RUNX transcription factors at the interface of stem cells and cancer

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Abstract

The RUNX1 transcription factor is a critical regulator of normal haematopoiesis and its functional disruption by point mutations, deletions or translocations is a major causative factor leading to leukaemia. In the majority of cases, genetic changes in RUNX1 are linked to loss of function classifying it broadly as a tumour suppressor. Despite this, several recent studies have reported the need for certain level of active RUNX1 for maintenance and propagation of AML and ALL cells, suggesting an onco-supportive role of RUNX1. Furthermore, in solid cancers RUNX1 is overexpressed compared to normal tissue, and RUNX factors have recently been discovered to promote growth of skin, oral, breast and ovarian tumour cells, amongst others. RUNX factors have key roles in stem cell fate regulation during homeostasis and regeneration of many tissues. Cancer cells appear to have corrupted these stem-cell associated functions of RUNX factors to promote oncogenesis. Here, we discuss current knowledge on the role of RUNX genes in stem cells and as onco-supportive factors in haematological malignancies and epithelial cancers.

1. Introduction

Core binding factors are a heterodimeric group of transcription factors consisting of a RUNX DNA binding subunit and their partner – the core binding factor beta (CBFβ) subunit. There are three RUNX genes in mammals, RUNX1-3, each of which encodes a protein with the highly conserved N-terminal Runt DNA binding domain and a C-terminal region containing transactivation and repressor domains that mediate interaction with a variety of regulatory factors (Figure 1). RUNX factors can both activate and repress a multitude of target genes in a context-dependent manner. The three members of the RUNX family display distinct, tissue-specific expression and lineage-restricted roles. RUNX1 is crucial for haematopoietic development, RUNX2 is a master regulator of osteogenesis, while RUNX3 has a central role in neural and T lymphocyte development (1-3).

Several critical domains are responsible for RUNX function with the N-terminal Runt homology domain (RHD) being responsible and sufficient for DNA binding and for heterodimerization with the CBFβ subunit (4, 5). The Runt domain contains a nuclear localization signal (NLS) and binds a consensus DNA motif 5'-PuACCPuCA-3' (6). The transactivation domain (TAD) is rich in proline, serine and threonine and is responsible for target gene transactivation. RUNX1 isoforms lacking TAD are found to act as suppressors and to compete with full-length RUNX1 for DNA binding (7). Proteins interacting with the TAD include the p300 acetyltransferase, MAD homologs (SMADs), Yes-associated proteins (YAPs) and C/EBPα among others (8-11). Downstream of the Runt domain a lower degree of homology is observed among the RUNX proteins, suggesting that this

may account for their functional differences. RUNX1 is by itself a weak transcriptional regulator and requires interaction with other factors to exert its activity as either a repressor or activator (12, 13). The majority of known RUNX1 partners are involved in haematopoiesis, such as the lymphoid-specific ETS1 TF, C/EBPα expressed in myeloid cells and PU.1 expressed in both lineages.

Numerous post-translational modifications (PTMs) were also found to modulate RUNX1 function and may explain how cells fine-tune RUNX1 activity in a context-dependent manner (reviewed in (14)). Briefly, phosphorylation leads to increased transcriptional activity either by disrupting interaction with co-repressors or by phosphorylating and stimulating the acetyltransferase activity of p300. Cyclin dependent kinases (CDKs) -1, -2 and -6 also induce RUNX1 phosphorylation thereby promoting degradation by the anaphase-promoting complex (APC) (15).

RUNX factors have been implicated as tumour suppressors or oncogenes in a variety of cancers (16). RUNX1 was first identified at the breakpoint of the t(8;21) translocation in acute myeloid leukaemia (AML) that results in fusion of the RUNX1 DNA binding domain to the ETO repressor protein, first highlighting the importance of this class of transcription factors in cancer (17). Subsequently, several mutational mechanisms have been identified to affect RUNX1, including chromosomal breakage, leading to the formation of novel fusion oncogenes, point mutations, found predominantly in AML and myelodysplastic syndromes (MDS), and increased dosage by acquisition of additional RUNX1 copies (18-21). The ETV6-RUNX1 fusion is found in ~25% of B-cell acute lymphoblastic

leukaemia (B-ALL) cases, while RUNX1-ETO is present in ~10% of AML patients. RUNX1 fusions commonly retain the Runt domain (Figure 1), and are suggested to act in a dominant repressive manner over the wild-type copy (18). Despite being the initiating event leading to leukaemia, RUNX1 fusions are by themselves insufficient to induce overt disease and require additional genetic changes. Point mutations in RUNX1 affect predominantly the Runt domain and are loss-of-function due to the inability of the TF to bind to DNA and/or to the CBFβ subunit (22). Based on the observations that inactivating mutations in RUNX1 are tumourigenic, this TF has largely been regarded as a tumour suppressor. However, both alleles of RUNX1 are rarely mutated in haematological malignancies, and some leukaemias exhibit amplification of RUNX1, suggesting that a certain level of activity is necessary and might be advantageous for disease progression. Recently, studies have revealed an oncogenic function of RUNX1 in a variety of different leukaemia types. Furthermore, RUNX1 is overexpressed in many solid cancers and RUNX factors have recently been implicated in promoting growth and survival of a variety of cancers. However, RUNX factors do not appear to act as dominant oncogenes but rather to support the proliferation, survival and migration of cancer cells. The oncosupportive function of RUNX in many cancers may represent an Achilles heel that may be exploited for novel cancer therapies. The recent development of compounds that disrupt the interaction between RUNX and CBFβ has opened up the exciting possibility of directly targeting RUNX factor function in cancer (23) (Figure 2).

In normal tissue homeostasis, RUNX factors are increasingly associated with the regulation of stem cell fate. RUNX1 was identified initially as a key regulator of

haematopoietic stem cell emergence in the embryo, but RUNX factors have now also been found to regulate the regenerative properties of blood, skin, neural, muscle, mammary and mesenchymal stem cells. Interestingly, the requirement for RUNX factors in cancer appears to mirror their involvement in stem cell regulation in those tissues. In this review, we discuss the role of RUNX factors, especially RUNX1, in regulating stem cell fate and how their function has been co-opted in cancer cells to promote carcinogenesis.

