RUNX in Invertebrates

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S. Hughes and A. Woollard

Abstract

Runx genes have been identified in all metazoans and considerable conservation of function observed across a wide range of phyla. Thus, insight gained from studying simple model organisms is invaluable in understanding RUNX biology in higher animals. Consequently, this chapter will focus on the *Runx* genes in the diploblasts, which includes sea anemones and sponges, as well as the lower triploblasts, including the sea urchin, nematode, planaria and insect. Due to the high degree of functional redundancy amongst vertebrate *Runx* genes, simpler model organisms with a solo *Runx* gene, like *C. elegans*, are invaluable systems in which to probe the molecular basis of RUNX function within a whole organism. Additionally, comparative analyses of Runx sequence and function allows for the development of novel evolutionary insights. Strikingly, recent data has emerged that reveals the presence of a *Runx* gene in a protist, demonstrating even more widespread occurrence of *Runx* genes than was previously thought. This review will summarize recent progress in using invertebrate organisms to investigate RUNX function during development and regeneration, highlighting emerging unifying themes.

Keywords

Runx • Runt • *rnt-1* • *C. elegans* • Planarian • Sea urchin • *Drosophila*

1.1 Introduction

Although the triploblasts (which include mammals, insects, nematodes and sea urchins) and the diploblasts (corals and jellyfish) diverged very early in evolution, there are striking similarities between both groups, suggesting that a simple genetic "toolkit" directed the development of the

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common ancestor (Schierwater et al. 2009). Indeed, developmentally important transcription factors originated early in evolution and underwent a rapid expansion in number during early eumetazoan evolution (Coffman 2009; Degnan et al. 2009; Sebe-Pedros et al. 2011).

Transcription factors play crucial roles in development, as evidenced by the fact that a large proportion of developmentally impaired mutants in model organisms such as *Drosophila* and *C. elegans* have lesions in transcription factor genes. RUNX transcription factors are known for their involvement in several different embryonic and adult developmental processes, centered on controlling developmental decisions between cell proliferation and differentiation via interaction with various signal transduction pathways (Duffy et al. 1991; Coffman 2003, 2009; Nimmo and Woollard 2008). In almost all cases, RUNX function has been shown to be dependent on binding to CBFbeta, which acts to increase the affinity and specificity of DNA binding to target genes (Golling et al. 1996; Adya et al. 2000; Kaminker et al. 2001; Kagoshima et al. 2007). RUNX factors are also associated with context-dependent regulation via interaction with co-activators (e.g. Core Binding Factor, CBF and acetyltransferases e.g. p300) and co-repressors (e.g. Groucho) (Ito 1999; Speck 2001; Coffman 2003; Durst and Hiebert 2004; Chang et al. 2013).

Although *Runx* genes have been identified in all metazoans (Fig. 1.1), this review will focus on *Runx* in invertebrates. The RUNX family of transcription factors is defined by the presence of a highly conserved 128 amino acid Runt domain (Kagoshima et al. 1993; Crute et al. 1996). The Runt domain contains sites that are required for DNA binding, dimerization of Runx proteins with their binding partners and a C-terminal WRPY motif that is required for the interaction with the Groucho/TLE co-repressor (Kamachi et al. 1990; Kagoshima et al. 1993; Ogawa et al. 1993; Ito 1999). Although *Runx* genes have been identified in all metazoa, the core WRPY motif is absent in the *Runx* homologs of the dermosponge, *Amphimedon queenslandica*, and one of the two planarian *Schmidtea mediterranea Runx* (Robertson et al. 2009). Surprisingly, although *Runx* has until recently been considered to be specific to metazoa, two *Runx* homologs (*Co_Runx1* and *Co_Runx2*) have been identified in the unicellular amoeboid halozoan *Capsaspora owczarzaki*, (Sebe-Pedros et al. 2011). This suggests that *Runx* genes may actually have evolved prior to the divergence of protists from metazoans (Sebe-Pedros et al. 2011). Intriguingly, *Capsaspora* lacks any evidence of a CBFbeta homologue, suggesting RUNX may function independently in this organism. However, it is possible that sequence divergence makes the identification of a *Capsaspora* CBFbeta homologue particularly difficult, as CBFbeta homologues tend to be associated with a greater level of sequence divergence than *Runx* homologues. The functional significance of *Capsaspora Runx* genes remains to be elucidated. Likewise, very little functional information has been obtained from the solo sponge (*Amphimedon queenslandica* and *Oscarella carmela*) and sea squirt (*Ciona intestinalis*) *Runx* genes (Robertson et al. 2009), although these do provide valuable insights into the evolution of this important transcription factor family.

In contrast, several invertebrate phyla have *Runx* genes that have been subjected to extensive functional analysis, offering significant insights into molecular mechanism, functional conservation and possible links with human disease. The two premier model organisms for studying *Runx* are *Drosophila* and *C. elegans* although other useful insights have been gleaned from the sea urchin *Strongylocentrotus purpuratus* and more recently from the planarian flatworm *Schmidtea mediterranea*.

