted in loss of proliferation. In total, our data showed that Rb pathway components are required for proper proliferation control and stem cell function in planarians. We next tested the effects of losing Rb, E2F4-1 or Dp on the maintenance of the stem cell population and the differentiation of stem cell progeny.

Rb, E2F4-1, and Dp are ultimately required for stem cell maintenance

The gross disruption of homeostasis and regeneration suggested that the Rb pathway is necessary for stem cell output in planarians (Fig. 2), however, the specific defects in lineage production can happen at multiple levels in the lineage hierarchy while yielding the same morphological phenotypes (Reddien et al., 2005b). Planarians offer significant advantages to visualize the entire stem cell population and stem cell progeny in whole animals during the time course of phenotypic progression. It has been shown that a large number of “early progeny” from planarian stem cells become post-mitotic and express the marker Smed-prog-1 within 24 h of their birth (formerly Smed-NB21.11e) and then transition to a “late progeny” fate within the next 24–48 h (Eisenhoffer et al., 2008). Combined with the stem cell marker piwi-1, we can examine the population dynamics of these three cell types, which can then provide mechanistic insight as to how lineage development is affected. For example, if we observe a decrease in stem cells and a concurrent increase in stem cell progeny, then we would interpret this as premature differentiation of the stem cell population. On the other hand, if all three cell populations decrease with similar kinetics, this would be indicative of a stem cell defect in the processes of cell division, self-renewal, or cell survival. Analyses of the stem cells and their progeny were performed for 15 days following RNAi for each gene, and a steady decrease in both stem cells and their early progeny was observed (Fig. 4). Furthermore, the late progeny marker, AGAT, followed a similar time course of loss (Figs. S2–S3) (Eisenhoffer et al., 2008). These data suggested that the Rb pathway is not necessary for the differentiation process, and instead, the stem cell population is specifically affected, consistent with our results for proliferation. Thus, the Rb pathway is either necessary for stem cell division/self-renewal or cell survival, which was tested next.

Planarian Rb is required for stem cell self-renewal and somatic cell survival

Roles of vertebrate p107 and p130 in promoting cell survival and differentiation are well known (Crosby and Almasan, 2004; Jiang et al., 2010; Manning and Dyson, 2011; van den Heuvel and Dyson, 2008). While we observed temporary stem cell hyper-proliferation in both E2F4-1 and Dp RNAi-treated worms, these knockdowns as well as Rb(RNAi) ultimately resulted in a loss of stem cells. Because SMED-RB is more closely related in sequence to p107/p130, we hypothesized that the loss of stem cells in

---

**Fig. 3.** Cell division (anti-H3P) during the course of Rb, Dp, or E2F4-1 RNAi phenotypes. (A) Each RNAi treatment was normalized to control worms and plotted above. At day 3 post-RNAi, E2F4-1 and Dp RNAi animals showed significant increases in cell division, while Rb(RNAi) animals showed mostly hypo-proliferation (Student’s t-test, error bars are standard error). (B) Representative stains for both the hyper- (day 3) and hypo- (day 15) proliferation phases are shown to the right. Images were taken from the dorsal side with anterior to the left. Significance indicators: *p < 0.05; **p < 0.01; ***p < 0.001.
Rb(RNAi) animals may be due to increased apoptosis of stem cells, as opposed to loss of self-renewal or increased exit from the cell cycle. To test this, we first measured global cell death by TUNEL labelling in Rb(RNAi) treated worms and then quantified cell corpses. Following Rb(RNAi), we observed significant increases in cell death during the time course of phenotypic progression (Fig. 5A).