2. RUNX factors as key regulators of stem cell fate

2.1. Haematopoietic stem cells

Runx1 is required for the development of definitive haematopoiesis in the embryo and homozygous loss of function results in embryonic lethality (1, 24). By conditionally deleting Runx1 in endothelial cells it was demonstrated that Runx1 is essential for the endothelial to haematopoietic transition that results in the emergence of haematopoietic stem cells (HSCs) from the ventral wall of the dorsal aorta and other arterial sites (25). However, specific excision of Runx1 in haematopoietic cells revealed that once HSCs are formed, Runx1 is then relatively dispensable for HSC self-renewal (25, 26). Functional assessment of long-term HSCs (LT-HSCs) revealed a small reduction in the number of LT-HSCs in these animals but relatively normal long-term self-renewal capacity (26). However, the differentiation of lymphoid and megakaryocytic lineages is impaired by Runx1 deletion and myeloid progenitors exhibit a mild expansion resulting in a myeloproliferative phenotype (27, 28).

Despite their normal self-renewal, Runx1-deficient HSCs have a slow growth phenotype characterized by an increase in cells in G1 and they are also smaller and metabolically less active (26, 29). Runx1 promotes cell cycle progression at the G1/S transition in haematopoietic cells at least partially through activation of *Cyclin D3* and *Cdk4* transcription and repression of *p21/CDKN1a* (30). In addition, Runx1-deficient HSCs were recently discovered to exhibit reduced ribosomal biogenesis resulting from a reduction in transcription of ribosomal RNA (rRNA) and ribosomal protein genes mediated by direct Runx1 regulation of their promoters, and this is likely to contribute to their slower growth (29). RUNX factors may have a general role in regulating ribosomal biogenesis as RUNX2 was previously found to bind to ribosomal DNA, although in this situation RUNX2 had a repressive effect on rRNA expression consistent with its inhibitory effect on osteoblast growth (31). Whether RUNX genes regulate ribosome biogenesis in other stem cell types, and the relevance to RUNX function in cancer has yet to be determined. However, it has been proposed that reduced ribosome biogenesis caused by RUNX1 loss of function mutations may mediate stress resistance and perdurance of pre-leukaemic stem cells during AML development (29).

2.2. Hair follicle stem cells

A wider role for RUNX factors in other tissue stem cells was not appreciated until Runx1 was discovered to promote hair follicle stem cell (HFSC) activation (32). The stem cells of the hair follicle reside in the bulge region and undergo cyclical organ transformation involving growth (anagen), and regression (catagen) with a period of intervening quiescence (telogen). Careful analysis of the hair cycle in

Runx1 epithelial conditional KO mice revealed that Runx1 is required for timely activation of hair follicle proliferation and anagen onset (32). Lineage tracing demonstrated that Runx1 is expressed in long-term self-renewing HFSCs and bulge stem cells have a cell-intrinsic requirement for Runx1 to promote proliferation during anagen (33, 34). Runx1 directly regulated exit from quiescence and entry into S phase through repression of cyclin dependent kinase inhibitor expression and p21 deletion rescued proliferation of Runx1 deficient keratinocytes (33, 35). Runx1 is also expressed in oral epithelial stem cells and co-localises with the stem cell marker, Lgr5, in cells in the base of the crypt, as well as transit amplifying cells in the upper crypt, suggesting a conserved role in different types of epithelial stem cells (34).

Using a Runx1 reporter and genetic manipulation of Runx1 expression, the Tumbar group demonstrated that cells in the hair germ either differentiate or revert back to HFSCs from an activated progenitor like state depending on the level of Runx1 expression. This analysis revealed that despite being required for proliferation at anagen onset, Runx1 is not sufficient to drive proliferation in quiescent cells (36). However, forced overexpression enhances proliferation of actively cycling cells, but also drives apoptosis resulting in stem cell exhaustion and senescence, reflecting an endogenous role of Runx1 upregulation in promoting the onset of programmed cell death during catagen (36). This illustrates the extreme dose-dependency of Runx1 action, which may be highly relevant to understanding its role in carcinogenesis, where apparently dichotomous tumour suppressor and oncogenic functions have been observed.

Runx1 is downregulated concomitant with cell division and differentiation of hair follicle progenitors, reminiscent of the downregulation of the Runx factor RNT-1 coincident with onset of mitosis in *Caenorhabditis elegans* seam cells, a stem like cell forming the skin of the worm (37, 38) (and Nimmo and Woollard, unpublished observations). RNT-1 is required for seam cell division and preventing RNT-1 downregulation after mitosis promotes an extra round of cell division in these cells (37). Strikingly, overexpression of RNT-1 and in conjunction with the CBFβ homologue BRO-1 drives more severe hyperplasia, suggesting that the expression of both Core Binding Factor subunits is rate-limiting for proliferation in these cells (38). In a variety of cell types RUNX factors have been shown to be subject to regulation dependent on the phase of the cell cycle (30). For example, Cyclin D directly binds and inhibits RUNX1 transactivation and Cdk-dependent phosphorylation of RUNX1 at S303 promotes degradation by the anaphase-promoting complex at G2/M (15, 39). It is likely that these feedback mechanisms have evolved to prevent excessive proliferation of stem/progenitor cells and ensure balanced proliferation and differentiation during homeostasis.