1.2 *Runx* Genes in the Fruit Fly, *Drosophila melanogaster*

Runx genes have been extensively studied in the fruit fly *Drosophila melanogaster*. In *Drosophila* as in other insects, four *Runx* genes have arisen as a consequence of gene duplication, independent of those that lead to the three vertebrate *Runx* genes (Rennert et al. 2003; Bao and Friedrich 2008). The first *Runx* family member to be

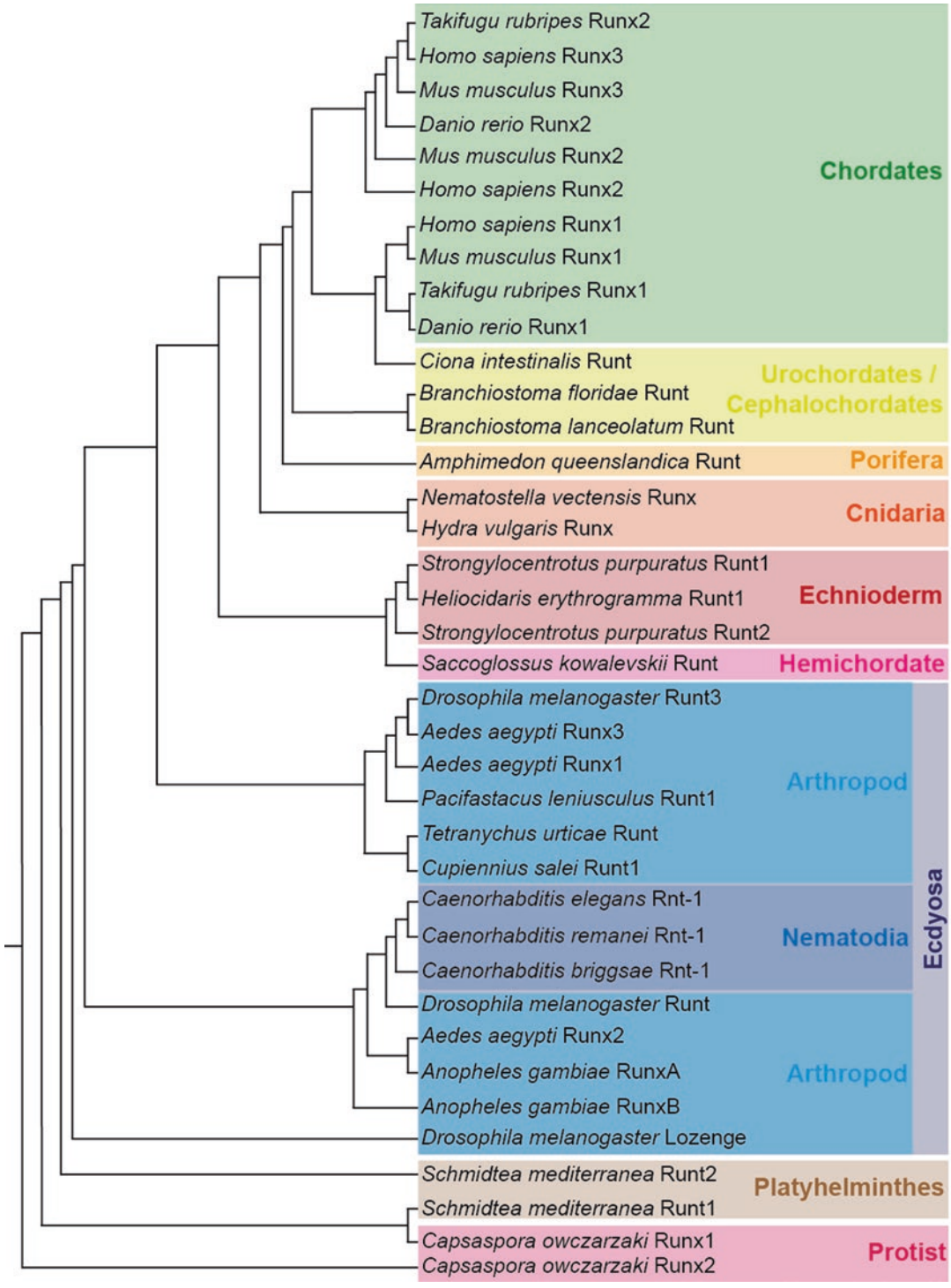

Fig. 1.1 *Runx* genes in the metazoa. *Runx* genes are represented in all major metazoan lineages, with a newly identified *Runx* gene in the unicellular protist *C. owczarzaki*. Alignments of whole Runx protein sequences were undertaken in MAFFT using Neighbor-joining, substitution model JTT and a bootstrap value of 1000 (Katoh et al. 2002)

extensively studied in *Drosophila* was *runt*, from which the whole gene family derived its name. *DmRunt* was isolated for its significant role in segmentation, with *runt* mutant flies being smaller due to the loss of segments (Nusslein-Volhard and Wieschaus 1980; Gergen and Wieschaus 1985). During *Drosophila* embryogenesis, at the mid-to-late blastoderm stage, the pair-rule genes form 7 stripes, whose precise pattern of expression will determine the one-cell-wide stripes of expression of the segment polarity genes (Klinger and Gergen 1993). *DmRunt* is a primary pair-rule gene, which regulates the spatial expression of other pair-rule genes, as well as controlling segment polarity genes. *DmRunt* positively regulates the secondary pair-rule genes, *fushi tarazu (ftz)*, and negatively regulates *hairy*, resulting in the resolution of stripes across the embryo such that *runt* and *ftz* are expressed in complementary stripes to *hairy* (Canon and Banerjee 2000). In addition, *runt* and *hairy* regulate each other independently of *ftz*. The result of this hierarchy, with *runt* at the top, is that the downstream segmentation genes convert positional information into patterns of gene expression, resulting in the generation of a regular and precise body plan.

