The *RUNX* Genes as Conditional Oncogenes: Insights from Retroviral Targeting and Mouse Models

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Abstract

The observation that the *Runx* genes act as targets for transcriptional activation by retroviral insertion identified a new family of dominant oncogenes. However, it is now clear that *Runx* genes are 'conditional' oncogenes whose over-expression is growth inhibitory unless accompanied by another event such as concomitant over-expression of MYC or loss of p53 function. Remarkably, while the oncogenic activities of either MYC or RUNX over-expression are suppressed while p53 is intact, the combination of both neutralises p53 tumour suppression *in vivo* by as yet unknown mechanisms. Moreover, there is emerging evidence that endogenous, basal RUNX activity is important to maintain the viability and proliferation of MYC-driven lymphoma cells. There is also growing evidence that the human *RUNX* genes play a similar conditional oncogenic role and are selected for over-expression in end-stage cancers of multiple types. Paradoxically, reduced RUNX activity can also predispose to cell immortalisation and transformation, particularly by mutant Ras. These apparently conflicting observations may be reconciled in a stage-specific model of RUNX involvement in cancer. A question that has yet to be fully addressed is the extent to which the three *Runx* genes are functionally redundant in cancer promotion and suppression.

Keywords

Cancer • Lymphoma • Retroviral mutagenesis • Senescence • Oncogene • Tumour suppressor

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16.1 The Murine *Runx* Genes as Targets for Insertional Mutagenesis

The discovery that the murine *Runx* genes can act as targets for transcriptional activation by murine leukaemia viruses (MLVs) provided evidence that they belong to the class of proto-oncogenes: genes normally involved in growth control that can be activated to play a dominant oncogenic role in cancer. MLVs are members of the gamma-retrovirus genus and induce tumours in mice primarily by insertional mutagenesis. Strong promoter/enhancer sequences in the long terminal repeats of these viruses affect the expression of genes near, or even at a considerable distance from, the insertion site. MLV induced tumours may display multiple insertions at complementing oncogenes; gene activation events predominate although tumour suppressor inactivation can also occur (reviewed in (Uren et al. 2005)). Completion of the mouse genome sequence has greatly increased the ease of mapping retroviral insertions in tumours, greatly extending the reach of mutagenesis screens for cancer –relevant genes (Hwang et al. 2002; Mikkers et al. 2002; Suzuki et al. 2002). While the process of retroviral integration into the host genome was previously thought to be random with regard to base sequence and location, it is now clear that some retroviruses display very significant and distinct biases. For MLV and related gamma-retroviruses, intrinsic integration preference arises at least in part from interaction of the viral integrase protein with the BET (bromodomain and extraterminal) host chromatin tethering factors Brd2,3 and 4 (De Rijck et al. 2013; Gupta et al. 2013; Sharma et al. 2013). This specificity is of interest with regard to oncogenic potential as BET binding is also a feature of 'super-enhancers' – highly cell-type specific tandem clusters of enhancer elements that appear to define cell identity and the cancer phenotype (Whyte et al. 2013). This intrinsic bias has to be allowed for, particularly when analysing and interpreting high throughput datasets (Cattoglio et al. 2010; LaFave et al. 2014). These findings led us to propose a two-stage model of gamma-retrovirus oncogenesis: selective integration at 'dangerous' sites, followed by clonal selection of collaborating mutations (Huser et al. 2014).

The first recorded example of *Runx* gene targeting by MLV arose from an early screen where a single case of Akv MLV insertion was recorded close to the P1 promoter of *Runx1* in a case of myeloid leukaemia in a BXH2 mouse (Suzuki et al. 2002). This appears to have been a fortuitous observation, as subsequent studies have shown a relatively low frequency of targeting of the *Runx* genes in end-stage tumours of wild-type mice. However, frequent activation of *Runx* genes has been observed in mice where predisposition to tumour development is conferred, for example, by a germ-line *MYC* oncogene over-expressed under tissue-specific transcriptional controls. While all three *Runx* genes have been shown to be capable of acting as targets in *MYC* transgenic mice, the frequency varies according to model. *Runx1* and *Runx3* are targeted in B-cell lymphomas accelerated by neonatal infection with Moloney MLV in the Eμ-Myc model (Mikkers et al. 2002; Uren et al. 2008). All three *Runx* genes have been observed as targets in virus-accelerated T-cell lymphomas of CD2-*MYC* but with frequency of targeting *Runx2*>*Runx3*>*Runx1* (Mikkers et al. 2002; Stewart et al. 2002; Stewart et al. 1997; Wotton et al. 2002). Moreover, a recent high throughput/NGS study confirmed the relative rarity of *Runx* gene insertions in clonally expanded cell populations in wild-type mice, and indicated that, in contrast, activation of a *Runx* family member is virtually obligatory in virus-accelerated CD2-*MYC* lymphomas (Huser et al. 2014).

A diagram summarising the location and orientation of recorded proviral insertions at the murine *Runx* genes is presented in Fig. 16.1. We have included only those examples where significant clonal expansion has provided corroborating evidence that these insertions played a causal role in tumour outgrowth. A track showing H3K27Ac 'enhancer' marks in mouse thymus is included for comparison. While there is substantial correspondence between H3K27Ac marks and the peaks of insertion, this overlap is not seen at the *Runx2* P1 promoter. However, it should be noted