We hypothesized that the stem cell loss we observed was specifically due to increases in stem cell death. To test this, we performed a double labeling for PIWI-1 protein and TUNEL, and then quantified levels of co-localization in RNAi-treated worms. It should be noted that while the PIWI-1 antibody labels all piwi-1\textsuperscript{+} stem cells, the PIWI-1 protein also perdures into and labels early stem cell progeny to some extent (Guo et al., 2006; Scimone et al., 2011). We focused our analysis on day 6 after RNAi feedings as Rb(RNAi) animals still possess a substantial stem cell population at this time point yet also exhibit increased TUNEL staining (Figs. 4 and 5A). Following quantification of PIWI-1\textsuperscript{TUNEL\textsuperscript{+}} cells, we made several interesting observations. First, we unexpectedly detected TUNEL\textsuperscript{+} PIWI-1\textsuperscript{+} cells in our control(RNAi) animals (Fig. 5C, E), which suggested that there is a basal level of cell death experienced by unperturbed PIWI\textsuperscript{+} cells in planarians. Second, we could not detect significant increases in cell death in the PIWI-1\textsuperscript{+} population of cells upon knockdown of Rb (Fig. 5E), which suggested that the stem cells may not require Rb for survival. Finally, in several more phenotypically-severe Rb(RNAi) animals at this time point, we observed high levels of TUNEL labeling in anterior regions of animals where the stem cells had already been lost (Fig. 5D). Thus, while we could not conclude that Rb is required for stem cell survival, the contribution to increased global cell death is due to apoptosis of non-stem cell types. Similar to the role of p107 in the survival of mouse cortical neurons (Vanderluit et al., 2007), these dying cells may be the neural cell types in the planarian brain that also express planarian Rb, which was supported using TUNEL, the neural marker anti-SYNAPSIN, and DAPI (Fig. 5F). From these data, we concluded that Rb was not required for stem cell survival, and thus the loss of cell division and stem cell population in Rb(RNAi) animals was likely due to defects in self-renewal.

Smed-HDAC1 and Smed-cyclinD are also required for stem cell self-renewal

Our results on the core Rb pathway components in planarians showed a requirement for the pathway in stem cell self-renewal. However, we sought to extend our analyses to include an upstream and downstream regulator of RB function in other systems. It is known that interaction with chromatin modifiers is another major branch of RB functional activity (Chae et al., 2012; Cui et al., 2006; Dufourcq et al., 2002; Ferreira et al., 1998; Lu and Horvitz, 1998). For example, RB is known in other systems to bind several chromatin remodelers, including histone deactylase.

---

**Fig. 4.** Stem cell lineage development requires the Rb pathway. All panels show WISH for either stem cell or stem cell progeny markers during the time course of RNAi phenotypes. All worms used were 2–3 mm in length. Even at the hyper-proliferation stage for E2F-1 and Dp RNAi animals (day 3), increases in either stem cells or stem cell progeny are not seen. By day 15 post-RNAi we observed severe reduction of the stem cells and stem cell progeny, suggesting that the Rb pathway is ultimately required for cell proliferation, self-renewal, or cell survival.
Fig. 5. Cell death quantification during the time course of the Rb(RNAi) phenotype. (A) We detected significant increases in TUNEL positive nuclei (TPN) at most time points of phenotypic progression (Student’s t-test, error bars are standard error). (B)–(D) Anterior is to the left. Confocal images of PIWI-1 (green) and TUNEL (magenta) double stains at day 6 post-RNAi. Asterisks denote position of the eyes, dashed line denotes body-edge. Any strong staining at the body-edge is considered background. (B) control(RNAi), head region; (C) control(RNAi), tail region, inset shows a double-positive cell; (D) Rb(RNAi), head region with minimal stem cells yet high TUNEL labeling in the brain region; (E) Quantification of TUNEL staining in PIWI-1+ cells between control and Rb RNAi animals; (F) A whole-mount stain on an Rb(RNAi) animal 9 days post RNAi, anterior is to the left. Using the pan-neural synaptic marker anti-SYNAPSin (green) and nuclei label DAPI (blue), the brain is easily visualized. TUNEL+ cells (red) were detected within the boundaries of the brain (white arrowheads), as well as peripheral neurons of the head (white arrows). Many other cells labeled near the margin of the brain, and could not be distinctly counted as being neural in position (yellow arrowheads). From these data, some of the increased cell death observed when Rb is knocked down, occurs in the brain. Significance indicators: * p < 0.05; ** p < 0.01; *** p < 0.001.