DmRunt also plays a key role in embryonic neural development (Gergen and Butlet 1988; Kania et al. 1990; Duffy and Gergen 1991; Duffy et al. 1991; Canon and Banerjee 2000). *Drosophila* neurogenesis begins during embryogenesis when the neuroectoderm enlarges and delaminates to form the neuroblast stem cells. These stem cells will divide asymmetrically giving rise to a new neuroblast (self-renewal) and a ganglion mother cell, GMC (differentiated daughter cell), that will further divide to form neurons and/or glial cells (Campos-Ortega and Jan 1991). Expression of *runt* is observed in the GMC and neurons with its activity necessary for the proper expression of *even-skipped* (*eve*) and the formation of EL (*even skipped* (*eve*)-expressing lateral) neurons (Kania et al. 1990; Duffy et al. 1991). *runt* is necessary and sufficient to induce *eve* expression in the *Drosophila* nervous system, however the precise role for *runt* in the development of EL neurons is not fully understood.

Of the three other *Drosophila Runx* genes, the most significant is *lozenge*, *lz*, which was identified via genetic analysis through its contribution to eye development and its involvement in hematopoiesis. The eye develops from an epithelial structure (the eye imaginal disk) during the third larval stage, where an indentation in the epithelium marks the onset of differentiation (Daga et al. 1996). Precursor cells localized anterior to the indentation (the furrow) express *eyeless* while those in the posterior express *lz* (Daga et al. 1996; Yan et al. 2003). *lz* negatively regulates *seven-up* and *deadpan* while simultaneously up-regulating *bar* and *prospero* expression, resulting in the photoreceptors adopting their correct fate (Daga et al. 1996; Canon and Banerjee 2000; Yan et al. 2003). Thus, *lozenge* is crucial for the regulation of cell fate within the equivalence group of cells in the developing *Drosophila* eye.

lz is also a key regulator of cell fate and identity in *Drosophila* hematopoiesis. Multipotent blood cell progenitors are produced during two distinct time points in *Drosophila* development giving rise to three types of differentiated blood cell, collectively called hemocytes. The first wave of hematopoiesis occurs during embryogenesis, where prohemocytes arise from the head mesoderm and form two lateral clusters of cells, which will ultimately differentiate into plasmatocytes or crystal cells. The second wave of hematopoiesis comes during later larval stages, when blood cell progenitors arise from the lymph gland (Waltzer et al. 2010; Gold and Bruckner 2014). The final cell type that contributes to the blood cell population are lamellocytes, which are only produced upon immune challenge when foreign bodies are too large to be phagocytosed (Markus et al. 2009).

During the larval stage of hematopoiesis, there are distinct populations of cells with different differentiation potentials. The medullary zone (MZ) contains undifferentiated quiescent prohemocytes while the adjacent cortical zone (CZ) comprises of differentiated maturing hemocytes derived from the prohemocytes from the MZ

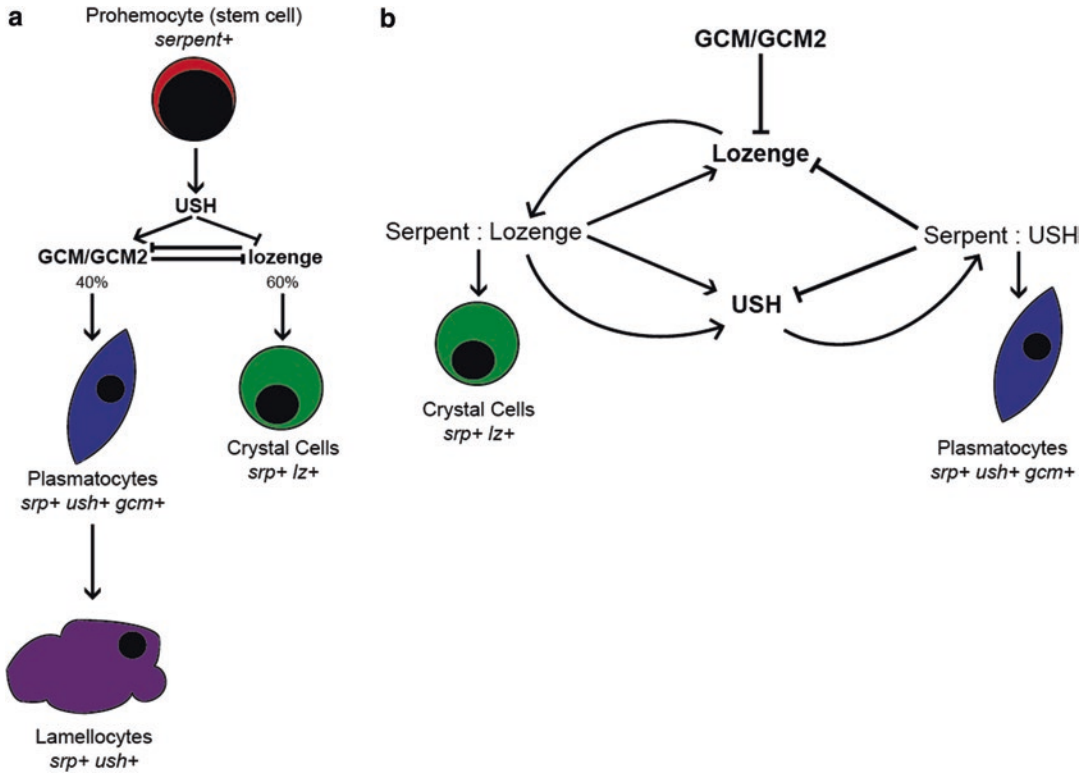

Fig. 1.2 Simplified diagram of the transcription factor network that controls cell fate in *Drosophila* hematopoiesis. (**a**) The prohemocytes are a stem cell population that express the GATA factor *serpent* (*srp*) that activates *ush* (*u-shaped*, friend of GATA (FOG) family) which will in turn function with *gcm/gcm2* (glial cells missing) to commit cells to the plasmatocyte lineage. In 60 % of the srp^+ prohemocytes, expression of *lozenge* (*lz*) will inhibit *gcm/gcm2*, and together with *srp*, will direct cells towards the crystal cell fate. (**b**) The regulation of cell differentiation by *lz/srp/ush* is dynamic, involving a bi-potential regulatory state that resolves two distinct cell populations; the crystal cells and the plasmatocytes. *srp* initiates and maintains *lz* expression. The SRP:LZ complex activates *ush* which will compete with LZ for binding to SRP. The SRP:USH complex negatively regulates both *lz* and *ush*, while GCM/GCM2 will independently suppress *lz* transcription (Adapted from Muratoglu et al. 2007; Braun and Woollard 2009; Wang et al. 2014)