Runx1 is expressed prior to the onset of proliferation in both worm and mouse skin progenitors and is required for cell division and exit from quiescence. However, forced expression of RUNX1 or RNT1 can promote increased proliferation only in cells that are already primed to cycle and is not sufficient to drive cell division in quiescent cells in either system (36-38). RUNX factors therefore appear to act as competency factors for proliferation in both worm and mammalian skin ensuring that the stem cells are ready and able to respond to

mitogenic signals occurring at defined stages. In support of this, the genes associated with RUNX upregulation in HFSCs include many metabolic genes that may promote cellular growth and thus prepare cells for proliferation (36). It will be interesting to investigate whether RUNX factors directly regulate ribosomal biogenesis in mammalian HFSCs and worm seam cells, as Runx1 does in HSCs, and if this mediates its function as a competency factor for cellular proliferation.

Runx1 upregulation is associated with migration of bulge cells from the niche into the outer root sheath during catagen and analysis of gene expression changes associated with forced Runx1 expression in HFSCs revealed enrichment of cell adhesion molecules in the down-regulated gene set (36). Runx1 may therefore directly regulate cell adhesion, as supported by the reduced migration of Runx1 deficient keratinocytes (40). RUNX factors also regulate migration and invasion of breast and ovarian epithelial cancer cells suggesting that these cancers have co-opted this physiological function of RUNX factors to promote metastasis of transformed epithelial cells (see sections 3.2.2 and 3.2.3).

2.3. Mammary epithelial stem cells

Mammary stem cells are multipotent cells that self-renew and give rise to both luminal and basal lineages of mammary epithelial cells. Runx2 was initially studied in breast cancer as it was found to promote the invasive, metastasic and osteolytic capacity of breast cancer cells (41-44). However, it was only recently discovered to have a role in normal mammary stem cells (MaSCs). Most studies of RUNX factor function in the mammary epithelium have used the MMTV-Cre system which predominantly targets the luminal compartment, but Ferrari et al

used a K14-Cre to generate Runx2 deletion in the basal mammary epithelial lineage including MaSCs (45). Conditional inactivation of RUNX2 resulted in a failure of excised MaSCs to regenerate new mammary glands in recipients (45). Furthermore, Runx2-deleted cells formed fewer and smaller primary and secondary mammospheres *in vitro* and had reduced colony-forming capacity, both surrogate assays for stem cells in this system (45). Embryonic mammary buds from mice with constitutive Runx2 KO form underdeveloped mammary glands after transplantation and MMTV-Cre deletion of Runx2 leads to reduced alveolar differentiation during pregnancy (46). However, conversely, forced expression of Runx2 from the MMTV promoter delays ductal elongation and inhibits lobular alveolar differentiation during late pregnancy and results in inappropriate cell cycling observed at lactation with over half of aged MMTV-Runx2 over-expressing mice developing hyperplasia (47). It is therefore possible that the apparent defects in alveolar differentiation in Runx2 KO mammary glands may result from reduced expansion of alveolar progenitors rather than a failure in lineage specification. Together, these data suggest that Runx2 may be involved in regulating the balance between proliferation and differentiation in mammary epithelial development.

Both Runx1 and Runx2 are expressed in mammary epithelial cells and both affect normal mammary gland development and differentiation, raising the possibility of partial redundancy in this tissue. However, there may be some lineage specificity and antagonistic functions as although Runx2 promotes alveolar fates, this is the only mammary epithelial cell type in which Runx1 is not expressed and Runx1 instead promotes luminal fates at least in part through

repression of the alveolar transcription factor Elf5 (48). Moreover, Runx1 deletion using MMTV-Cre results in a decrease in mature luminal cells. Interestingly this loss can be rescued by loss of Rb or p53, and p53-related gene sets were enriched in Runx1-deficient luminal cells suggesting a role for cell cycle and survival pathways downstream of Runx1 (48).

Both Runx1 and Runx2 are preferentially expressed in basal cells (containing MaSCs) and so it will be interesting to investigate whether Runx1 has a role in MaSCs in addition to Runx2. If they act redundantly, the compound KO deletion of Runx1 and 2 in MaSCs using the K14-Cre may reveal a more severe stem cell defect in these animals.

In summary, RUNX factors have a role in both the regenerative potential of MaSCs (Runx2) and in promoting differentiation of mature mammary epithelial cells (Runx1 and Runx2). This is similar to the observation in haematopoietic and hair follicle stem cell lineages where RUNX factors have stem cell supportive functions in primitive cells as well as promoting differentiation of particular cell lineages derived from these stem cells.

2.4. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal and differentiation into cartilage, bone and adipose tissues. In prostate cancer myofibroblasts promote tumour formation and are produced from tissue resident MSCs in response to TGFβ secreted by the tumour cells. RUNX1 was identified as a key transcription factor induced by TGFβ in prostate cancer

274 associated MSCs (49). Although TGFβ promotes myofibroblast differentiation,
275 RUNX1 overexpression actually promotes MSC proliferation and delays MSC
276 differentiation. Conversely, knockdown of RUNX1 in human prostate and bone
277 marrow-derived MSCs prevented their proliferation due to cell cycle arrest and
278 promoted myofibroblast differentiation (49). During MSC differentiation,
279 induction of RUNX1 may therefore act to link differentiation signals to onset of
280 proliferation ensuring that MSCs undergo expansion prior to terminal
281 differentiation into myofibroblasts. Since myofibroblasts are part of a tumour-
282 promoting reactive stroma in cancer, this data suggests that therapeutic
283 targeting of RUNX1 could abrogate tumour growth by preventing the cancer
284 from remodeling its niche through secretion of TGFβ.
285
286 **2.5. Neural stem cells**
287 RUNX factors are intimately linked with TGFβ signaling in a variety of contexts.
288 In neurogenic regions of the adult brain - the hippocampal dentate gyrus (DG)
289 and the forebrain subventricular zone (SVZ) - TGFβ signaling is induced by injury
290 along with upregulation of Runx1, both in the microglia and neural
291 stem/progenitor cells, and is associated with increased proliferation of these
292 cells (50). Runx1 is not normally detectably expressed in the neural
293 stem/progenitor cells (NSPCs) in the DG or SVZ , but it is rapidly induced in
294 Nestin+ progenitors after injury (50). In neurosphere cultures of NSPCs
295 inhibition of Runx1 reduced their proliferation but overexpression increased
296 differentiation, predominantly down the neuronal lineage (51). It was also
297 previously shown that Runx1 promotes proliferation in embryonic olfactory bulb
298 progenitors (52). Runx1 therefore has a developmental role in promoting neural

progenitor proliferation and may also act in neural stem/progenitors to promote the repair of neural tissue after injury. It will be interesting to investigate whether Runx1 has a role in brain tumours such as glioblastoma, in which neural stem cell self-renewal mechanisms are corrupted to promote malignant growth.