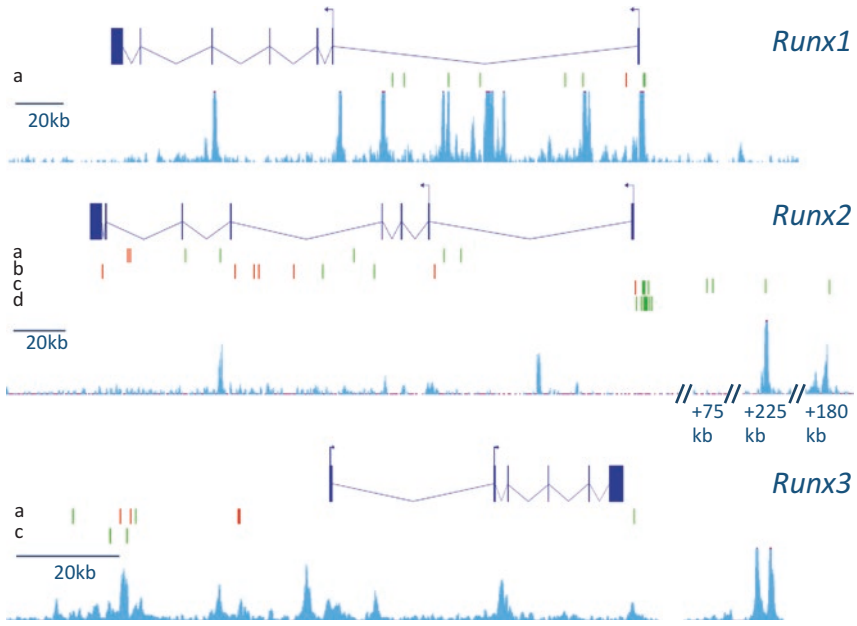

Fig. 16.1 *In vivo* clonally expanded insertions of MLV and transposons at the *Runx* gene loci in murine tumours. The results of multiple studies have been collated. The *top* of each track shows gene structure, with *solid vertical bars* representing exons and *lines* representing introns, with *arrows* showing transcriptional start sites. Proviral insertions in the forward direction are coloured *green* while those in the reverse direction are coloured *red*. Sources of data for tracks (**a–d**) are as follows: (**a**) CD2-*MYC* transgenic T-cell lymphomas analysed by high throughput/NGS analysis (read number >100) (Huser et al. 2014). (**b**) CD2-*MYC* transgenic T-cell lymphomas from restriction mapping and direct sequence analyses (Cameron et al. 2003; Stewart et al. 2007; Wotton et al. 2002) (**c**) Common insertion sites from the retroviral tagged cancer gene database (ref) (**d**) Sleeping beauty transposon insertions in leukaemia/lymphomas from *Rassf1a* deficient mice (van der Weyden et al. 2012). Insertions with more than 100 copies are shown. The *bottom* of each track shows H3K27ac intensity in adult mouse thymus, obtained from the mouse ENCODE repository

that available ChIP-Seq datasets may not reflect the target cell at the stage of development when retroviral integration occurred.

In the CD2-*MYC* system it is clear that MLV induces transcriptional activation of *Runx* family genes, as cell lines established from these lymphomas show very high level expression of the targeted gene with no mutational changes in coding sequence (Stewart et al. 2002; Stewart et al. 1997; Wotton et al. 2002). Moreover, the strong statistical bias of proviral orientation where insertions have been observed at the *Runx2* P1 promoter fits with the 'enhancer mode' of retroviral insertion, where the viral long terminal repeat is upstream and backwards with regard to the targeted promoter (Huser et al. 2014). Expanded clonal insertions far upstream of *Runx2* in CD2-*MYC* lymphomas suggest that regulation of this gene in early T-cell development also involves distant cis-acting enhancer elements that serve as targets for long-range activation by retroviral insertions (Huser et al. 2014).

The pattern is somewhat less clear for *Runx1*. In the 6i lymphoma cell line derived from CD2-*MYC* lymphoma, MLV insertion is in the 'promoter insertion' mode and the result has been established as over-expression of the P1 isoform of RUNX1 (Wotton et al. 2002). Other examples from the RTCGD (retroviral tagged cancer gene database; http://variation.osu.edu/rtcgd/index.html) show insertions between the P1 and P2 promoters mainly in the opposite orientation to the gene, and the consequences for activation of either promoter have not been investigated.

In the case of *Runx3*, insertions appear to cluster at an upstream 'super-enhancer.' In the 1i

CD2-*MYC* lymphoma cell line MLV insertion at this site is in the upstream and backwards mode and is associated with over-expression of the P1 isoform of RUNX3 (Stewart et al. 2002) and a similar location and orientation is evident in other lymphomas from the CD2-*MYC* series (Fig. 16.1). While other RTCGD database examples are less clearly biased in orientation, multiple insertions were reported at the homologue of this site in MLV-induced lymphomas in the rat. This site was originally designated *Dsi1* before the discovery of the *Runx* genes, and it was noted that all insertions were in the same orientation, which we can now read as upstream and backwards with respect to *Run*x3. Notably, in the index case from which Dsi1 was cloned, there was also a proviral insertion at rat c-*Myc* (Vijaya et al. 1987). The *Runx3* gene was also identified as a target for MLV insertion in two independent transplanted B-ALLs in a mouse model of *BCR-ABL* under selection for imatinib resistance. In this system over-expression of either RUNX3 or RUNX1 was shown to diminish imatinib-induced apoptosis (Miething et al. 2007).

The *Runx2* gene has also been reported as a target for retroviral insertional mutagenesis in myeloid leukaemias of *Cbfb-MYH11* (Inv16) mice where it was initially considered a candidate tumour suppressor on the basis that the insertions were intragenic and potentially disruptive (Castilla et al. 2004). However, further study revealed that reduced dosage of *Runx2* suppressed disease in this model, while ectopic expression of full-length *Runx2* cooperated with *Cbfb-MYH11* in transplantation assays (Kuo et al. 2009), suggesting that the intragenic insertions may have been activating events. This conclusion appears to conflict with a recent report of intragenic *Runx2* insertions of the transposon *Sleeping Beauty* in leukaemias/lymphomas of *Rassfs1a*$^{-/-}$ mice which were also interpreted as inactivating events (van der Weyden et al. 2012). However, in view of the location and orientation of these insertions in *Runx2* (Fig. 16.1) and the lack of corroborating evidence that the gene is up-regulated by these insertions, it is difficult to judge which interpretation is correct. As discussed later (Fig. 16.6), other lines of evidence suggest that both may have credence, if reduced Runx expression is favoured in the early tumour development while high expression drives later stages.

16.2 The Human *RUNX* Genes Act as Frequent Targets for Retroviral Vector Insertion in CD34+ Cells

The development of vector-induced leukaemias in gene therapy trials (Hacein-Bey-Abina et al. 2008; Howe et al. 2008) demonstrated the mutagenic potential of murine gamma-retroviruses in human subjects and focused attention on the factors affecting this adverse event (Scobie et al. 2009). High throughput studies of MLV vector integration in human CD34+ cells revealed pronounced hot-spots or hyper-clusters, including the *LMO2* gene that was the most frequently activated target in gene therapy-related leukaemias (Cattoglio et al. 2010). The selective targeting of MLV integration to regions of active chromatin marked by acetylated histones due to direct interaction of the viral integrase protein with Brd/BET chromatin tethers sheds light on these observations (Gupta et al. 2013; Sharma et al. 2013). As shown in Fig. 16.2, the human *RUNX* genes also serve as preferential targets for integration in CD34+ and K562 cells *in vitro*, with a distribution that mirrors H3K27Ac chromatin marks. There is an evident difference between the targeted sites for each *RUNX* gene. For *RUNX3*, the upstream 'super-enhancer' is most frequently targeted, while most insertions in *RUNX1* and *RUNX2* are intragenic. The lack of orientation bias of these insertions is consistent with the interpretation that the clustered pattern results from preferential integration and that no significant post-integration clonal selection has occurred during the limited culture period prior to harvesting for analysis (Huser et al. 2014).

