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Yoram Groner
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James C. Neil
Nancy A. Speck
Andre van Wijnen *Editors*

RUNX Proteins in Development and Cancer

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Editors

RUNX Proteins in Development and Cancer

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About the Editors

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P. Paul Liu M.D., Ph.D., is a senior investigator in the Genetics and Molecular Biology Branch at the National Human Genome Research Institute, USA. Dr. Liu identified the *CBFB* gene as a target of the inv Puig-Kroger and Corbi (2006) in acute myeloid leukemia in 1993. Dr. Liu studies the normal and oncogenic RUNX and CBFβ proteins in hematopoietic development and leukemia using both mouse and zebra fish models.

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John D. Gross Ph.D., is principal investigator and director of the NRM Lab at the University of California, San Francisco, USA. He was one of the first to show that CBFβ is an essential cofactor of the HIV-1 protein Vif, which together regulate the APOBEC3 family of retroviral restriction factors.

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Motomi Osato M.D., Ph.D., is an associate professor at the National University of Singapore and a professor at Kumamoto University, Japan. He was one of the first to identify somatic mutations in *RUNX1* in patients with myeloid malignancies. He has since studied the functions of RUNX proteins in hematopoiesis and leukemogenesis with an emphasis on stem cells. His current interests also include the identification of *cis*-regulatory elements in the RUNX family genes.

Antonino Passaniti Ph.D., is a professor of pathology at the University of Maryland in Baltimore, USA. He discovered that Runx2 collaborates with the YAP protein in promoting cell transformation. He currently studies the role of Runx2 in angiogenesis and of the TAZ gene in breast cancer.

Takaomi Sanda M.D., Ph.D., is a principal investigator at the Cancer Science Institute of Singapore, National University of Singapore. Dr. Sanda identified the transcriptional regulatory circuit involving the *RUNX1* gene in T-cell acute lymphoblastic leukemia when he was a postdoctoral fellow. He is currently studying transcriptional regulatory elements aberrantly activated by oncogenic transcription factors in T-cell leukemia.

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Stefano Stifani Ph.D., is a professor at McGill University, Montreal, Canada. Dr. Stifani identified Runx1 based on its interaction with the mammalian hairy and enhancer of split protein, Hes-1. Dr. Stefani studies the role of Runx1 in the development of the nervous system.

Tahir H. Tahirov Ph.D., is a professor at the Eppley Institute for Research in Cancer and Allied Diseases at the University of Nebraska Medical Center, USA. Dr. Tahirov published the first X-ray crystal structure of the ternary complex consisting of the DNA-binding domain of RUNX1 and CBFβ bound to DNA and more recently determined the structural basis for the cooperative DNA binding of RUNX1 and its frequent partner ETS1.

Ichiro Taniuchi M.D., Ph.D., is a group director at the Riken Center for Integrative Medical Sciences, Japan. Dr. Taniuchi first described the contribution of RUNX proteins to silencing of the *Cd4* gene. His research centers on the mechanism of lineage choice in T-cell development, with a continued focus on RUNX.

Jean Paul Thiery Ph.D., is the Toh Chin Chye visiting professor in the Yong Loo Lin School of Medicine at the National University of Singapore and a

principal investigator in the Institute of Molecular and Cell Biology, A*STAR, in Singapore. Dr. Thiery was the first to propose the crucial contribution of the epithelial-mesenchymal transition (EMT) to the progression of carcinoma and has made seminal contributions to the study of minimal residual disease in breast cancer. He also discovered the first cell-cell adhesion molecule, N-CAM, and developed new physical approaches to measure the strength of intercellular adhesion.

Dominic Chih-Cheng Voon Ph.D., is an associate professor at the Institute for Frontier Science Initiative, Kanazawa University, Japan. Dr. Voon discovered that *Runx3* safeguards gastric epithelial cells against epithelial-mesenchymal transition-induced cellular plasticity and tumorigenicity. His current interests include the role of RUNX proteins in epithelial inflammation and plasticity.