2.6. Muscle stem cells

Muscle satellite cells (SCs) are stem cells responsible for muscle regeneration and Runx1 is required to promote stem/progenitor cell expansion in response to injury. SCs regenerate muscle by proliferating, differentiating and fusing to form new myofibres. Runx1 is highly expressed in myopathic muscles, including satellite cells, although it is apparently not expressed homeostatically in embryonic or adult muscle tissue. In a mouse model of Duchenne muscular dystrophy (DMD) muscle-specific deletion of Runx1 revealed a pronounced defect in muscle regeneration leading to reduced life span, weight loss and impaired muscle performance (53). Consistent with a role for Runx1 in satellite cell regeneration, the mice had fewer Pax7-expressing satellite cells and a reduced number of proliferating myoblasts. Culturing the Runx1-deleted primary myoblasts revealed they had lower proliferation and higher rates of spontaneous differentiation, and conversely overexpression of Runx1 delayed differentiation and reduced numbers of multinucleated myofibres (53). Runx1 therefore regulates the balance between proliferation and differentiation of satellite cells during muscle regeneration.

2.7. Summary: Stem cells

RUNX genes are associated with stem cell function in many tissues and in general it appears that they function to promote the high levels of proliferation needed to regenerate tissues either during homeostasis or repair. However, their proliferative functions are intimately linked with differentiation as RUNX factors act as rheostats for cellular proliferation and are often downregulated in differentiating cells. Forced expression delays but does not completely block differentiation, perhaps explaining why wild type RUNX factors do not act as dominant oncogenes but rather as competency factors for oncogenesis – leading us to define them as "onco-supportive" (see section 3). Furthermore, in many lineages RUNX factors also have a role in promoting cell type-specific differentiation in a lineage-dependent manner. They may therefore ensure balanced tissue regeneration by directly tethering progenitor expansion to exit from the progenitor state into post-mitotic mature effector cells. This may explain why Runx1 also has a tumour suppressive role. Inactivating mutations and translocations in RUNX1 in luminal breast cancer and haematological malignancies may lead to a block in differentiation and formation of an aberrant progenitor that retains a wild-type copy of RUNX1 to support its continued proliferation.

3. Onco-supportive effects of RUNX factors in cancer

3.1. Haematological malignancies

The idea that RUNX proteins can have an oncogenic role was first suggested by the discovery that all three RUNX members are targets for murine leukaemia virus (MLV) insertional mutagenesis (54, 55), and ectopic expression of RUNX1 in a Eμ-Myc lymphoma model was found to drive lymphomagenesis and promote

B-cell survival (56). However, it was not clear from these studies if endogenous RUNX1 was required for lymphomagenesis but it has now been shown that basal expression of normal RUNX1 is critical for the maintenance of primary Myc-driven lymphoma *in vivo,* although this dependence is partially attenuated in p53-deficient cells (57).

In leukaemia, although translocation and point mutations in core binding factor genes are frequent events, complete loss of RUNX1 in leukaemias bearing RUNX1 fusion genes is very rare. Instead, the normal copy of RUNX1 is retained and even amplified, suggesting its possible requirement for leukaemogenesis (58-60) (summarized in Figure 3). In addition, increased dosage of RUNX1, either by acquisition of an additional chromosome copy (trisomy 21) or by intrachromosomal amplification of one copy of chromosome 21 (iAMP21), has been linked to increased risk of leukaemia (21, 61-63). The extent and mechanism behind RUNX1 involvement in these malignancies is not completely understood and requires further investigation.

3.1.1. Acute myeloid leukaemia (AML)

RUNX1 was first identified as the gene at the breakpoint of the t(8;21) translocation found in around 10% of AML patients. In this translocation, the Runt DNA binding domain of RUNX1 is fused to the ETO protein, producing a fusion protein that was originally proposed to act as constitutive repressor of Runx1 targets. RUNX1-ETO knockin causes early embryonic lethality and haematopoietic defects similar to those in Runx1 knockout mice suggesting that RUNX1-ETO acts as to dominantly inhibit normal Runx1 function (64). Another

chromosomal rearrangement, inv(16) fuses the CBFβ and MYH11 genes to produce the CBFβ -SMMHC oncoprotein which is also thought to act as an inhibitor of normal Runx1 function by sequestration of RUNX1 (65). Further evidence that Runx1 has a tumour suppressive role in myeloid cells comes from the finding that inactivating mutations of Runx1 are frequently found in myelodysplastic syndromes and AML (66, 67). However, these mutations are usually heterozygous, and mutation of the remaining allele of Runx1 is not found in patients with CBF or MLL rearrangements suggesting that wild type Runx1 activity is important for leukaemic growth and propagation.

Several studies in AML have now reported a role of native RUNX1 in supporting leukaemic development. Inhibition of RUNX1 either by shRNA depletion or expression of dominant negative RUNX1 mutants in human cord blood cells expressing AML-ETO or MLL-AF9 had a growth-inhibitory effect due to cell cycle arrest and increased apoptosis. (68). Furthermore, RUNX1 was also essential *in vivo* for engraftment of primary MLL-rearranged leukaemia cells suggesting that RUNX1 activity is required for the growth of these leukaemias. BCL2 was identified as an important mediator of the survival effect exerted by RUNX1, but could not on its own rescue RUNX1-depletion phenotype, suggesting that other factors are contributing to this oncosupportive phenotype. The oncosupportive role of RUNX1 was also revealed in a mouse model expressing *Cbfb-MYH11* in which a dominant negative form of RUNX1 rescued differentiation defects and delayed leukaemia development (69).