1 (HDAC1), which can mediate the transcriptional repression of RB target genes and also has roles in regulating embryonic stem cell self-renewal and differentiation (Dovey et al., 2010; Ferreira et al., 1998; van den Heuvel and Dyson, 2008). In addition, CyclinD-CDK4/6 and CyclinE-CDK2 complexes are well known to phosphorylate and modify RB activity (Malumbres and Barbacid, 2005). Similar to our analysis for the other components of the Rb pathway, we searched the planarian genome and transcriptome for HDAC1, cyclinD, cyclinE, and CDKs 2, 4, and 6. Interestingly, planarians were found to have 20 homologs to cyclinE genes, though no homologs to cyclinD could be detected (their expression analysis is summarized in Table S2). To ensure that this sequence was not simply missing in planarians, 3 other flatworm genomes were examined and no cyclinE genes could be found, while 2 non-flatworm Lophotrochozoans had clear cyclinE genes. Thus, it appeared that the loss of an activating E2F in flatworms coincides with loss of cyclinE (see Discussion). For the CDK gene family, 30 CDK homologs were detected and a best reciprocal BLAST hit to CDK2, 4, and 6 were all found (their expression analysis is summarized in Table S3). Importantly, we observed that the CyclinD-binding CDKs (4 and 6) were highly enriched in stem cells, whereas the predicted CyclinE-binding CDK (2) had virtually no expression in any cell compartment.

cycD1, cycE1, and cycD2 transcripts showed a lower expression based on RNAseq, suggesting that it may no longer alter them. Here we demonstrated that despite conservation from plants to animals, the Rb pathway can be substantially modified. In the first study to examine Rb pathway function and gene complement in Lophotrochozoans, we showed that freshwater planarians have conserved the ancestral roles for Rb in cell division and cell death. However, flatworms as a phylum have undergone loss of an activating E2F homolog. Further analysis showed that flatworms have also lost a cyclinE homolog, which is a critical regulator of RB activity in other systems. It is interesting to hypothesize that the loss of either cyclinE or the activating E2F was an initial trigger that selected for the rapid loss of the other gene to balance pathway activity (Fig. 7). On the other hand, perhaps another cyclin has taken up the role of cyclinE in planarians, or another molecule has substituted for the loss of an activating E2F. Interestingly, the canonical binding partner of CyclinE, CDR2, still exists in planarians, but has extraordinarily low expression based on RNAseq, suggesting that it may no longer function in the cell cycle (Fig. 7).

Our analysis of planarian Rb has shown that it has more sequence similarity to p107/p130 in vertebrates, and our functional analyses support this conclusion. Similar to p107 in mice, we show that Smed-Rb does not behave like a classical tumor suppressor, but rather is required for maintaining stem cell populations in the planarian nervous system. Interestingly, the Rb pathway is evolutionarily conserved across multiple phyla, from flatworms to insects, suggesting that it may play a conserved role in regulating stem cell proliferation and regeneration.
Fig. 6. Functions of HDAC1 and cycD in planarians. (A) Similar to Rb, HDAC1 showed a stem cell specific expression pattern, which was ablated by 24 h following 60 Gy of irradiation. Also observed was brain-specific staining once the stem cell pattern was ablated (blue arrowheads). Asterisks mark the pharynx. (B) Effects of HDAC1 and cycD RNAi on stem cell lineage development showed dramatic reduction of both the stem cell population and stem cell progeny by day 6 post-RNAi. (C) When knocked down by RNAi, HDAC1 and cycD animals showed stem cell defective phenotypes during both intact homeostasis and during head regeneration (white arrowheads mark dorsal lesions or amputation plane). (D) As stem cells are lost, cell division goes down as expected, as measured by anti-H3P (Student’s t-test, error bars are standard error). *** p < 0.001.

Fig. 7. Model of Rb pathway function in planarians. (A) Planarian Rb functions at multiple levels in stem cell lineage development. Our data suggested that Rb and HDAC1 are specifically expressed in both stem cells and post-mitotic neurons in the brain. When knocked down by RNAi, Rb and HDAC1 were required for stem cell self-renewal. Some of the increase in cell death in Rb(RNAi) animals occurs in the brain, and Rb may have a pro-survival role in neurons. (B) Shown is the canonical regulation of Rb activity by phosphorylation by cyclin/CDK complexes. When Rb is inactivated, activating E2F1-3/Dp complexes activate transcription of genes that drive entry into the cell cycle. (C) Based on the data, shown is a graphical hypothesis how planarian Rb functions in adult stem cell proliferation control. Despite no activating E2F, and no cyclinE, the core Rb pathway in planarians still controls cell division. We hypothesize that HDAC1 and CyclinD are still involved in this process, but may function slightly differently to compensate for cyclinE and E2F1-3 loss. Red line through cyclinE denotes gene loss, while red line through CDK2 denotes no expression. We hypothesize that with no activating E2F1-3, planarians may use an unknown activating transcription factor to transcribe cell cycle genes and enter the cell cycle.
suppressor: it is highly transcribed in cycling cells, is required for cell-cycle progression, and promotes cell survival. Unlike p107, it does not appear that Smad-Rb is required for differentiation because stem cell progeny were produced during the entire time course of the Rb(RNAi) phenotypic progression (Fig. 4). Overall, our results support the previous hypothesis that the ancestral function of RB-like molecules was in cell division and cell survival, similar to p107 and p130 in vertebrates, and when combined with the plant and vertebrate data, also suggests that RB evolved for specific roles in stem cell biology. It will be interesting to further test whether the Drosophila Rb genes have any unappreciated role in adult stem cell compartments such as intestinal stem cells or neural stem cells.