Michelle J. West Ph.D., is a professor of tumor virology at the University of Sussex, UK. Her work focuses on the regulation of cellular and viral gene expression and B-cell transformation by Epstein-Barr virus.

Owen Williams Ph.D., is a principal investigator in the Developmental Biology and Cancer Programme at the University College London, UK. Dr. Williams studies the ETV6-RUNX1 fusion protein and the mechanism by which it contributes to the development of pediatric B-cell acute lymphoblastic leukemia.

A. Woollard Ph.D., is an associate professor in the Department of Biochemistry at the University of Oxford, UK. Dr. Woollard described a role for the *C. elegans RUNX1* and *CBFB* genes (*rnt-1* and *bro-1*) in stem cell proliferation.

Overview

The year 2016 is the 25th anniversary of the cloning of the first mammalian RUNX gene and its simultaneous association with human disease (Miyoshi et al. 1991). It is thus a fitting occasion to assemble a compendium of articles on the RUNX proteins. Although the RUNX genes gained their notoriety based on their involvement in human disease, they were first identified in *Drosophila* by Nüsslein-Volhard and Wieschaus in their historic screen for mutations that affected the basic body plan of the embryo (Nüsslein-Volhard and Wieschaus 1980). The mutant *runt*, named for the diminutive embryo, was categorized as one of six pair-rule mutants. Later cloning of the *Drosophila runt* gene by Gergen and colleagues revealed that it encoded a nuclear protein, with no known function (Kania 1990). The human *RUNX1* gene (originally named *AML1*) was the next to be discovered based on its location on chromosome 21 at the breakpoint of the 8;21 translocation in acute myeloid leukemia (Miyoshi et al. 1991). The homology of *RUNX1* to the *Drosophila runt* gene was noted, but the protein's function remained obscure. Only upon the purification and cloning of the mammalian RUNX proteins based on their biochemical properties, i.e., their ability to bind a specific sequence of DNA, did it become apparent that the RUNX proteins were transcription factors (Kamachi et al. 1990; Wang and Speck 1992; Wang et al. 1993; Meyers et al. 1993; Ogawa et al. 1993a, b). Also revealed by the biochemical studies was that the RUNX proteins constituted one subunit in a heterodimeric complex that also contained a non-DNA-binding partner, core-binding factor beta (CBFβ) (Kamachi et al. 1990; Wang et al. 1993; Ogawa et al. 1993a). At around the same time, the gene encoding CBFβ (*Cbfb*) was cloned; the human *CBFB* gene was identified as one of the genes disrupted by the inv(16) in acute myeloid leukemia (Liu et al. 1993). This convergence of information from multiple independent lines of inquiry and in particular the identification of translocations in *RUNX1* and *CBFB* in leukemia was an extremely exciting time in the field.

The field that began with a handful of laboratories has grown into a large and varied enterprise; as of today, a PubMed search for RUNX proteins yields >8000 references. It has become evident that the RUNX proteins are involved in many different areas of biology, ranging from basic cellular processes such

as cell cycle and DNA repair, cell specification during development, stem cell biology, pathogenesis of solid and liquid tumors, and virology. This variety of functions is reflected by the diverse topics covered in this compendium.

Several investigators who were involved in the early days of discovery have contributed to this compendium, along with many other outstanding investigators who later joined the field and who have greatly broadened our knowledge about the RUNX proteins and their many biological and biochemical roles. It is impossible to cover all of the various developmental, biochemical, and disease processes in which these proteins play a part. Nevertheless, it is our hope that this volume will be a useful resource to those in the field or interested in the RUNX proteins and will stimulate further research on these fascinating molecules.

Genomic and Protein Structure of RUNX Family

For readers new to the field, here are the basics. There are three RUNX genes, *RUNX1*, *RUNX2*, and *RUNX3*, in mammals. Figure 1 shows genomic structure of three mammalian RUNX genes and the exon-intron structure of RUNX mRNAs including major splice variants. Note that there are two promoters in each gene, P1 (distal) and P2 (proximal). RUNX genes are evolutionarily very old, dating back to unicellular organisms (see Chap. 1 by Hughes and Woollard). Figure 2 shows an alignment of amino acid sequences of three human RUNX proteins. Amino acid sequences of RUNX proteins in many animal species are also well conserved. For example, Fig. 3 shows the amino acid sequence comparison between *Homo sapiens RUNX3* and *Caenorhabditis elegans Rnt-1*.