It is becoming increasingly evident that a fine balance exists between mutant and wild-type CBF complexes in AML. RUNX1 silencing in leukaemia cells expressing either RUNX1-ETO or CBFβ-SMMHC induces caspase-dependent apoptosis and cell cycle arrest, while double knockdown of the fusion protein and wild type RUNX1 rescues this phenotype (70, 71) suggesting that RUNX1 counteracts the inherent proapoptotic activity of the fusion protein (72, 73). A close investigation of direct target genes by global gene expression analysis and ChIP-Seq demonstrated that target genes dysregulated upon knockdown of either the fusion or RUNX1 alone are inversely correlated and the two proteins compete for common target gene binding sites resulting in dynamic interplay between these transcription factors at key targets such as those involved in myeloid differentiation and apoptosis (70, 74).

Altogether, these findings indicate that RUNX1 dependency is valid across many different leukaemias and suggest that RUNX1 may present an attractive target for therapeutic intervention.

3.1.2. Acute lymphoblastic leukaemia (ALL)

The ETV6-RUNX1 (TEL-AML1) fusion protein is the most common chromosomal translocation in B-ALL, found in ~25% of all paediatric cases and ALL (60, 75). The translocation brings together the N-terminal end of ETV6 (1-336aa), including the pointed domain (PD) required for oligomerization and the repression domain to almost all of the RUNX1 protein (22-480aa) (76, 77). The general assumption is that the fusion, as other RUNX1 translocations, acts in a dominant negative manner by hijacking and corrupting the endogenous RUNX1

programme (18). However, the remaining allele of RUNX1 is not mutated in these leukaemias and on the contrary is often amplified. Furthermore, increased RUNX1 copy number is observed in other types of ALL without the ETV6-RUNX1 translocation, most notably in the iAMP21 group in which a small region including the *RUNX1* locus is amplified but also arising from polyploidy of chromosome 21 in hyperdiploid and Down's syndrome ALL.

To investigate mechanism by which ETV6-RUNX1 promotes leukaemogenesis, a conditional ETV6-RUNX1 mouse model was generated. ETV6-RUNX1 has weak oncogenic potential and was unable to transform fetal liver B cells and induce overt leukaemia (78). However simultaneous induction of the ETV6-RUNX1 fusion and homozygous RUNX1-deletion resulted in a synthetic lethal phenotype with 100% of tested animals dying within 8 days due to severe anaemia following complete loss of HSCs and progenitors. Although not the main focus of the study, this phenotype emphasized an essential requirement of native RUNX1 for maintenance and propagation of ETV6-RUNX1-positive cells. Further investigation will be necessary in order to accurately define and segregate effects of the fusion and native RUNX1.

An onco-supportive role of RUNX1 in B-ALL was further highlighted in a study aiming to characterize the molecular basis underlying MLL-AF4 B-ALLs (79). The t(4;11) translocation fuses Mixed Lineage Leukaemia (MLL) protein with the AF4 gene resulting in a novel protein causing an aggressive form of B-ALL with poor prognosis. Wilkinson et al found that MLL-AF4 is highly enriched at the RUNX1 promoter and RUNX1 levels were significantly higher in MLL-AF4 leukaemias

compared to other B-ALL subtypes including other MLL-rearrangements. RUNX1 knockdown in MLL-AF4 cell lines reduced clonogenic ability, suggesting that similarly to the ETV6-RUNX1 mouse model, the MLL-AF4+ cells are dependent on RUNX1 for their growth and proliferation. Considering this and the correlation between higher RUNX1 levels and worse clinical outcomes observed in MLL patients in the COGP9906 clinical trial, it is tempting to suggest that targeting RUNX1 activity would present a novel strategy for targeting aggressive and poor-prognosis B-ALL subtypes. It will be important to define which ALL subtypes may be RUNX1 addicted and to determine the mechanisms underlying RUNX1-dependency in both AML and ALLs.

3.2. Epithelial cancers

RUNX1 is overexpressed in many solid tumours compared to normal tissue and many studies have now implicated RUNX factors in promoting and supporting oncogenic properties of epithelial cancer cells (34).

3.2.1. Skin and oral cancers

In a chemically induced mouse model of skin cancer, Runx1 deletion severely reduced the numbers of tumours formed (33). Runx1 was expressed at high levels in the papillomas in these mice and was also abnormally expressed in interfollicular epidermis. Lineage tracing revealed that Runx1 expressing HFSCs are the cell of origin for chemically induced skin tumours in mice and BrdU incorporation was reduced in Runx1-deficient bulge cells suggesting that Runx1 is required for the proliferation of stem cells in these tumours. Critically, deletion of Runx1 in established papillomas resulted in a shrinkage of the tumour

revealing that Runx1 is required for both initiation and maintenance of tumour growth in skin cancer (34). However, Runx1 does not appear to be sufficient for tumourigenesis as it is upregulated by injury in other cell types in the hair follicle and epidermis but these do not give rise to tumours. Strikingly, tumour cells display a more stringent requirement for Runx1 than normal tissue stem cells as Runx1 is essential for tumour formation but normal bulge stem cell proliferation *in vivo* is reduced, but not prevented by Runx1 deletion (33).

The relevance of these findings for human epithelial cancers was underscored by the finding that RUNX1 is significantly overexpressed in many cancers compared to normal tissue (34). It is particularly highly expressed in skin and oral (head and neck) squamous cell carcinomas and knockdown of RUNX1 revealed it is essential for growth of cell lines derived from these cancers (34). RUNX1 may therefore be a promising therapeutic target for epithelial cancers since it is not required for normal HFSC maintenance but was found to be essential for tumourigenesis in a mouse skin cancer model, and for growth and survival of human epithelial cancer cells.