(Jung et al. 2005). *lz* is only expressed in the CZ by prohemocytes adopting the crystal cell fate (Lebestky et al. 2000; Gajewski et al. 2007). Although *lz* expression is activated in all prohemocytes, only 60 % of these lz^+ cells will maintain *lz* expression via a feedback loop and differentiate into crystal cells while the remaining 40 % of cells are $lz^{â^{\wedge}}$ and thus differentiate into plasmatocyes (Fig. 1.2a) (Bataille et al. 2005). The molecular mechanism by which *lz* expression translates to the lineage commitment of prohemocytes to either crystal cells or plasmatocytes involves a complex transcriptional circuit (Muratoglu et al. 2006, 2007). *lz* expression is regulated by a feedback loop involving the pan-hematopoietic GATA factor *serpent*, promoting crystal cell differentiation (Bataille et al. 2005), while expression of *ush* (friend-of-GATA family of transcription factors, *u-shaped*) in lz^+ prohaemocytes is required, together with *serpent*, to direct plasmatocyte cell fate (Fig. 1.2b) (Muratoglu et al. 2007). The complex regulation of *lz, srp* and *ush* is dynamic and results in two distinct cell populations, the plasmatocytes (srp^+ush^+) and crystal cells (srp^+lz^+). Several aspects of this circuitry remain to be elucidated, including the mechanism by which *ush* is turned off in crystal cells.

Additional antagonists of *lz* which direct crystal cell fate are the transcription factors *gcm* (glial cells missing) and its homologue *gcm2*, which act with reciprocal asymmetry with *lz* limiting the expression of *lz* and therefore reducing the production of crystal cells (Alfonso and Jones 2002; Bataille et al. 2005). The mechanism by which *gcm/gcm2* and *ush* act in combination to regulate *lz* expression and maintenance is unclear, but recent work has identified other candidates in the regulation of lineage commitment. Through the Salvador-Warts-Hippo pathway, *yorki* acts in a complex with *scalloped* to control the expression of *lz* and therefore regulate the proliferation and terminal differentiation of progenitor cells into crystal cells (Milton et al. 2014). Thus, *lz* is at the hub of an increasingly complex transcriptional network directing *Drosophila* hematopoiesis.

1.3 *Runx* Genes in the Nematode, *Caenorhabditis elegans*

The single *C. elegans Runx* homolog, *rnt-1*, is an important regulator of the balance between proliferation/self-renewal and differentiation in the lateral neuroectodermal seam cells (Kagoshima et al. 2005; Nimmo et al. 2005; Xia et al. 2007). The seam cells are a group of multipotent stem-cell like cells formed during embryogenesis that divide in a stereotypical pattern throughout larval development. Animals hatch with 10 seam cells per lateral side of the animal, most of which proceed through a re-iterative series of asymmetric divisions, interspersed by the odd symmetrical division in order to expand the number of progenitor cells. In this sense, the seam cells provide a useful paradigm for the stem cell mode of division. In general, at each larval molt there is an asymmetric division producing a posterior daughter cell that retains the ability to self-renew, and an anterior daughter cell that differentiates into either a hypodermal cell, a glial cell or a neuronal cell (Fig. 1.3a) (Sulston and Horvitz 1977). In addition, there is a single symmetrical (proliferative) division at the L2 stage whereby both daughter cells retain the proliferative ability and consequently expand the pool of seam cells so that adult worms have 16 seam cells per side (Fig. 1.3b). At the last larval stage (L4), after the final round of cell division, the seam cells terminally differentiate and fuse into a syncytium. However, although the terminal differentiation of the seam cells occurs at the start of adulthood, the cells are capable of further divisions under certain circumstances, as evidenced in heterochronic mutants (Nimmo and Slack 2009; Harandi and Ambros 2015).

The regulation of this division pattern is controlled by *rnt-1*. In *rnt-1* mutant animals, there are fewer seam cells due to the failure of divisions, specifically the symmetrical L2 division (Nimmo et al. 2005). A similar phenotype was observed in *bro-1* mutants, *bro-1* being the sole *C. elegans* homolog of CBFbeta necessary for correct RNT-1 function (Kagoshima et al. 2007; Xia et al. 2007). BRO-1 enhances the binding affinity and specificity of RNT-1, and is itself regulated by the GATA transcription factor, ELT-1 which acts as a direct activator of *bro-1* to promote seam cell proliferation (Brabin et al. 2011).

In contrast to the mutant phenotype of fewer seam cells at adulthood, overexpressing *rnt-1* and *bro-1* leads to seam cell hyperplasia at the expense of other differentiated cell types (Kagoshima et al. 2007). This is in large part due to the symmeterisation of normally asymmetric divisions, leading to the production of two proliferative daughters rather than a single one, and resulting in the tumourous appearance of the seam tissue (Nimmo et al. 2005; Kagoshima et al. 2007).