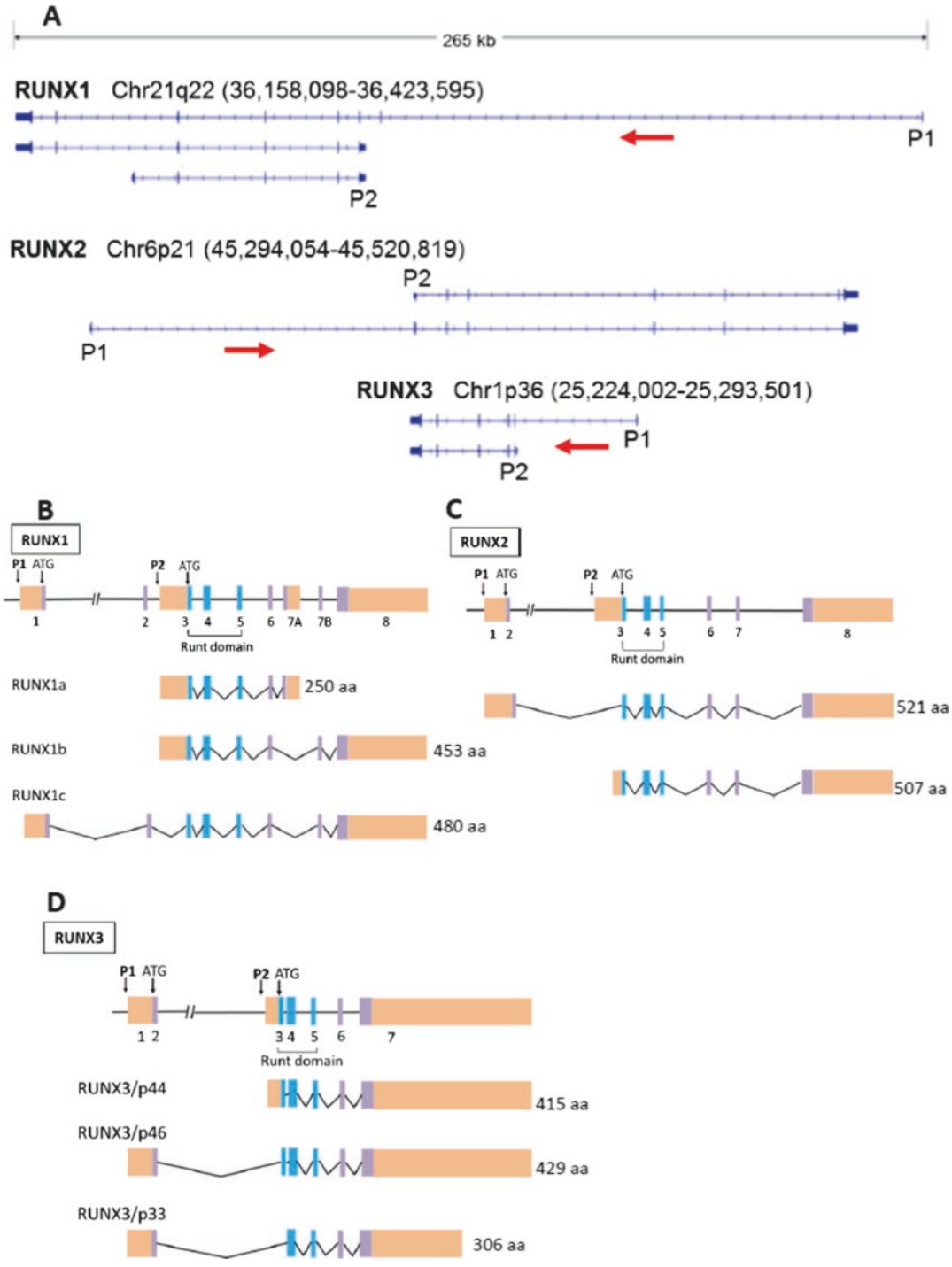

Fig. 1 Comparison of the genomic structure of and mRNA variants produced from the human *RUNX1*, *RUNX2*, and *RUNX3* genes. (**a**) Genomic structure of the three human RUNX genes. The positions of P1 and P2 promoters are indicated. *Red arrows* show the direction of transcription. Note the large size differences between the three genes. RUNX3, believed to be the ancestral gene, is the smallest (http://hgdownload.soe.ucsc.edu/downloads.html). (**b**–**d**) Exon-intron structure of *RUNX1* (**b**) (Levanon et al. 2001; Osato 2014), *RUNX2* (**c**) (Terry et al. 2004), and *RUNX3* (**d**) (Bangsow