3.2.2. Breast cancers

Mutations and deletions in RUNX1 and CBFβ have recently been identified specifically in luminal breast cancers (80-82). It was shown in mice that loss of Runx1 function results in a block in differentiation of luminal progenitors (48) and so RUNX1 is likely to be tumour suppressive in this type of breast cancer due to its normal function in promoting luminal fate. However, in basal-like and

triple negative breast cancers a variety of evidence points to an oncogenic role of RUNX factors.

RUNX2 has long been suggested to have a tumour-promoting role in breast cancer as it is upregulated in breast cancer cell lines and promotes tumour growth, invasion and osteolytic disease (41-44). However, its role in primary breast cancer has only recently been studied using mouse models. Overexpression of Runx2 with the MMTV promoter disrupts normal mammary gland development and causes pre-neoplastic hyperplasia in older animals (47). Furthermore, Runx2 deletion reduced proliferation, delayed tumour formation and prolonged survival in the MMTV-PyMT mouse model (46). Hyperplastic lesions in the MMTV-Runx2 overexpression model were negative for ER, PR and HER2 and high RUNX2 expression was significantly associated with triple negative breast cancers suggesting a link between RUNX2 and this type of poor prognosis breast cancer (47). Furthermore, WNT/B-catenin activation is associated with triple-negative breast cancer and Runx2 was found to be specifically upregulated in WNT driven mouse models of breast cancer (45).

RUNX1 is also upregulated in breast cancer cells compared to normal tissue (34, 83) and high RUNX1 expression is associated with poor prognosis in triple negative breast cancer (84). In the mouse MMTV-PyMT tumour model it was upregulated during tumour development and metastasis, and knockdown of Runx1 reduced invasive and migratory properties of cancer cells (83). To what extent RUNX1 and RUNX2 act redundantly in breast cancer is not yet known and will require compound knockout of these two genes in mouse breast cancer

models. Furthermore, it will be of interest to examine the effect of RUNX depletion in different types of breast cancer including basal-like, triple-negative and WNT-driven breast cancers. Triple negative breast cancers currently have a poor prognosis due to a lack of targeted therapies for this type of breast cancer and so it will be important to investigate whether CBF inhibitors may be effective for treating this disease.

3.2.3. Ovarian and prostate cancer

RUNX3 is expressed in 30-40% of ovarian cancer cells of serous carcinoma and endometroid types but not in clear cell carcinomas and knockdown of RUNX3 in ovarian cancer cell lines reduced cell proliferation (85). RUNX1 also was found to be overexpressed in ovarian cancers compared to normal tissue using both gene expression data and tissue microarrays and depletion of RUNX1 reduced growth and colony forming capacity of ovarian cancer cell lines (34, 86, 87). Furthermore, invasion and migration of ovarian cancer cells was reduced by RUNX1 knockdown and genes associated with cell adhesion and cellular movement pathways were enriched in the differentially expressed genes (87). RUNX1 is upregulated in part through reduced expression of mir-302b and acts through activation of Stat3 and downstream effectors including Cyclin D and BCL2 (86).

It is likely that when co-expressed, RUNX factors have partially redundant functions and cancer cells often co-express multiple RUNX family members but this redundancy can be partially overcome by inhibiting CBFβ expression. Using a double transduction strategy, >95% knockdown of CBFβ was achieved in

546 serous ovarian cancer cells and this completely blocked growth of these cells
547 (88). Interestingly, there was no obvious defect in cell cycle progression and the
548 growth defect was instead attributed to decreased viability resulting from non-
549 apoptotic cell death mediated by elevated ceramide levels, enhanced autophagy
550 and increased oxidative stress. RUNX1 has been found to promote cell survival
551 through direct regulation of genes involved in sphingolipid metabolism including
552 Sgpp1 and Ugcg (89) and these were downregulated after CBFβ knockdown in
553 ovarian cancer cells suggesting that they may be responsible in part for the
554 elevated ceramide levels in these cells (88). A similar effect on cell growth was
555 also observed in prostate cancer cells (88, 90). In prostate cancer, the effect of
556 RUNX1 depletion may be mediated in part through RUNX1-dependent Androgen
557 receptor (AR) signaling as AR induces RUNX1 expression and directly interacts
558 with RUNX1 to regulate many target genes (91). The fact that abrogating CBFβ is
559 highly effective at blocking cell growth and killing cancer cells suggests that
560 targeting CBF using novel small molecule inhibitors may be an effective
561 treatment for ovarian and prostate cancers.
562
563 **3.3. Neural cancers**
564 Neurofibromas are benign Schwann cell tumours found in patients with loss of
565 the tumour suppressor gene Neurofibromatosis type I (*NF1*). RUNX1 was
566 recently identified as a gene that was upregulated in neurofibromas and the
567 Runx1/ CBFβ interaction inhibitor Ro5-3335 or knockdown of RUNX1 reduced
568 sphere formation by murine neurofibroma Schwann cell progenitors (92).
569 Furthermore, deletion of Runx1 in neurofibroma progenitors delayed tumour
570 formation in mice. Increased numbers of Runx1+ progenitors are present in the

dorsal root ganglion of Nf1-/- mice and the number and size of spheres formed by Nf1 deficient progenitors was reduced by deletion of Runx1 suggesting that Runx1 is a key player in neurofibroma stem/progenitor cells (92). RUNX1 is also required for growth and survival of neuroblastoma cells but overexpression of either RUNX1 or RUNX3 also arrests cell cycle and promotes cell death suggesting that RUNX factor expression must be tightly controlled in order to maintain neuroblastoma growth (93).