Expression of *rnt-1* is observed in the seam cells during embryogenesis and throughout larval development, where it is normally restricted to the proliferative (posterior, seam) daughter and not the hypodermal (anterior, differentiated) daughter cell (Kagoshima et al. 2005, 2007). Thus *rnt-1* expression is closely associated with, and crucial for, the promotion of the proliferative fate, at the expense of the differentiative fate. The molecular mechanism by which *rnt-1* promotes proliferation likely involves repression of the CIP/KIP CDK inhibitor *cki-1* in the posterior daughter destined to proliferate further (Nimmo et al. 2005).

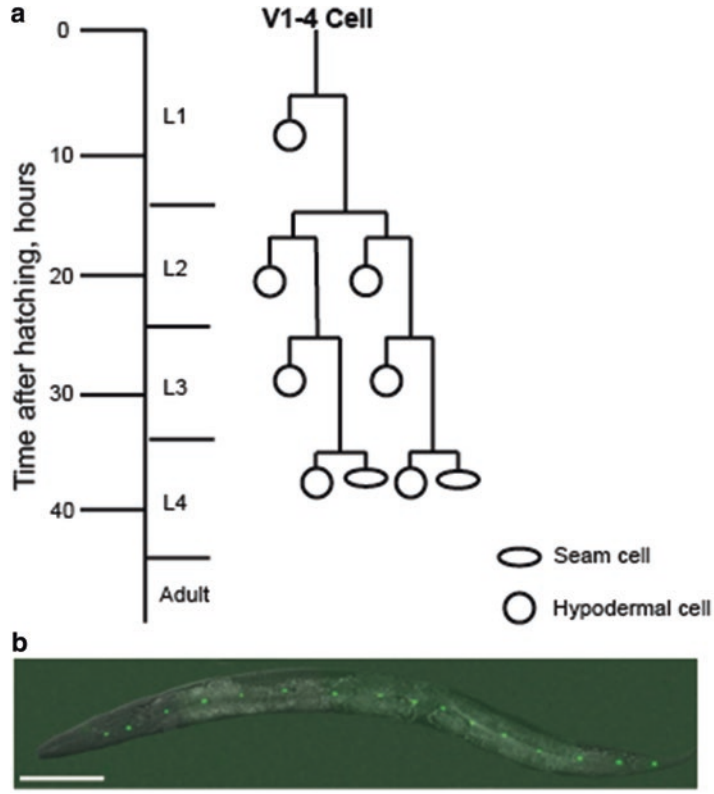

Fig. 1.3 Seam cells in *Caenorhabditis elegans*. (**a**) Lineage diagrams of the anterior V seam cells, which most obviously display the stem-like mode of division. The asymmetric divisions occur at each larval stage with an additional symmetric division at the L2 stage. In general, at adulthood, each V cell will have given rise to seven hypodermal nuclei and two seam cells that will terminally differentiate in adulthood. (**b**) An image of an early adult *C. elegans* which expresses a seam cell marker, *scm::gfp* (Strain, *JR667*). There are 16 seam cell nuclei running along each side of the animal at the end of development. Scale bar is 100 Î¼m

A major player in *rnt-1* regulation in *C. elegans* is the *ceh-20/unc-62* transcriptional partnership (homologous to the Pbx/Meis complex in mammals). Both *ceh-20* and *unc-62* mutants display seam cell hyperplasia, caused, like *rnt-1/bro-1* overexpression, by the symmetrisation of seam cell divisions such that both daughters adopt the proliferative fate (Hughes et al. 2013). *ceh-20/unc-62* seam hyperplasia is completely supressed in *rnt-1/bro-1* mutants, suggesting that *rnt-1* likely operates downstream of *ceh-20/unc-62* to promote proliferation. The fact that *rnt-1* expression appears to be de-repressed in anterior daughters (that would normally differentiate) when *ceh-20/unc-62* are silenced, suggests that *ceh-20/unc-62* function upstream to repress *rnt-1* expression in cells that normally quit the cell cycle in order to differentiate (Hughes et al. 2013).

The expression of *rnt-1* has also been observed in intestinal cells. Although RNT-1::GFP is undetectable in the intestine at adulthood, *rnt-1* mRNA is present in the adult intestine, suggestive of post-transcriptional regulation (Lee et al. 2012). Indeed, RNT-1 has been shown to be stabilized in the intestine following oxidative stress, with *rnt-1* mutants displaying increased sensitivity to these conditions (Lee et al. 2012). Given that the intestine is the first line of defence against the environment, it is possible that the post-transcriptional control of RNT-1 provides a mechanism for a rapid response to environmental changes. The p38 MAP kinase pathway plays an important function in stress response in *C. elegans* (Inoue et al. 2005) and acts to directly phosphorylate RNT-1, stabilising it via inhibition of degradation (Lee et al. 2012).

1.4 *Runx* Genes in the Sea Urchin, *Strongylocentrotus purpuratus*

Strongylocentrotus purpuratus has two *Runx* genes with the sole characterized *Runx*, *SpRunt-1*, expressed during embryogenesis and

transiently expressed in adult coelomocytes as a consequence of immune challenge (Coffman et al. 1996; Pancer et al. 1999; Robertson et al. 2002). During embryogenesis, *SpRunt-1* promotes the expression of a number of zygotically induced *Wnt* genes, in particular *wnt6* and *wnt8* (Robertson et al. 2008). Indeed, morphillino-antisense silencing of *SpRunt-1* results in impaired cell proliferation during late blastula development and widespread apoptosis as a consequence of the down regulation of these *Wnts* (Coffman et al. 2004; Dickey-Sims et al. 2005; Robertson et al. 2008). The reverse of this, where *wnt6* and *wnt8* are silenced, phenocopies the proliferation defect of the *SpRunt-1* morphant. Evidence for the direct regulation of *Wnt* by Runt-1 comes from mutational analysis of a *wnt8 cis*-regulatory module (Minokawa et al. 2005). SpRunt-1 cooperates with the effectors Tcf/Lef and Krox/Blimp-1 at the *cis*-regulatory region ('module C') of *wnt8*, which is necessary for the beta-catenin dependent maintenance of *wnt8* activity in the endomesoderm (Minokawa et al. 2005; Robertson et al. 2008). Additionally, GSK-3beta (the sole sea urchin glycogen synthase kinase that targets mitogenic proteins for ubiquitination), which itself is negatively regulated by Wnt signaling, is able to stabilize SpRunt-1 when inhibited, highlighting the complex interplay between RUNX and Wnt (Fig. 1.4) (Robertson et al. 2008).