et al. 2001). The Runt domain is encoded in exons 3, 4, and 5 (*blue*). Sizes of exons and introns are not to scale. The splice variants of RUNX proteins suggest complex cellular mechanisms regulate and fine-tune RUNX activities

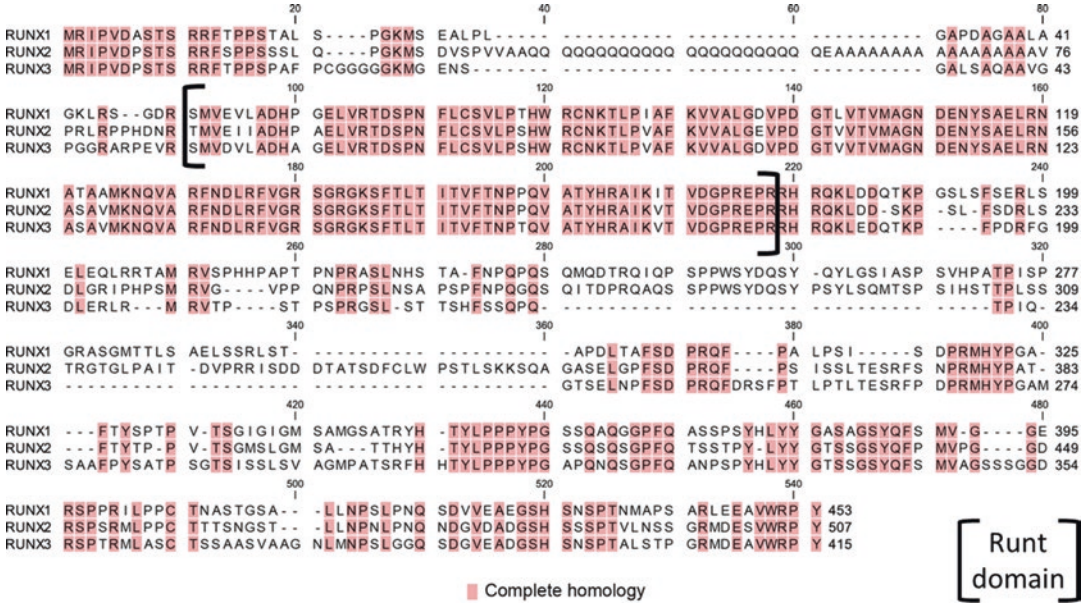

Fig. 2 Amino acid alignment of human RUNX1, RUNX2, and RUNX3. The highly conserved 128-amino acid region (*bracketed*) called the Runt domain is required for DNA binding and heterodimerization with CBFβ. The 5 amino acids at the C terminus, VWRPY, are also conserved. The VWRPY motif is required for binding to the corepressor Groucho (transducin-like enhancer of split 1)