4. Summary: Corruption of RUNX stem cell-associated functions in cancer

RUNX1 has a key role in promoting proliferation of many different types of stem and progenitor cells during homeostasis and regeneration. It appears to act to provide competency to respond to mitogenic signals and promote cell cycle progression, in part through direct regulation of cell cycle regulators and growth-related pathways including ribosomal biogenesis. However, forced expression is insufficient to drive uncontrolled proliferation in stem/progenitor cells and RUNX1 also promotes differentiation of stem cells down particular lineages. It does not therefore have traditional dominant oncogenic properties but in the context of other more powerful oncogenic drivers is required for proliferation and survival of cancer cells. It therefore represents an example of non-oncogene addiction resulting from the cellular context in which the cancer arises, whereby the endogenous stem cell activation machinery is co-opted to drive malignant expansion. The overexpression of RUNX1 (and in some cases RUNX2) observed in cancer, may arise from an increase in the number of RUNX-expressing stem/progenitor cells in the tumour compared to normal tissue, or epigenetic changes resulting in upregulation of RUNX gene expression. Cells

596 overexpressing RUNX factors are likely to be selected during cancer progression
597 as these cells have stem-like properties that enable them to proliferate rapidly.
598 RUNX factors therefore act as oncosupportive, competency factors for
599 oncogenesis presumably as a result of their normal functionality in promoting
600 stem cell proliferation and survival.
601
602 The apparently dichotomous observation of Runx1 mutations/translocations in
603 cancers such as luminal breast cancers, AML and ALL that also show dependency
604 on residual RUNX function, may arise from the dual role of RUNX factors. As part
605 of the mechanism by which stem cells become activated during either
606 homeostatic or injury driven regeneration, RUNX factors mediate cellular
607 proliferation but also have key roles in promoting the differentiation of many
608 different cell lineages. RUNX1 can therefore acts as a haploinsufficient tumour
609 suppressor and loss of function mutations in RUNX1 are likely to promote
610 oncogenesis through disruption of differentiation. However, RUNX1 is very
611 rarely subject to biallelic mutations in cancer and on the contrary has an onco-
612 supportive role in many cancers, presumably due to a requirement for residual
613 RUNX1 to promote proliferation and survival of cells trapped in an oncogenic
614 progenitor-like state. Therefore, loss-of-function mutations or translocations
615 affecting one allele of RUNX or CBFβ in breast cancers and leukaemias may set
616 up a pre-cancerous state through blockage of differentiation and perhaps
617 promoting a stress-resistant low metabolic phenotype associated with lower
618 ribosomal biogenesis that establishes a long-lived clone able to then acquire
619 secondary mutations leading to malignant transformation. However, the second
620 allele of RUNX1 is maintained to support growth and survival of the transformed

cells. RUNX1 also regulates pathways that may mediate resistance to chemotherapy, migration and metastasis and so high RUNX expression may be selected during tumour progression.

5. Prospects for therapy

The fact that RUNX factors are not essential to maintain stem cells in blood, skin, breast, muscle and brain, but are required for the proliferation and survival of many cancers arising in these tissues suggests that RUNX1 may be an excellent target for cancer therapies. RUNX1 inhibition would be expected to specifically eradicate cancer cells without depleting normal stem cells thus allowing re-establishment of normal tissue development post-treatment. Although traditionally classified as "undruggable", new methods for targeting transcription factor function are under development. Novel compounds that allosterically inhibit the interaction between CBFβ and RUNX subunits and thus prevent binding of RUNX1 to DNA have recently been identified. These CBF inhibitors were found to severely inhibit growth and survival of a range of myeloid leukaemia cell lines, and completely ablated colony formation in a basal-like breast cancer cell line at 1μM concentration (23). However, to fully harness the therapeutic potential of RUNX-addiction in cancer, and to specifically target its tumour-promoting roles, it will be important to perform systematic analysis of gene networks mediating RUNX-dependency in cancer cells in order to identify further druggable targets.

644

645 **Figure Legends**

646 **Figure 1. Structure of the RUNX proteins and the two most common**
647 **translocations of RUNX1.** P1 (distal) and P2 (proximal) promoters regulate
648 expression of RUNX genes and produce multiple isoforms differing in their
649 structure and function. The Runt domain (purple) is highly conserved in the
650 RUNX family and is responsible for DNA binding and heterodimerization with
651 CBFβ. It is present in the most common RUNX1 translocations – AML1-ETO (in
652 AML) and ETV6-RUNX1 (in ALL), which are proposed to function as repressors
653 of RUNX1 target genes. All three proteins have the transactivation domain (TAD
654 – red box) and the C-terminal VWRPY found to interact with Groucho family co-
655 repressors. Blue box in RUNX2 depicts the unique QA region, consisting of
656 tandem repeats of glutamine and alanine amino acids. CDK1 and 6 were found
657 to phosphorylate RUNX1 at the N- and C-termini.

658

659 **Figure 2. CBF inhibitors.** A) The CBF complex can act as a repressor or activator
660 of transcription in a context-dependent manner. B) Small molecule inhibitors
661 blocking the interaction between RUNX1 and CBFβ have been developed leading
662 to a diminished binding of RUNX1 to DNA and aberrant gene expression (23).

663

664 **Figure 3. Oncosupportive role of RUNX1 in haematological cancers**. RUNX1
665 is a frequent target for loss-of-function point mutations found in T-ALL, FPD and
666 AML. Increased dosage of RUNX1 has been associated with a specific ALL
667 subtype – iAMP21, characterized by an amplification of a 5.1MB region of
668 chromosome 21, encompassing RUNX1. It is diagnosed routinely by FISH and