Recent evidence has implicated the serine/threonine kinase, AKT, as a key mediator of mitogenic RUNX function in sea urchin, via phosphorylation and inhibition of GSK-3 (Robertson et al. 2013), with *akt-2* morphant animals phenocopying *SpRunt-1* morphants (Dickey-Sims et al. 2005; Robertson et al. 2013). In a further complication it is thought that RUNX also activates PKC in a positive feedback loop to inhibit GSK-3beta (Dickey-Sims et al. 2005; Robertson et al. 2008, 2013). Overall, SpRunt-1 appears to have a number of distinct roles depending on developmental stage, but as in *C. elegans*, with an emphasis on promoting cell proliferation.

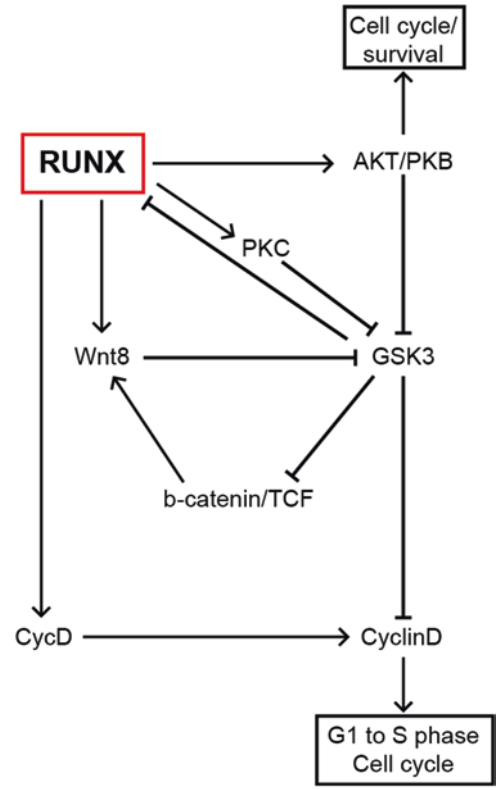

Fig. 1.4 Regulatory circuit through which *runx* regulates cell proliferation in the sea urchin embryo. The transcription factor Runx directly activates embryonic *wnt8* that is necessary for the beta-catenin dependence maintenance of *wnt8* activity. *SpRunt-1* is as an anti-apoptotic factor that, together with AKT functions through the direct regulation of PKC and GSK-3. RUNX and GSK-3 function in a mutually antagonistic regulatory pathway suggesting that, in sea urchin, RUNX promotes somatic cell proliferation by activating genes, including *pkc*, in a positive feedback loop to inhibit GSK-3 (Adapted from Robertson et al. 2002, 2008; Dickey-Sims et al. 2005, 2013)

1.5 *Runx* Genes in the Planarian Flatworm, *Schmidtea mediterranea*

Planarians are relatively simple free-living platyhelminthes that lie at an important juncture of the evolution of the basal metazoans (Newmark and Sanchez-Alvarado 2002). Planarians such as *Schmidtea mediterranea*, have amazing developmental plasticity due to the presence of a large

population of pluripotent stem cells called neoblasts, with the striking ability to regenerate missing body parts following injury (Newmark and Sanchez-Alvarado 2002; Reddien and Sanchez-Alvarado 2004; Sanchez-Alvarado and Tsonis 2006; Forsthoefel and Newmark 2009; Salo et al. 2009; Wagner et al. 2011). After wounding, the neoblasts respond by undergoing proliferation, followed by migration to the wound site and finally local differentiation into the specific cell types required to generate new tissue (Eisenhoffer et al. 2008; Wenemoser and Reddien 2010; Lapan and Reddien 2011; Scimone et al. 2014).

Transcriptome analysis has revealed a number of genes that are significantly upregulated during the period of neoblast self-renewal as a response to damage (Sandmann et al. 2011; Wenemoser et al. 2012). *runt-1* is one such gene, being expressed within 30 min of wounding, likely as an immediate response to the injury. A second wave of *runt-1* expression is induced 3-12 hours post wounding (Wenemoser et al. 2012; Wurtzel et al. 2015). The role of *runt-1* in the planarian response to injury is to firstly direct the proliferation of cells, followed by the differentiation of these cells into lineage restricted precursors. Following wounding, knockdown of *Smed-Runt-1* by RNAi results in defects in cell positioning and photoreceptor phenotypes in the eye (Sandmann et al. 2011; Wenemoser et al. 2012), indicative of *Smed-Runt-1* promoting the formation of fate restricted neoblasts in the anterior of the animal following wounding to form eyes.

1.6 *Runx* Genes in the Cnidaria

A similar upregulation of *runt-1* has been observed following injury and during regeneration in the sea anemone (*Nematostella vecterisis*) (DuBuc et al. 2014) where *NvRunt-1* is localized to the pluripotent progenitors of the sensory neurons in ectodermal cells of the tentacle tips (Sullivan et al. 2008). Hydra, like sea anemones, are members of the phylum Cnidaria and are freshwater polyps with a symmetrical tubular body. As in *S. mediterranea*, a pool of heterogeneic stem cells have been identified in hydra (Govindasamy et al. 2014). These stem cells are quiescent until they become activated to enter the cell cycle following removal of the head (Govindasamy et al. 2014) with *runt-1* upregulated following decapitation (DuBuc et al. 2014; Petersen et al. 2015).