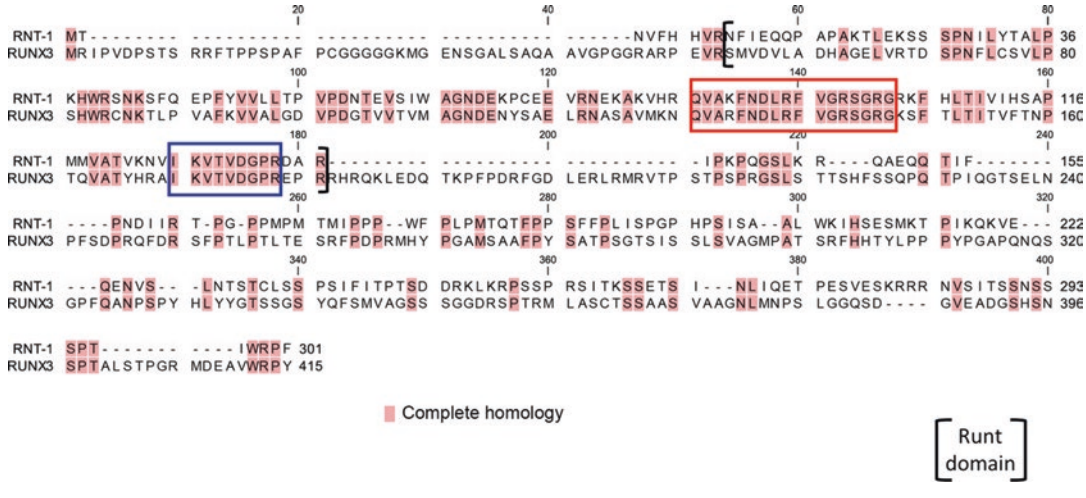

Fig. 3 Amino acid alignment of *Caenorhabditis elegans Rnt-1* (RNT-1: B0414.2) and *Homo sapiens RUNX3*. Two particularly highly conserved regions are indicated in boxes. The conserved sequences from aa131 to aa147 (*red box*) (the amino acid numbers correspond to *RUNX3*) contain a Walker motif A and AKT phosphorylation site, the significances of which are not known. The second conserved sequence is from aa170 to aa178 (*blue box*). These regions are likely to be important for evolutionarily conserved functions

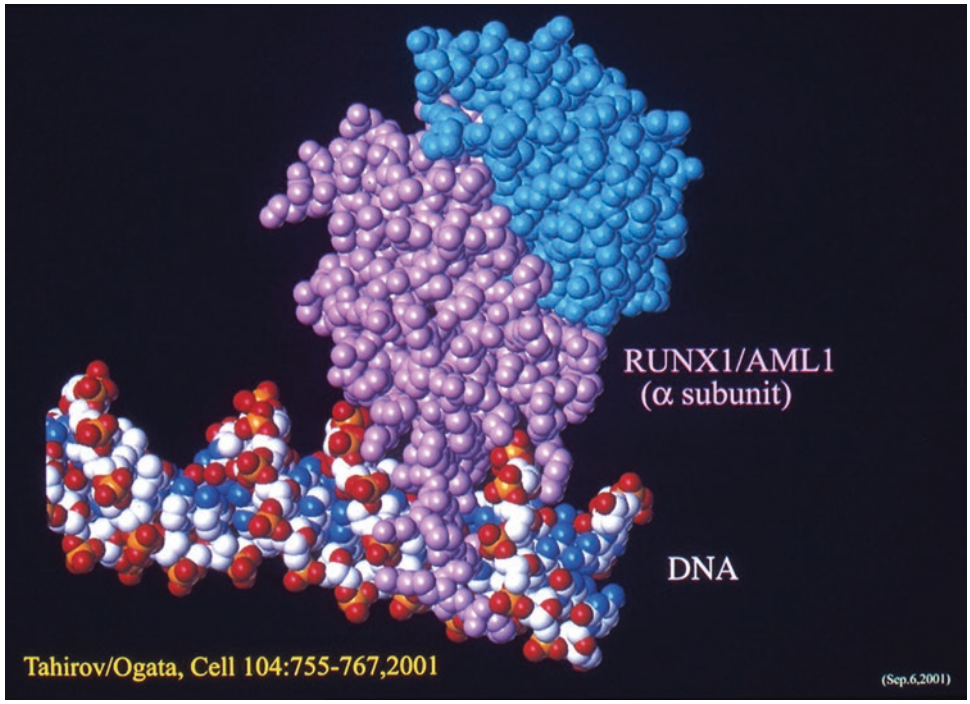

Fig. 4 Crystal structure of the Runt domain heterodimerized with a 134-amino acid region of CBFβ bound to DNA (Figure from Tahirov et al. 2001)