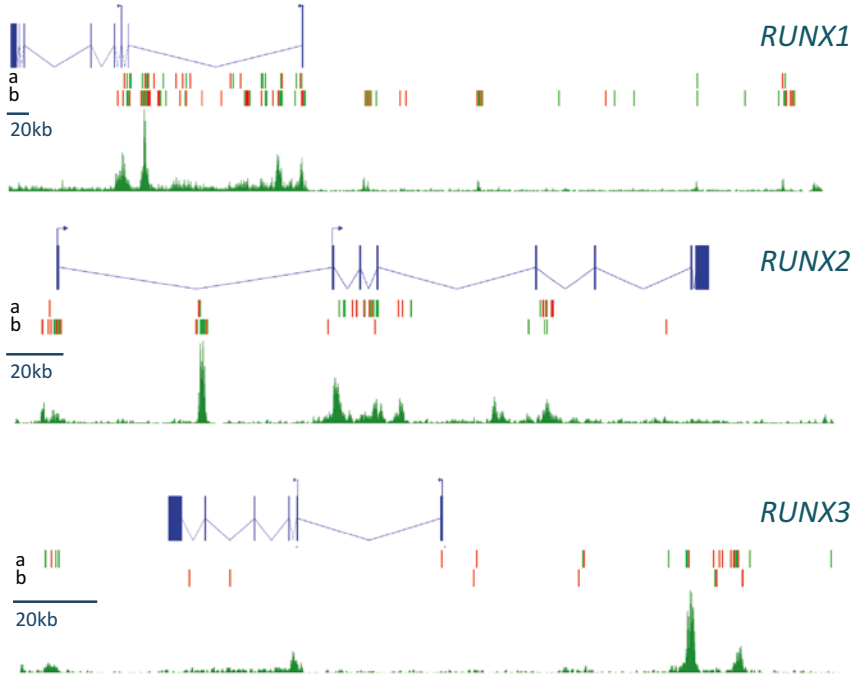

Fig. 16.2 Insertions at the *RUNX* gene loci in human haematopoietic cells transduced with MLV vector or infected with MLV/VSV pseudotypes. The *top* of each track shows gene structure, with *solid vertical bars* representing exons and lines representing introns, with *arrows* showing transcriptional start sites. Insertions in the forward direction are coloured *green* while those in the reverse direction are coloured *red*. Tracks (**a**–**b**) represent datasets: (**a**) MLV vector insertions in CD34+ cells (Cattoglio et al. 2010). (**b**) MLV Insertions in K562 cells (LaFave et al. 2014). The *bottom* of each track shows H3K27ac intensity in CD34+ primary cells, obtained from the human Epigenetics Roadmap

The frequent targeting of all three *RUNX* genes by retroviral vectors in CD34+ cells is also consistent with the fact that they are all transcriptionally active in early haematopoiesis, including *RUNX2*, providing a parallel with the targeting of *Runx2* by retroviral insertion in the Inv16 leukaemia model (Castilla et al. 2004). Moreover, the sites of preferred integration overlap to some extent with those selected in clonal end-stage murine lymphomas induced by MLV (Fig. 16.1). Despite these common features, end-stage patient leukaemias have not yet revealed the *RUNX* genes as targets, nor have the *RUNX* genes emerged from monitoring of insertion sites in the blood of healthy trial subjects (Hacein-Bey-Abina et al. 2008; Schwarzwaelder et al. 2007). This is perhaps not surprising in light of the requirement for activation of MYC or loss of p53 to facilitate the oncogenic effects of RUNX overexpression in murine models.

16.3 Switching the *Runx* Genes from Growth Suppressors to Oncogenes In Vivo: The Roles of MYC and p53

The potent synergy between RUNX2 and MYC in transgenic mice over-expressing both genes in the T-cell compartment (CD2-*MYC*/CD2-*Runx2* mice) confirmed the dominant oncogenic potential of RUNX2 (Vaillant et al. 1999). While the generality of the MYC/RUNX synergy was later confirmed by synergistic induction of B-cell lymphomas in *vav-Runx1*/Eμ-*Myc* mice (Blyth et al. 2009), most studies to date have been conducted with the CD2-*Runx2* model. Under the control of the CD2 LCR, RUNX2 was shown to accelerate lymphoma development in Eμ-Pim1 and CD2-v-Myb transgenic mice as well as in $p53^{null}$ mice (Blyth et al. 2001; Cameron et al. 2003), indicating a non-redundant, unique role for RUNX in

tumour development. However, the selective targeting of *c-Myc* and *N-Myc* in virus-accelerated tumours of CD2-*Runx2* mice was a further indication of a special relationship between RUNX and MYC oncogenic functions (Blyth et al. 2001).

An unusual feature of the CD2-*MYC* model is the undetectable expression of the CD2-*MYC* transgene in the majority of mice that remain healthy (Stewart et al. 1993). To account for this phenotype it has been suggested that variegated expression of the hCD2 locus in early lymphoid development (Williams et al. 2008) leads to MYC-induced apoptosis in expressing cells and that compensatory expansion of transgene non-expressing cells leads to replenishment and generation of an apparently normal lymphoid compartment (Blyth et al. 2006). It seems likely that the potent oncogenicity of the CD2-*MYC*/CD2-*Runx2* combination is at least partially explained by the simultaneous activation of both oncogenes at the same stage of early lymphoid development. Similarly, the efficiency of virus acceleration in CD2-*MYC* mice may be explained by the activation of collaborating genes by viral infection and integration in lymphoid progenitor cells that have yet to activate the CD2-*MYC* transgene (Stewart et al. 1993). The remarkably low apoptotic index of spontaneous CD2-*Runx2* lymphomas and reduced apoptosis in compound transgenics compared to CD2-*MYC* alone in this setting indicates that the major selective advantage of RUNX2 in this context is survival and ablation of MYC-induced apoptosis (Blyth et al. 2006).