Thus, a role for *runt-1* in regeneration in planarians and cnidarians such as sea anemone and hydra appears to be associated with the stimulation of both cell proliferation and subsequent differentiation following injury. In this way, RUNX may play a key role in the transition of undifferentiated cells into committed lineage precursors, and therefore provide new insights into the control of regenerative processes.

1.7 Comparative Analysis Delineates Emerging Themes in RUNX Biology

Establishing functional relationships between genes in very diverse organisms is a daunting, yet appealing task, beset with problems of interpretation and translation between systems. Nevertheless, any systematic examination of RUNX biology throws up some immediate areas of commonality, both in terms of biological processes as well as molecular pathways, and it is these areas of commonality that may hold the key to unlocking a broader understanding of RUNX biology in increasingly complex organisms.

1.8 Conserved RUNX-Associated Biological Processes

1.8.1 Regulation of the Transition from Quiescence into Proliferation

Runx genes have an obvious role in promoting cell proliferation in many species. The function of *rnt-1* in *C. elegans* seam cells to promote proliferation bears a remarkable similarity to the role of mammalian *Runx1* in hair follicle stem cells (HFSC). Both stem cell systems are comprised of

epidermal cells where divisions occur after long quiescent phases. In the worm, seam cells are quiescent until the molt preceding each larval transition when the cells divide in a *rnt-1*-dependent manner. Similarly, in mammals, Runx1 activates quiescent stem cells in the hair follicle, with *Runx1* mutant mice having an extended quiescent phase and defects in HFSC colony formation (Osoiro et al. 2008). Further invertebrate examples of *Runx* genes functioning in cell proliferation include the sea urchin, where inactivation of *SpRunt-1* is associated with proliferation defects and both hydra and planaria, where *runt-1* appears to be involved in promoting cell proliferation following injury (Sandmann et al. 2011; Wenemoser et al. 2012; DuBuc et al. 2014; Govindasamy et al. 2014; Petersen et al. 2015). These latter observations support the idea that *Runx* genes may have a general role to play in regeneration. An additional example of RUNX-dependent proliferation in mammals is in the nervous system, where Runx1 is required to sustain the proliferation of olfactory receptor neuron (ORN) precursors (Theriault et al. 2005). Indeed, in this example, over-expression of *Runx1* increased the number of proliferating cells, much like the over-expression of *rnt-1* in *C. elegans* seam cells, causing hyperplasia of this cell type (Nimmo et al. 2005; Kagoshima et al. 2007).

Moving from quiescence into proliferation involves transduction of growth factor signalling, and *Runx* genes appear generally to have an important role in this process. For example, in the HFSC system, *Runx1* mutants do not respond properly to a growth signal, thus proliferation fails. Intriguingly, in *C. elegans* which are starved, the *rnt-1* mutant phenotype is enhanced (Nimmo et al. 2005), and *rnt-1* was found to be one of the most highly up-regulated genes following re-feeding after starvation (Baugh et al. 2009), consistent with an important role for *Runx* genes in transducing environmental information to achieve properly coordinated growth and development. Furthermore, the role of *C. elegans rnt-1* in regulating stress response (Lee et al. 2012) is intriguing in the light of recent data suggesting that mammalian *Runx1* deficient hematopoietic stem cells (HSC) display increased stress resistance (Cai et al. 2015), together with lower rates of translation, attenuated p53 signalling and a decrease in ribosome biosynthesis. Understanding the molecular pathways that result in the altered metabolic profile of *Runx1*-deficient HSC will have significant implications in treating leukaemia.

Finally, the important role for *Runx* genes in controlling cell number in invertebrate models by promoting, or even repressing in some examples, (Kramer et al. 2006; Murthy et al. 2014) cell proliferation resonates strongly with the well-characterised role of *Runx* genes as oncogenes or tumour suppressors, depending on context (Strom et al. 2000; Cameron and Neil 2004; Ito 2004; Wotton et al. 2004; Keita et al. 2013; Wysokinski et al. 2015). This suggests that invertebrate model systems have useful contributions to make the field of Runx-associated carcinogenesis.

1.8.2 Lineage Commitment and Cell Fate Determination

Runx genes have been described as molecular switches coordinating the developmental balance between proliferation and differentiation (Nimmo and Woollard 2008). There are certainly many examples of *Runx* genes acting to promote proliferation, as we have seen, and there are several examples of *Runx* genes acting in lineage commitment and cell fate decisions; there are two examples of *Runx* genes being required for eye development (planaria and fly), two examples of a requirement during haematopoiesis (mammals and fly) and several examples of a role in neurogenesis (fly, worm, mammals, planaria). But whether these shared functions are orthologous, in the sense that they indicate an ancient origin, or whether they are examples of *Runx* genes being co-opted during evolution for different purposes, some common between different organisms and some not, is difficult to determine.