RUNX proteins have a conserved, obligate non-DNA-binding partner, CBFβ. CBFβ allosterically regulates DNA binding by the RUNX proteins (see Chap. 2 by Tahirov and Bushweller). Figure 4 shows crystal structure of the Runt domain together with CBFβ bound to DNA (courtesy of Dr. Tahir H. Tahirov).

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Part I

Evolution of RUNX Genes

RUNX in Invertebrates

1

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

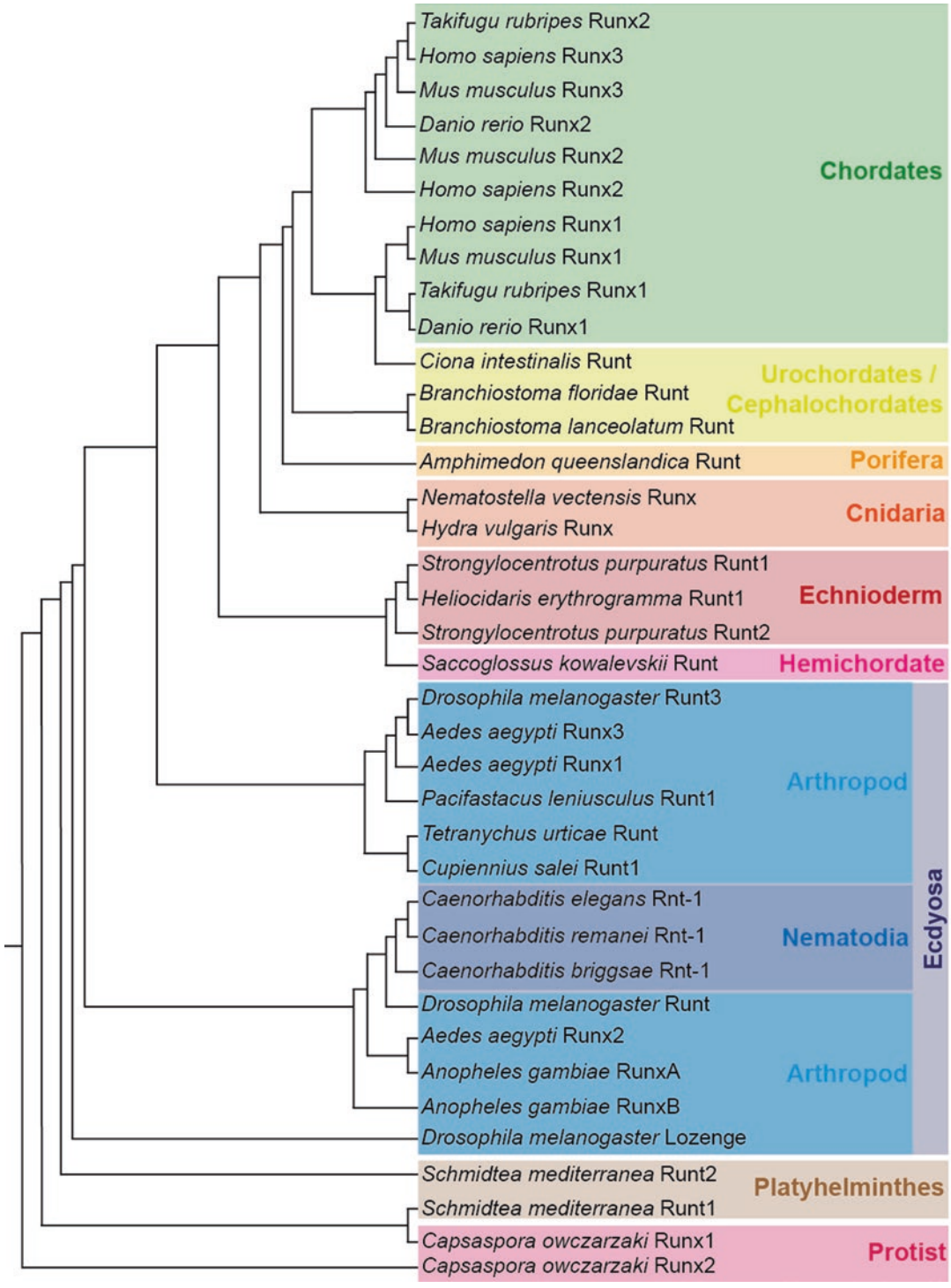

Fig. 1.1 *Runx* genes in the metazoa. *Runx* genes are represented in all major metazoan lineages, with a newly identified *Runx* gene