In the CD2-*Runx2* model, where RUNX2 expression is initiated in early lymphoid development, there is a gene dose-dependent predisposition to thymic lymphoma development (Vaillant et al. 1999). However, analysis of thymic development in the transgenic mice revealed a marked deficit in fetal thymocyte numbers rather than a preneoplastic expansion, indicating that the initial response to ectopic RUNX2 expression is growth suppressive. Moreover, analysis of the immature CD8+ subset (CD8ISP) which was expanded in these mice showed that these were small cells with a markedly lower proliferation rate than CD8ISP from healthy mice (Vaillant et al. 2002). A similar though less marked phenotype was observed in *vav-Runx1* mice which displayed reduced proliferation in haematopoietic stem/progenitor cells and B-cells, although with enhanced survival (Blyth et al. 2009). The ability of MYC to counteract the growth suppressive potential of ectopic RUNX expression is evident from the rapid onset of tumours in vav-Runx1/Eμ*Myc* and in CD2-*Runx2*/CD2-*MYC*, where the CD8ISP population observed in CD2-*Runx2* mice is transformed to large blastic cells in the premalignant thymus (Blyth et al. 2009; Blyth et al. 2006; Vaillant et al. 1999). The rapid onset of lymphomas in CD2-*Runx2*/p53^{null} mice shows that loss of the p53 pathway can also synergise with ectopic Runx expression (Blyth et al. 2001). A diagram summarising the collaboration of *Myc* and *Runx* genes in the context of T-cell lymphomas under the influence of CD2 LCR-driven gene expression is summarised in Fig. 16.3.

16.4 Over-Expression of RUNX and MYC Combine to Disarm the p53 Response In Vivo: A Dual Signal Hypothesis

While *MYC* and *Runx* transgenes are potently synergistic in lymphomagenesis, they also collaborate independently with germline inactivation of p53 (Blyth et al. 1995; Hsu et al. 1995). In accord with this observation, both MYC and RUNX induce p53 responses when ectopically expressed (Wolyniec et al. 2009; Zindy et al. 1998). Remarkably, the combination of transgenic MYC and RUNX2 appears to neutralise p53 activation, abolishing the selection to lose the wild-type *Trp53* allele in primary and transplanted lymphomas on a CD2-*MYC*/ *Runx2*/ Trp53+/− background. The fact that the wild-type allele is rapidly lost on *in vitro* establishment of cell lines argues that it was intact but not active in the primary lymphomas (Blyth et al. 2006). These observations suggest that, *in vivo*, RUNX expression modifies the effects of MYC on the p53 pathway and vice versa. While the underlying mechanism has yet to be uncovered (Fig. 16.3b), it is notable that other oncogenes identified as potent MYC collaborators in retroviral mutagenesis screens impinge on the p53 pathway, including Bmi1, a repressor of Ink4a/Arf (Jacobs

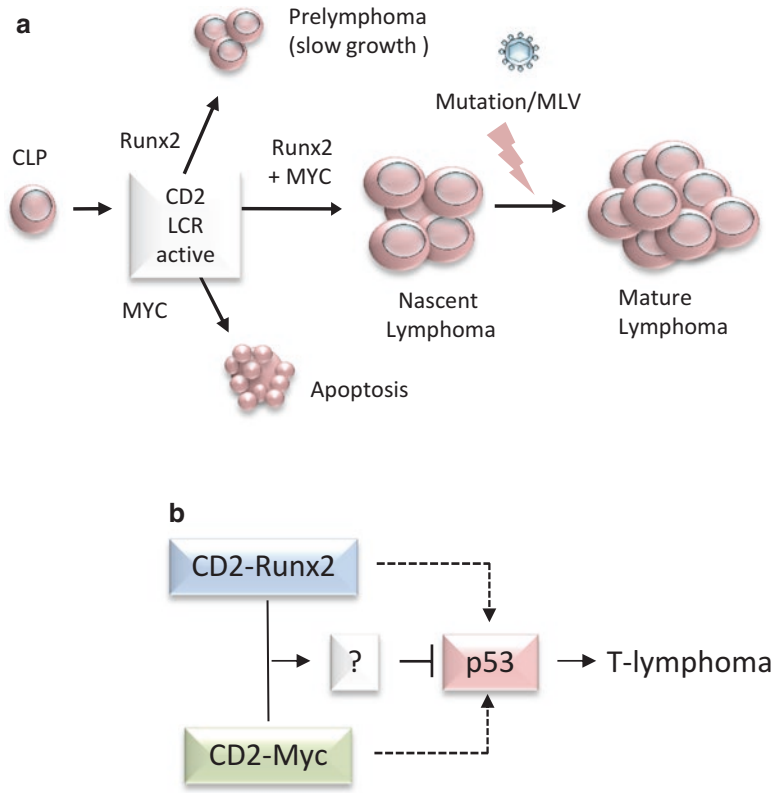

Fig. 16.3 Model of RUNX/MYC collaboration in lymphomagenesis. See text for explanation and supporting references. Panel *A* shows the key features of premalignant phenotype and disease in CD2-*MYC*, CD2-*Runx2* and compound transgenic mice. Panel *B* shows the interaction between RUNX2, MYC and p53 in the early T-cell compartment. Both CD2-*Runx2* and CD2-*MYC* transgenes are independently synergistic with germ-line inactivation of p53, suggesting that the oncogenic activity of both genes is antagonised by the tumour suppressor function of p53, while the combination of both oncogenes appears to overcome p53. We hypothesise that this phenomenon entails co-activation one or more genes that neutralises p53 function in T-lymphoma cells. CLP: common lymphoid progenitor

et al. 1999), and Gfi1, an indirect regulator of p53 activity via LSD1 mediated demethylation (Khandanpour and Moroy 2013).

Induction of MYC expression in primary fibroblasts leads to apoptosis unless pre-empted by a 'dual signal' provided by survival factors (Harrington et al. 1994) or relieved by inactivation of p53 (Hermeking and Eick 1994). It is interesting to compare this phenomenon to dual signalling hypotheses that evolved to account for shaping of the T-cell repertoire by positive and negative selection, and the proliferation of T-cells in response to foreign antigens (Zinkernagel et al. 1978). Notably, MYC expression enhances positive selection of thymocytes with functional T-cell receptors (Rudolph et al. 2000), while early studies showed that ectopic expression of RUNX1 could block TCR-signalling induced apoptosis of T-cell hybridomas (Fujii et al. 1998). It is tempting to suggest that the extremely potent synergy between *Runx* and *Myc* genes in driving lymphoma development is not merely a disordered response in the context of cancer, but an inherent feature of a signalling network that normally licenses lymphoid cells to proliferate in response to exogenous signals. The occurrence of autoimmune disease and hypersensitivity in *Runx*-deficient mice also provides indirect support for this hypothesis (Brenner et al. 2004; de Bruijn and Speck 2004; Wong et al. 2012).