The most intriguing shared function is surely haematopoiesis. Runx1 has long been known to regulate the differentiation of hematopoietic stem cells (HSCs) from myeloid precursors in mam-

mals (Tanaka et al. 1995; Ahn et al. 1998; Yokomizo et al. 2001). In fact, RUNX proteins are expressed throughout all hematopoietic lineages, being necessary for the emergence of the first HSCs through to their terminal differentiation. In *Drosophila*, the hemocytes formed during larval development (in a process resembling vertebrate definitive hematopoiesis) most closely resemble vertebrate myeloid linages (Waltzer et al. 2010), with the plasmatocytes having a similar function to vertebrate macrophages (Lanot et al. 2001; Wood and Jacinto 2007). The parallels between the complex network of transcription factors regulating lineage commitment in *Drosophila* crystal cells and human thymocytes are striking, with co-factors such as GATA factors figuring prominently in both cases. In recent years, there is increasing evidence for a role of *RUNX* in the immune system beyond haematopoiesis (Ito et al. 2008; Kitoh et al. 2009; Wong et al. 2011, 2012, 2014; Lotem et al. 2013). It has long been known that sea urchin *Runx* is expressed as a consequence of immune challenge, and more evidence is emerging for the function of *RUNX* in the mammalian immune system (reviewed in Voon et al. 2015) that may allow for future comparative analysis.

1.9 Conserved RUNX Molecular Pathways

Evaluating conservation of molecular mechanisms involving RUNX is perhaps even more difficult than assessing conserved processes. Firstly, experiments may be difficult to translate between organisms. Secondly, transcription factors can be co-opted into many different signalling pathways over the course of evolution, and adopt many different target genes depending on the context of their precise role. Finally, *Runx* genes have emerged, been lost, multiplied and diverged, so that evolutionary history presents many molecular fossils that are hard to interpret, and there is the additional confounding factor of convergent evolution. Nevertheless, certain similarities in the molecular architecture of RUNX function across highly divergent groups appear to stand out. One example is the interaction of RUNX with cell cycle genes, and other examples include interactions with Wnt signalling and GATA factors.

1.9.1 Interaction with Cell Cycle Genes

The role of *Runx* genes in the transition from quiescence to proliferation is associated in several cases with the direct regulation of the cell cycle. In *C. elegans rnt-1* mutants, expression of *cki-1* (cyclin dependent kinase inhibitor of the CIP/KIP family) is upregulated in seam cells, and depleting *cki-1* in these animals rescues the seam cell proliferation defect (Nimmo et al. 2005). RNT-1 is therefore acting (directly or indirectly) to repress the expression of *cki-1* in seam cells destined to divide. With striking similarity, RUNX1 and RUNX2 have been shown to repress the cyclin-dependent kinase inhibitor p21 in mammalian cell culture (Strom et al. 2000; Bernardin and Friedman 2002; Westendorf et al. 2002; Bernardin-Fried et al. 2004). Similarly, in sea urchin, RUNX induces cyclinD during embryogenesis leading to cell cycle progression (Coffman et al. 2004; Dickey-Sims et al. 2005; Robertson et al. 2008).

1.9.2 Interaction with Wnt Signalling

In the sea urchin, experiments show that SpRunt1 binds directly to *wnt6* and *wnt8* in the late blastula stage of embryogenesis (Robertson et al. 2008), and depletion of SpRunt1 is associated with a decrease in Wnt signalling. This is also the case in mammals where *wnt4* gene expression is reduced in *Runx1* knockout mice (Naillat et al. 2015), although the mechanism in this latter case likely involves TCF/LEF (T-cell factor/lymphoid enhancer factor) binding to RUNX1 in order to attenuate Wnt signalling. Indeed, there are several examples of TCF interactions with *Runx* genes, including the binding of TCF1 to RUNX2 during osteoblast development (Kahler and Westendorf 2003), the interaction of TCF7 and

RUNX1 in haematopoiesis (Wu et al. 2012) and the interaction of TCF4 with RUNX3 to regulate Wnt signalling, which has been linked to gastric cancer (Ito et al. 2008, 2011). Overall, RUNX, TCF/LEF and Wnt signalling have been shown to act together in a context dependent manner to activate or repress transcription of genes to control cell fate choice in a variety of tissues. However, although interactions between Wnt signaling and Runt have been demonstrated in sea urchin, there is little to support this in *Drosophila* or *C. elegans*. Indeed, in nematodes it is likely that, at least in the stem cell-like seam cells, *rnt-1* acts in a parallel pathway to Wnt (Gleason and Eisenmann 2010; Hughes et al. 2013; Gorrepati et al. 2015).

1.9.3 Interaction with GATA Factors

In the nematode RNT-1 and BRO-1 regulate the proliferation of seam cells, with the GATA transcription factor ELT-1 directly regulating *bro-1* (Brabin et al. 2011). The function of RNT-1, BRO-1 and ELT-1 in the worm directly reflect the roles of RUNX, CBFbeta and GATA in stem cells in other systems. The interaction of these transcription factors is reminiscent of the situation in *Drosophila* and mammalian haematopoiesis where GATA/Serpent, RUNX/Lozenge and CBFbeta/Brother tightly control cell fate choice (Li and Gergen 1999; Waltzer et al. 2003; 2010; Pencovich et al. 2011).

1.10 Conclusion

There are intriguing connections between RUNX functions in mammals and invertebrates, centering on the regulation of cell proliferation and lineage commitment. Invertebrate models such as *C. elegans*, *Drosophila* and the sea urchin are useful in the study of RUNX function because they offer unique options in relation to genetic manipulation and ease and speed of experimentation. Work in *C. elegans* offers the particular advantage of the lack of functional redundancy issues, as it contains a solo *Runx* homologue. However, it does not appear to be the case that research in invertebrate models will necessarily uncover a single ancestral function of *Runx* genes that explains the range of functions documented in mammals. On the contrary, different invertebrate models have proved invaluable to highlight and investigate specific functions of *Runx* genes reported in vertebrates. Taken together, studies of invertebrate RUNX biology provides a wealth of information that will be instrumental in our understanding of the importance of *Runx* genes in developmental control and in the fight against disease.

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