in the unicellular protist *C. owczarzaki*. Alignments of whole Runx protein sequences were undertaken in MAFFT using Neighbor-joining, substitution model JTT and a bootstrap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

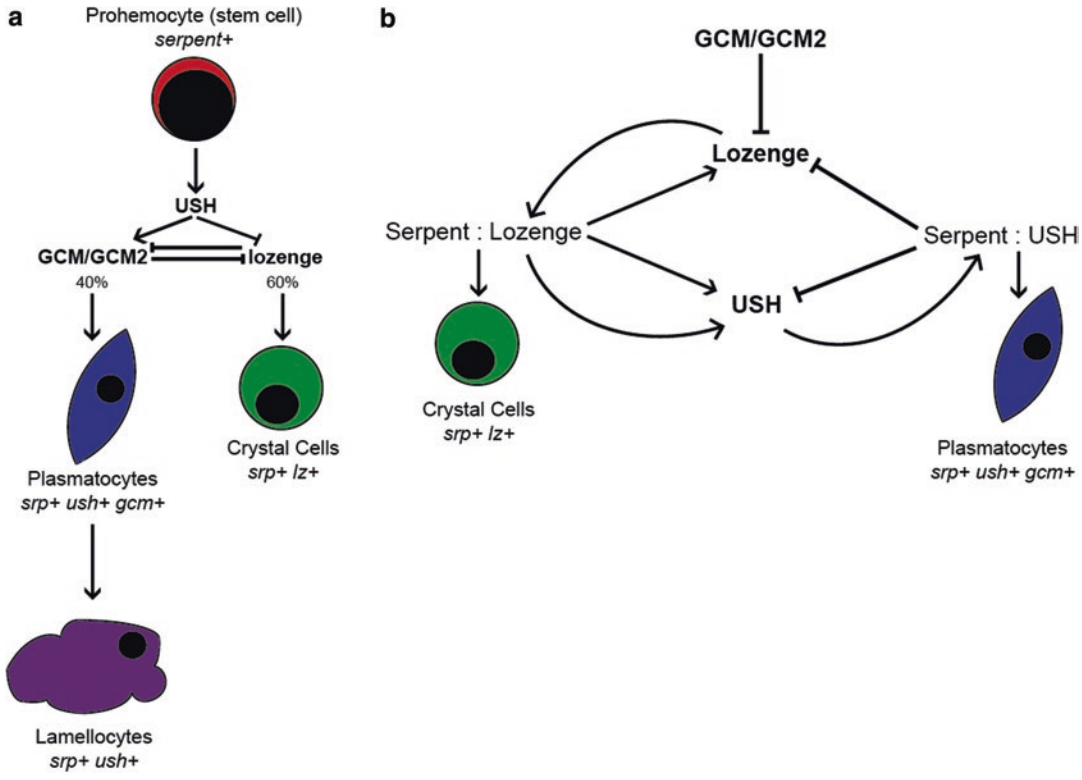

Fig. 1.2 Simplified diagram of the transcription factor network that controls cell fate in *Drosophila* hematopoiesis. (**a**) The prohemocytes are a stem cell population that express the GATA factor *serpent* (*srp*) that activates *ush* (*u-shaped*, friend of GATA (FOG) family) which will in turn function 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).