16.5 Lymphoma Progression: Identification of Third Hit Genes in *MYC/Runx2* Lymphomas

Analysis of rapid onset tumours arising in CD2-*MYC*/CD2-*Runx2* transgenic mice showed that these are clonal outgrowths as indicated by their unique patterns of T-cell receptor rearrangement (Vaillant et al. 1999). This observation suggested that a further selective step is required to drive the end-stage lymphomas and led us to conduct further retroviral mutagenesis screens to identify the key target genes. Neonatal infection of CD2-

Table 16.1 Analysis of MYC/RUNX collaboration by retroviral insertional mutagenesis

Transgene	Disease		Preferred RIM targets
	−MLV	+MLV	
CD2-MYC	T-cell lymphoma Low incidence	T-cell lymphoma Rapid onset 100 %	*Runx2, Runx3, Runx1*
CD2-*Runx2*	T-cell lymphoma Low incidence, later onset	T-cell lymphoma Rapid onset 100 %	*C-Myc, N-Myc, Ikzf1*
CD2-*MYC/Runx2*	T-cell lymphoma Rapid onset 100 %	T-cell lymphoma 100 % Accelerated onset and dissemination	Multiple: TCR, PI3K, JAK-STAT signalling pathways, chemokine receptors, G1 checkpoint controls

Huser et al. (2014) and Stewart et al. (2007)
T-cell lymphoma onset is accelerated markedly in CD2-*MYC* and CD2-*Runx2* mice by neonatal infection with Moloney MLV, but with complementary patterns of insertional mutagenic targets that show a strong reciprocal relationship between *Myc* and *Runx* oncogenes. *Ikzf1* is a target for intragenic insertions that can generate dominant negative isoforms that may relieve MYC repression as an alternative mechanism of activation. Retroviral acceleration of tumour onset in highly tumour-prone CD2-*Runx2*/CD2-*MYC* mice reveals multiple target genes. A common selective advantage predicted for these insertions is the ability to grow in the absence of exogenous growth signals

MYC/CD2-*Runx2* mice with Moloney MLV leads to even more rapid tumour onset, increased clonal complexity and increased dissemination of primary thymic lymphomas to peripheral lymphoid tissues (Stewart et al. 2007). As outlined in Table 16.1, analysis of the preferred RIM targets in the progressing lymphomas showed a strong bias towards G1 checkpoint genes (D cyclins) and other genes that overcome the requirement for exogenous growth factor signals (e.g. Pim kinases). A further deep profiling analysis of many thousands of insertion sites in these tumours (Huser et al. 2014) confirmed these core progression genes as part of a broader set enriched for T-cell receptor, PI3K and JAK-STAT pathways along with selected chemokine receptors involved in T-cell homing to thymus (*Ccr7*, *Ccr9*). Most of these genes were frequently targeted in tumours of wild type mice suggesting that this gene set is frequently recruited in the normal course of viral infection where unscheduled proliferation of the target cell provides a selective advantage. While the consequence in otherwise normal cells might be self-limiting proliferation, activation of these genes in a lymphoma stem cell transformed by MYC and RUNX over-expression is sustained proliferation in the absence of exogenous signals (Huser et al. 2014).

Potential parallels with this 'three-hit' model are emerging from recent studies on human cancer where a recent review highlighted evidence for RUNX2 synergy with PI3K/AKT signalling in multiple cancer types (Cohen-Solal et al. 2015). Moreover, it may be interesting to re-evaluate evidence for similar oncogene combinations that may have been overlooked e.g. in osteosarcomas where *RUNX2* and *CCND3* on chromosome 6p21 are frequently co-amplified and over-expressed (Lu et al. 2008) and *MYC* is also frequently over-expressed (Gamberi et al. 1998).

16.6 Evidence of RUNX Addiction in Lymphoma Development

Evidence that endogenous RUNX activity is important for lymphoma development was provided by the delayed onset of T-cell lymphomas in *Runx1*$^{+/-}$ mice, whether these are induced by Moloney MLV infection or by the potent CD2-*MYC*/CD2-*Runx2* combination. Moreover, the lack of evidence of loss of heterozygosity in these lymphomas argued strongly that the *Runx1* gene was acting to promote tumour development rather than as a tumour suppressor (Wotton et al. 2002). Even more strikingly, the frequently

occurring T-cell lymphomas in *Trp53*^{null} mice are virtually abolished on a *Runx1*$^{+/-}$ background (Shimizu et al. 2013). While these observations suggested an important pro-oncogenic role for basal RUNX1 expression, it could not be excluded that the gene plays an essential role in T-cell development and influences lymphomagenesis only indirectly by controlling the size of the target cell population.

A recent study in Eμ-*Myc*/*Runx1cKO* mice has provided direct evidence of addiction to RUNX1 in primary lymphoma cells which, in contrast to normal splenic lymphocytes, resist even mono-allelic deletion *in vivo*. While established lymphoma cell lines that have lost p53 function become permissive for complete loss of *Runx1*, the *Runx1*^{null} cells display a proliferative disadvantage and become markedly more sensitive to chemotherapeutics including doxorubicin and dexamethasone (Borland et al. 2016). These findings validate the *Runx* genes and their downstream effectors as targets for lymphoma therapies. Another notable feature of this study is that the transcriptional signature conferred by deletion of *Runx1* in these cells is enriched for genes involved in B-cell survival, proliferation and differentiation but does not include the 'ribosomal biogenesis' signature seen in *Runx1*^{null} haematopoietic progenitors (Cai et al. 2015) or the mitotic checkpoint signature observed in human AML cells after RUNX1 knockdown (Ben-Ami et al. 2013). While these findings again emphasise the cell context-dependent roles of RUNX, it should be noted that there are other players that may influence the outcome of loss of RUNX expression. In Eμ-*Myc* lymphomas, the cells overexpress MYC, a major driver of ribosome biogenesis that may be able to rescue loss of RUNX1 expression (Borland et al. 2016), while Kasumi AML cells express RUNX1-ETO, a potential antagonist of RUNX-dependent gene expression (Ben-Ami et al. 2013).