**E2F/Dp-independent roles of planarian Rb?**

In other systems, RB proteins have been reported to have approximately 100 binding partners in addition to E2F and DP proteins (van den Heuvel and Dyson, 2008). Thus, similar to other systems (Chi and Reinke, 2006), it is likely that planarian RB may have roles independent of E2F4-1/DP and cell cycle regulation, which is supported by the differences in phenotypic progression between Rb(RNAi) and E2F4-1 or Dp RNAi worms. For example, the very different kinetics of cell division suggests that RB may have a different role in the stem cell from E2F4-1 and DP. Perhaps this role is in chromatin remodeling to repress a different set of target genes than the E2F4-1 targets. A recent report of another RB-binding chromatin remodeler, RbAp48, also showed a stem cell defective phenotype in the planarian Dugesia japonica, although lineage markers were not used to analyze the phenotype (Bonuccelli et al., 2010). Another well-known binding partner of RB proteins, which is also involved in chromatin remodeling, is histone deacetylase 1 (HDAC1) (Ferreira et al., 1998). We find both RbAp48 and HDAC1 to be very highly expressed specifically in planarian stem cells (Table S1), and our results upon knockdown of HDAC1 are consistent with the self-renewal and proliferation defects observed for Rb(RNAi). In future studies, it will be interesting to determine biochemically what other proteins bind RB in planarians to elucidate complexes that function independently of cell cycle regulation. Given the expression of Rb and HDAC1 in the planarian brain and cell death observed in the brain of Rb(RNAi) animals, one possibility is that RB functions in a specific, pro-survival protein complex in planarian neurons.

**p53 and Rb tumor suppressor pathways in stem cell lineage development**

In mammals, Rb and p53 pathways are known to converge and synergize in many tumor types, such that double mutants of both genes are more severe than either single mutants (Beck et al., 2000; Berman et al., 2008; Chow et al., 2011; Meuwissen et al., 2003; Miyamoto et al., 1996; Moon et al., 2008; Schwartzman et al., 2011; Zhou et al., 2006). We have previously shown that planarian p53 primarily functions in newly-born stem cell progeny (prog1+ population) to promote differentiation and cell cycle exit. Therefore, a simple model for loss of stem cells in Rb(RNAi) animals would be the de-repression of p53 in stem cells, which would cause cell cycle exit and premature differentiation. When we tested Rb(RNAi) animals for de-repression of p53, we did not observe increases in p53 expression, and therefore we believe that crosstalk between these pathways may not be occurring in planarians (Fig. S4). In addition, p53 is expressed in a different spatial domain than Rb, and p53(RNAi) results in dramatically different effects on lineage production than knockdown of the Rb pathway (Pearson and Sanchez Alvarado, 2010). Finally, we did not observe increases in differentiating cells in Rb(RNAi) animals (Fig. 4). Together this suggests that both pathways serve fundamentally different roles in stem cell lineage determination and cell cycle control (Fig. 7A). This is not entirely unexpected because while the Rb and p53 pathways converge in mammalian neural stem cell lineage development and tumorigenesis, they appear to have differing roles in the hematopoietic stem cell compartment (Liu et al., 2009; Walkley and Orkin, 2006). In future experiments, it will be critical to understand what the genomic targets of each pathway are in order to further understand their roles in stem cell lineage development and proliferation control.

**Acknowledgements**

We would like to thank Alejandro Sánchez Alvarado (Stowers Institute, HHMI) for advice and reagents, and Eric Ross for aligning RNAseq data to specific transcripts. Thanks to S. Hutchinson and K. Gurely for comments on the manuscript. This work was supported by the Ontario Institute for Cancer Research (OICR) New Investigator award (#IA-026).

**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.10.025.

**References**