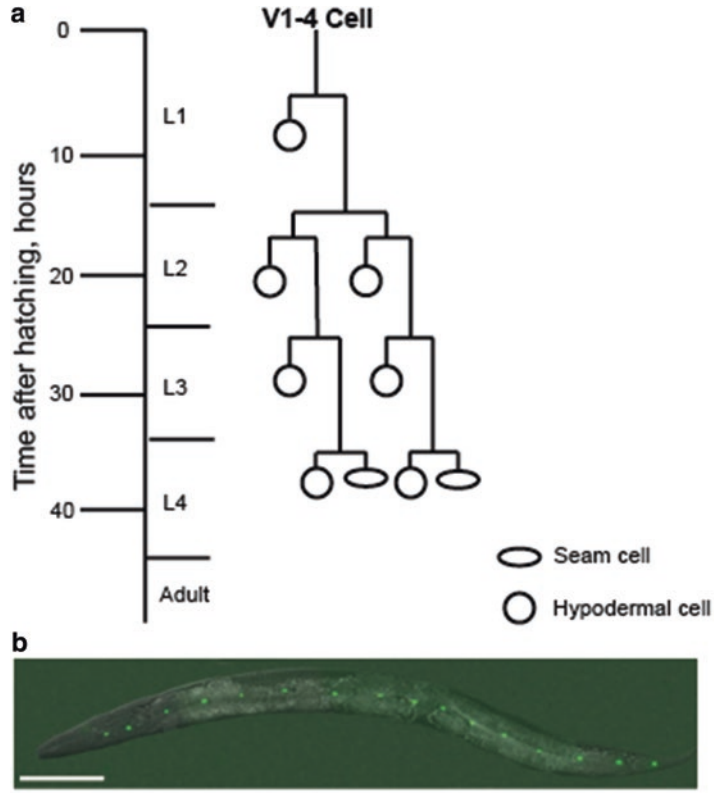

Fig. 1.3 Seam cells in *Caenorhabditis elegans*. (**a**) Lineage diagrams of the anterior V seam cells, which most obviously display the stem-like mode of division. The asymmetric divisions occur at each larval stage with an additional symmetric division at the L2 stage. In general, at adulthood, each 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.

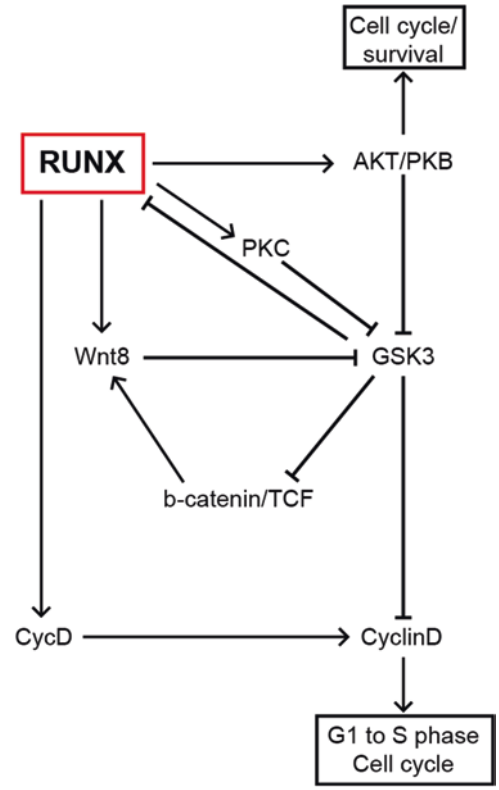

Fig. 1.4 Regulatory circuit through which *runx* regulates cell proliferation in the sea urchin embryo. The transcription factor Runx directly activates embryonic *wnt8* that is necessary for the beta-catenin dependence maintenance of *wnt8* activity. *SpRunt-1* is as an anti-apoptotic factor that, together 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