16.7 RUNX, p53 and Senescence: Insights from Primary Cells

Primary fibroblasts have provided many useful insights into the activities of cancer-relevant genes in the absence of the many genetic and epigenetic changes that affect the responses of established cancer cell lines (Etzold et al. 2016; Serrano et al. 1997). In primary murine embryonic fibroblasts (Fig. 16.4) or human foreskin

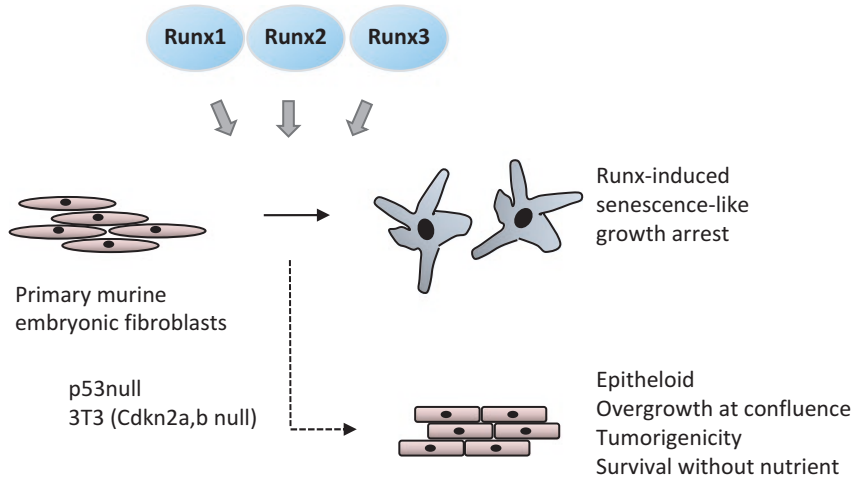

Fig. 16.4 Contrasting effects of ectopic RUNX expression in wild-type and established mouse fibroblasts. In primary mouse embryonic fibroblasts, RUNX overexpression leads to a profound growth arrest with flattened morphology and accumulation of senescence-associated β-galactosidase. In p53 deficient MEFs or NIH3T3 cells, ectopic RUNX expression leads instead to a morphological change resembling mesenchymal to epithelial transition along with enhanced growth and/or survival (Kilbey et al. 2007; Wotton et al. 2008, 2004)

fibroblasts (Hs68), ectopic expression of any of the three *Runx* genes (P1 or P2 isoforms) leads to senescence-like growth arrest (SLGA) (Kilbey et al. 2007; Wotton et al. 2004). Unlike cells undergoing Ras-induced SLGA which develops as a response to unscheduled proliferation and DNA damage (Di Micco et al. 2006), cells with enforced RUNX expression do not display nuclear DNA damage foci, and have a phenotype that instead resembles the effects of ectopic p53 expression. The requirements for induction of this failsafe response are not fully understood, but appear to engage both p53 and p16 (Cdkn2a) pathways. In primary MEFs lacking p53, the effect of ectopic RUNX1 is not growth arrest, but over-proliferation at confluence and increased tumorigenicity in nude mice (Wotton et al. 2004). In NIH3T3 cells, which have an abnormal p53 pathway response and lack p16 expression due to a large genomic deletion encompassing *Cdkn2a/Cdkn2b* (Wotton et al. 2004), the effect of ectopic RUNX expression is to promote an epitheloid morphology resembling mesenchymal to epithelial transition, and markedly enhanced survival under stress conditions (Wotton et al. 2008). This survival phenotype is associated with direct RUNX modulation of multiple enzymes involved in sphingolipid metabolism (Sgpp1, Ucgc, St3gal5), shifting the 'rheostat' from pro-apoptotic ceramides to pro-survival sphingosine-1-phospate (Kilbey et al. 2010). In human Hs68 cells, RUNX1 expression induces p53 despite the absence of detectable p14ARF expression, while the induction of SLGA is blocked by HPV E6. Moreover, Leiden fibroblasts (CDKN2A mutant) are resistant to RUNX1-induced SLGA (Wolyniec et al. 2009).

The strongly growth suppressive effect of RUNX over-expression in normal cells provides an important caveat to the interpretation of cancer cell inhibition by ectopic RUNX as evidence of a tumour suppressor function. This problem potentially compromises many publications on the *RUNX* genes and their putative roles in human cancer where in general only established cell lines are available for study. Moreover, the fact that the RUNX1-ETO fusion oncoprotein is also a potent inducer of senescence-like growth arrest in fibroblasts (Wolyniec et al. 2009) argues against a yin-yang interpretation of its functional relationship to RUNX1 mediated by regulation of p14/p19ARF (Linggi et al. 2002).

16.8 Translational Relevance: *RUNX* Genes as Dominant Oncogenes in Human Cancer

The relative rarity of copy number gains affecting the *RUNX* genes in human cancer and the widespread assumption that they are predominantly tumour suppressors has until recently diverted attention from their capacity to act as dominant oncogenes. Amplification of a large domain of chromosome 21 that encompasses *RUNX1* has been observed in a poor prognostic subset of B-ALL, presenting an exception to this rule (Harrison et al. 2014). Although it has been argued that these leukaemias do not significantly over-express *RUNX1* mRNA compared to ALLs lacking *RUNX1* amplification (Strefford et al. 2006) it is conceivable that other leukaemias over-express RUNX1 by different mechanisms, as *RUNX1* mRNA is highly elevated in many ALLs (Niini et al. 2002). Notably, gains of chromosome 21 and *RUNX1* copies are also evident in progressing t(12;21) leukaemias that express the TEL-RUNX1 fusion oncoprotein, in contrast to the frequent loss of the normal, non-translocated *TEL* allele (Lilljebjorn et al. 2010). The requirement for activity of the RUNX1 protein expressed from the untranslocated *RUNX1* allele for survival and proliferation of leukaemia cell lines harbouring RUNX1 fusion oncoproteins (Ben-Ami et al. 2013; Zaliova et al. 2011) also argues against a simple tumour suppressor role /dominant negative inhibitor relationship. It should also be noted that there are many ways in which RUNX expression can be dysregulated, including post-translational modification and translational controls via miRNA. Examples of RUNX oncogenic activity apparently mediated by such mechanisms have emerged from recent studies on human cancer cells of multiple types (Bledsoe et al. 2014; Browne et al. 2016; Shin et al. 2016).

16.9 The *Runx* Genes as Tumour Suppressors in Haematopoietic Cancers: Evidence from Mouse Models

The severe development defects resulting from germ-line deletion of the *Runx* genes has delayed assessment of their tumour suppressor activity *in vivo*, requiring the development of conditional knockout strains. As reviewed recently (Chin et al. 2015), conditional knockouts of *Runx1*, *Runx3* or *Cbfb* have revealed mainly myeloproliferative or myelodysplastic disease and/or haematopoietic stem cell expansions of varying degree. Deletion of *Runx1* in HSPC mediated by vav-Cre was shown to lead to reduced cell size as a result of diminished ribosome biogenesis, along with reduced apoptosis and resistance to genotoxic and ER stress, and it was suggested that this phenotype provides a selective advantage for null cells (Cai et al. 2015). Dual deletion of *Runx1* and *Runx3* using the Mx1-Cre system resulted mainly in bone marrow failure although this was preceded by expansion of haematopoietic stem/progenitor cells (HSPC) and almost 20 % of the mice developed fatal myeloproliferative disorder (Wang et al. 2014). While there are few reports of the development of spontaneous malignant disease in knockouts (Chin et al. 2015), *Runx1* inactivation collaborates strongly with other oncogenic insults such as FLT3-ITD or N-Ras to induce AML-like disease (Mead et al. 2013; Motoda et al. 2007). The predisposition towards MDS/AML rather than other malignancies in familial platelet disorder due to *RUNX1* mutation (Owen et al. 2008) suggests that the unique sensitivity of the myeloid lineage to *RUNX1* mutation and loss of function is conserved from mouse to human.

However, *RUNX1* mutations have also been found in some human lymphoid malignancies, notably in early T-ALLs (18 %) and a small subset of B-ALL also carrying BCR-ABL (Grossmann et al. 2011). Moreover, on the basis of a systems biology approach dubbed 'reverse engineering' of transcription networks, RUNX1 was predicted to act as a tumour suppressor in this lineage (Della Gatta et al. 2012). Evidence from mouse models in support of this designation is rather limited although one study of Mx1-Cre mediated deletion of *Runx1* reported thymic lymphoma in a proportion of *Runx1KO* mice. However, the major phenotype observed in these mice was myelodysplasia and a block in T-cell development and it is unclear from the report whether the lymphomas actually arose from $Runx1^{null}$ cells (Putz et al. 2006). Another early study in chimeric mice showed that *Runx1KO* cells were preferentially targeted in T-cell lymphomas induced by chemical mutagenesis (Kundu et al. 2005). As highlighted earlier, reduction to a single functional allele in $Runx1^{+/-}$ slows onset of MLV-induced T-cell lymphomas (Wotton et al. 2002) and virtually ablates spontaneous T-cell lymphomas in p53-deficient mice (Shimizu et al. 2013), arguing for a pro-oncogenic role. Moreover, a recent study has provided evidence that primary B-cell lymphomas in the Eμ-*Myc* model are addicted to RUNX1, while established cell lines lacking p53 become permissive to Cre-mediated deletion and display *Rag* gene de-repression, providing a potential explanation for the apparent oncogene/tumour suppressor paradox in the lymphoid compartment (Borland et al. 2016).

16.10 RUNX2 and Oncogene-Induced Senescence: A Temporal Model for RUNX Function in Cancer

Murine primary embryonic fibroblasts and osteoblasts lacking RUNX2 are prone to spontaneous immortalisation and tumorigenic conversion (Kilbey et al. 2007; Zaidi et al. 2007). Both cell types display reduced basal expression of a number of negative regulators of cell cycle progression that have been implicated as effectors of oncogene-induced failsafe responses (p16Ink4a, p19Ink4a, p53 and p21Waf1). These observations provide a rationale for the failure of primary fibroblasts and osteoblasts to undergo early growth arrest in the oxidative conditions of cell culture (Parrinello et al. 2003) and suggest a non-

redundant role for RUNX2, which is perhaps not surprising as RUNX2 is the predominant expressed family member in both cell types. *Runx2* null MEFs also resist H-Ras oncogene-induced senescence and become tumorigenic (Kilbey et al. 2007). However, despite their failure to arrest in response to mutant H-Ras, failsafe effectors are induced in *Runx2* null fibroblasts at levels comparable to wild-type cells. The basis of their continued proliferation in the presence of failsafe effector expression is not fully understood, but is associated with altered expression of chromatin remodelling factors that regulate cyclin gene expression (Kilbey et al. 2008). It has also been reported that loss of *Runx1* impairs N-Ras-induced failsafe responses in haematopoietic progenitors (Motoda et al. 2007), while *Runx3*cKO mice show accelerated lung tumour development in a K-Ras knock-in model (Lee et al. 2013) suggesting that this may be a wider feature of Runx oncogenesis (Fig. 16.5).

However, at this point knockout mouse models have supported a pro-oncogenic rather than a suppressor role for RUNX2 *in vivo* (Ferrari et al. 2015) as have many recent studies of human cancer. A rare co-occurrence of cleido-cranial dysplasia and AML suggested a possible loss-of-function scenario but instead the authors of that study found that RUNX2 was actually over-expressed, a phenomenon they suggested might be explained by compensatory up-regulation of the wild-type allele (Schnerch et al. 2014). As the *RUNX* genes can cross-regulate (Brady et al. 2009) it should also be kept in mind that functional loss of one family member may have consequences for other members, and that the point mutations of RUNX1 in AML and a handful of other cancers may affect more than merely RUNX1. These observations also invite us to propose a temporal model to explain the dualistic behaviour of the *Runx* genes in cancer. We hypothesise that reduced levels of RUNX expression may act early to promote cancer development in a number of ways; by facilitating the growth of cells carrying initiator mutations (including mutant Ras alleles), preventing exit

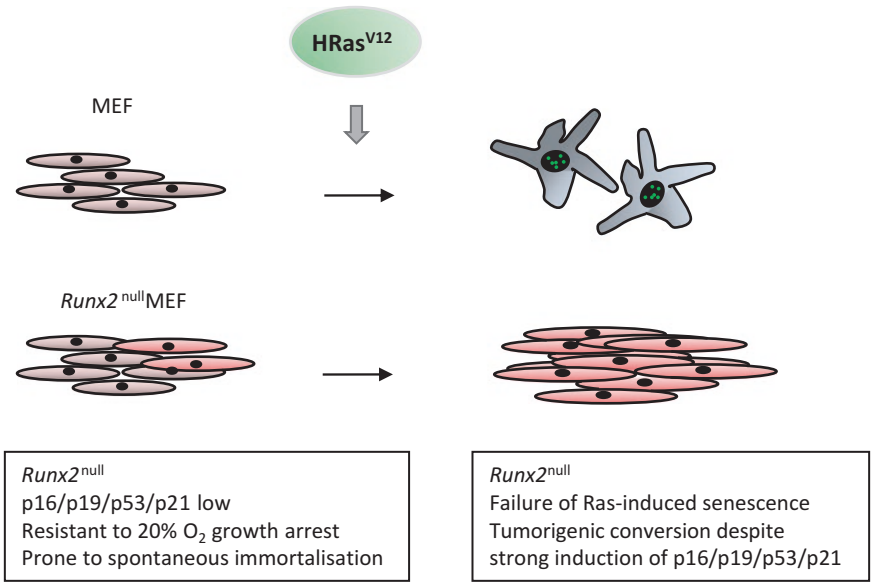

Fig. 16.5 *Runx2*^{null} mouse fibroblasts are prone to spontaneous immortalisation and resist Ras-induced senescence. *RUNX2* deficient primary embryonic fibroblasts express low levels of markers of aging and failsafe senescence (p16/p19^{Cdkn2a}, p53, p21^{Cdkn1a}), resist early growth arrest in normoxic culture and have an increased propensity for spontaneous immortalisation after 3T3 passage. Introduction of HRas^{V12} results in senescence-like growth arrest with nuclear DNA damage foci (*green*) in wild-type MEFs while *Runx2*^{null} cells proliferate and become tumorigenic, despite apparent induction of failsafe mediators (Kilbey et al. 2007)

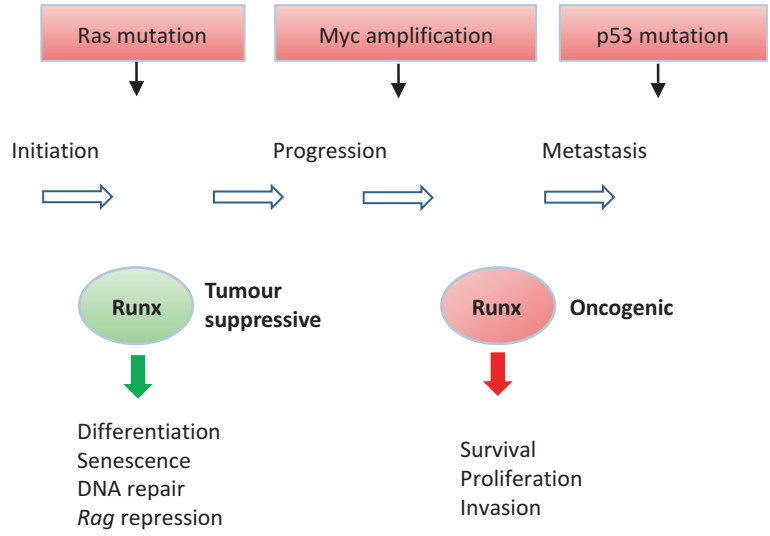

Fig. 16.6 A temporal model for RUNX function in cancer. In this model, RUNX expression is required for tumour suppressive cell fate decisions and protection against mutational damage in early tumorigenesis where compromised expression increases the probability of transformation. At later stages, over-expression of MYC and/or loss of p53 function unmask the latent oncogenic potential of RUNX and increased expression is selected in end-stage tumours

from stem cell/progenitor compartments, promoting genomic instability, impairing DNA repair and finally by de-repressing potentially mutagenic *Rag* genes. In contrast, in the later stages of cancer where Myc drives proliferation and the p53 pathway is compromised, *Runx* gene activity drives tumour cell growth and metastatic potential. This temporal model is also compatible with the apparent addiction of AML cells with RUNX1-ETO to expression of the wild-type allele of *RUNX1* (Ben-Ami et al. 2013) and the copy number gains of the unaffected *RUNX1* allele in progressing TEL-RUNX1 leukaemias (Lilljebjorn et al. 2012) (Fig. 16.6).

16.11 The *RUNX* Genes: Isoforms or Functionally Divergent Genes?

For historical reasons, the field has focused heavily on *RUNX1* in haemato-oncology, *RUNX2* in bone development, and *RUNX3* in immune cell function and tumour suppression. However, given the evidence of functional overlap as well as cross-regulation between family members (Spender et al. 2005) maintaining a singular focus on one family member while ignoring its relatives appears myopic. The fact that only *RUNX1* has emerged as a common target for chromosomal translocation events in human leukaemia appears suggestive of a unique function for this family member. However, it should be noted that this bias could arise instead due to the relatively high expression of *RUNX1* in haematopoietic progenitors where initiating events occur and/or to specific features of the *RUNX1* locus on chromosome 21 that confer susceptibility to rearrangement (Levanon et al. 2001).

Despite their unique roles in specific biological niches, indications of functional overlap between family members can be seen in the T-cell lineage where RUNX1 and RUNX3 act sequentially to silence CD4 (Taniuchi et al. 2002) and in bone where both RUNX1 and RUNX3 are required in addition to the bone 'master regulator' RUNX2 for full osteoblast function and skeletal development (Bauer et al. 2015; Liakhovitskaia et al. 2010). These observations prompt the question of redundancy and whether the products of all *Runx* genes should be regarded as isoforms that play unique roles in development only because of their tissue-specific expression patterns. An analogy may be drawn from the Myc gene family where the entire *N-Myc* coding sequence can replace *c-Myc* in murine development despite their significant sequence divergence (Malynn et al. 2000). While this type of experiment has

not been fully recapitulated for the *Runx* family, substitution of the C-terminus of RUNX1 with equivalent domains of RUNX2 and RUNX3 to create chimeric proteins led to at least partial rescue of haematopoietic development *in vivo* (Fukushima-Nakase et al. 2005). Moreover, all three genes appeared equally efficient in rescuing haematopoietic development of *Runx1*^{null} cells in an *in vitro* co-culture system (Goyama et al. 2004).

Direct comparison of all three genes by ectopic expression in murine fibroblasts and gene expression microarray analysis showed a very high degree of redundancy, with a strong overlap in the signature gene expression changes and no examples of opposing regulation. However, there were clearly differences with regard to the relative potency of regulation for individual target genes that could conceivably translate into functional differences in specific niches *in vivo* (Wotton et al. 2008). In our view the degree of RUNX redundancy and the biological contexts in which it may operate largely remains to be addressed.

16.12 Conclusions and Prospects

There is growing evidence that the oncogenic potential of the *Runx* gene family revealed by their powerful co-operation with MYC overexpression or p53 loss in mouse models is highly relevant to human cancer, where a growing body of literature attests to the important roles that RUNX family members play in supporting the oncogenic phenotypes of end-stage cancers and cell lines. The tumour suppressor features of the *Runx* genes have been less amenable to dissection in *in vivo* models, but are now being elucidated using conditional knockout models. Evidence that the *Runx* genes operate in a complex integrated regulatory network suggests that future studies should address effects on all three genes where any single gene is affected.

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