669 defined by the presence of 3 or more extra copies of RUNX1. Increased dosage of
670 RUNX1 might be a factor predisposing to leukaemia also in Down's Syndrome
671 (trisomy 21). The exact involvement of RUNX1 and the leukaemogenic
672 mechanism in these diseases is not yet clear. In leukaemias with CBF or MLL
673 translocations, a certain level of RUNX1 expression is necessary to support the
674 leukaemogenic phenotype. Suppression of native RUNX1 in AML1-ETO, MLL-AF9
675 and MLL-AF4 leukaemias leads to cell cycle arrest and apoptosis. Decreased
676 RUNX1 activity in a CBFβ -MYH11 mouse model delayed leukaemic progression
677 and rescued CBFβ -MYH11 induced defects. Simultaneous ETV6-RUNX1
678 induction and RUNX1-disruption in an ETV6-RUNX1 mouse model led to severe
679 anaemia due to complete loss of HSPCs and caused death in 100% of animals
680 tested.
681
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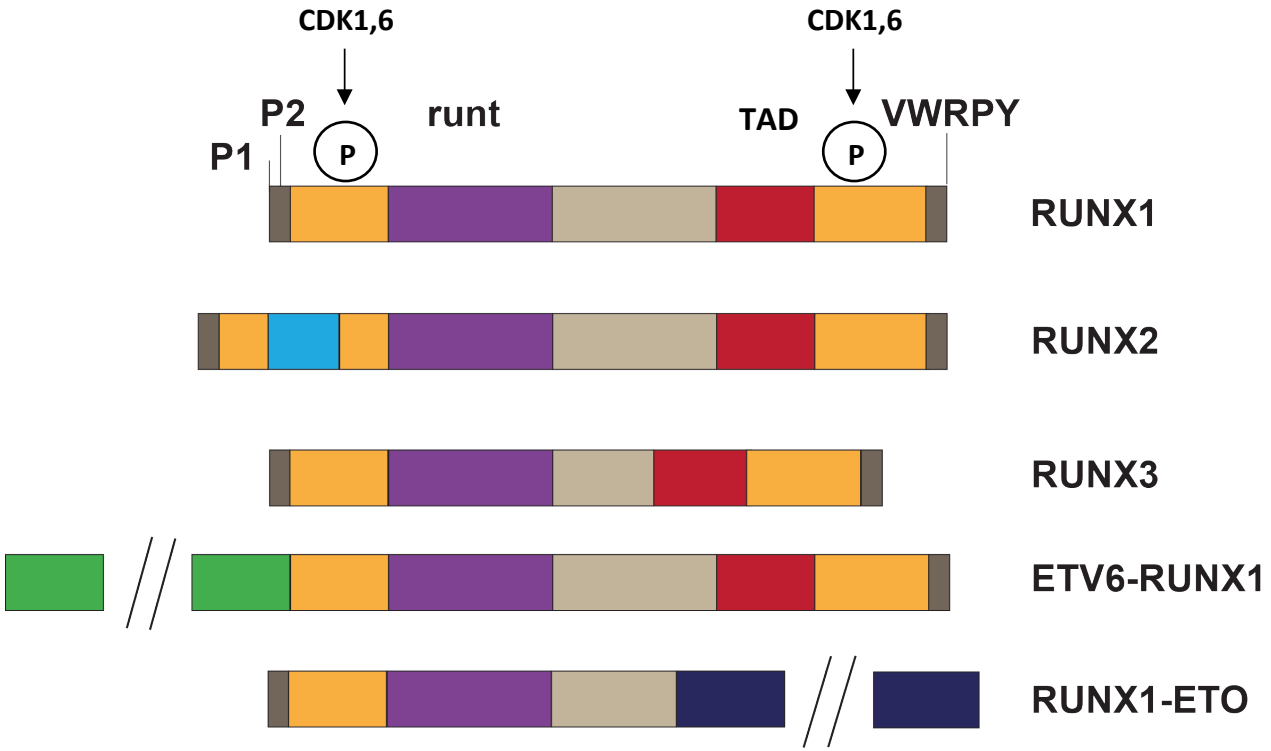
CDK1,6
CDK1,6
P2
runt
TAD
VWRPY
P1
P
P
RUNX1
RUNX2
RUNX3
ETV6-RUNX1
RUNX1-ETO

Figure 1

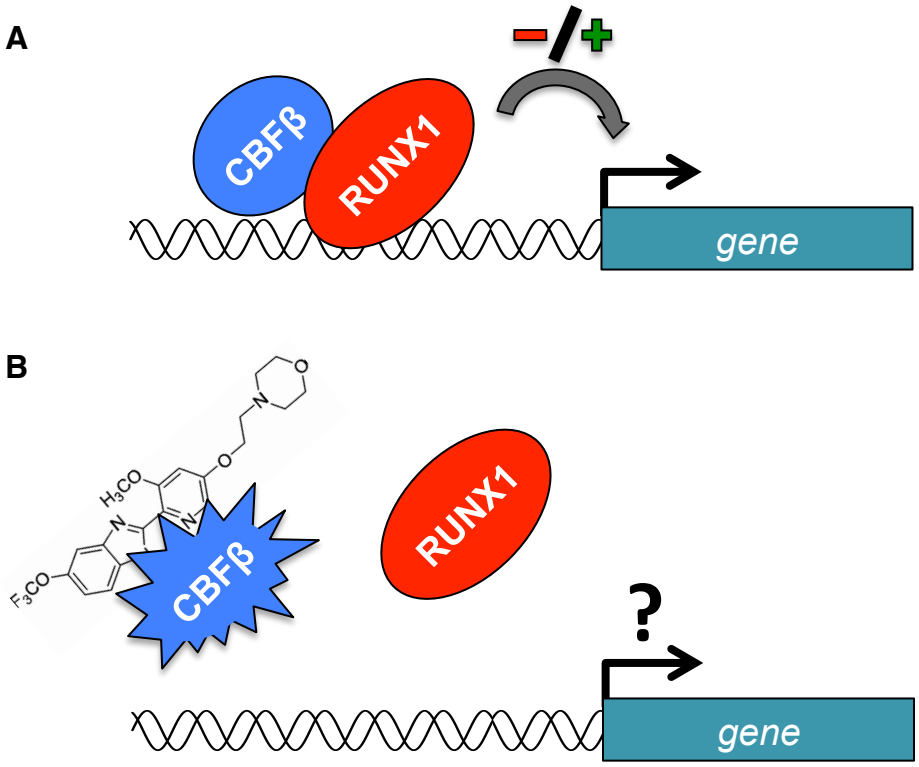

Figure 2

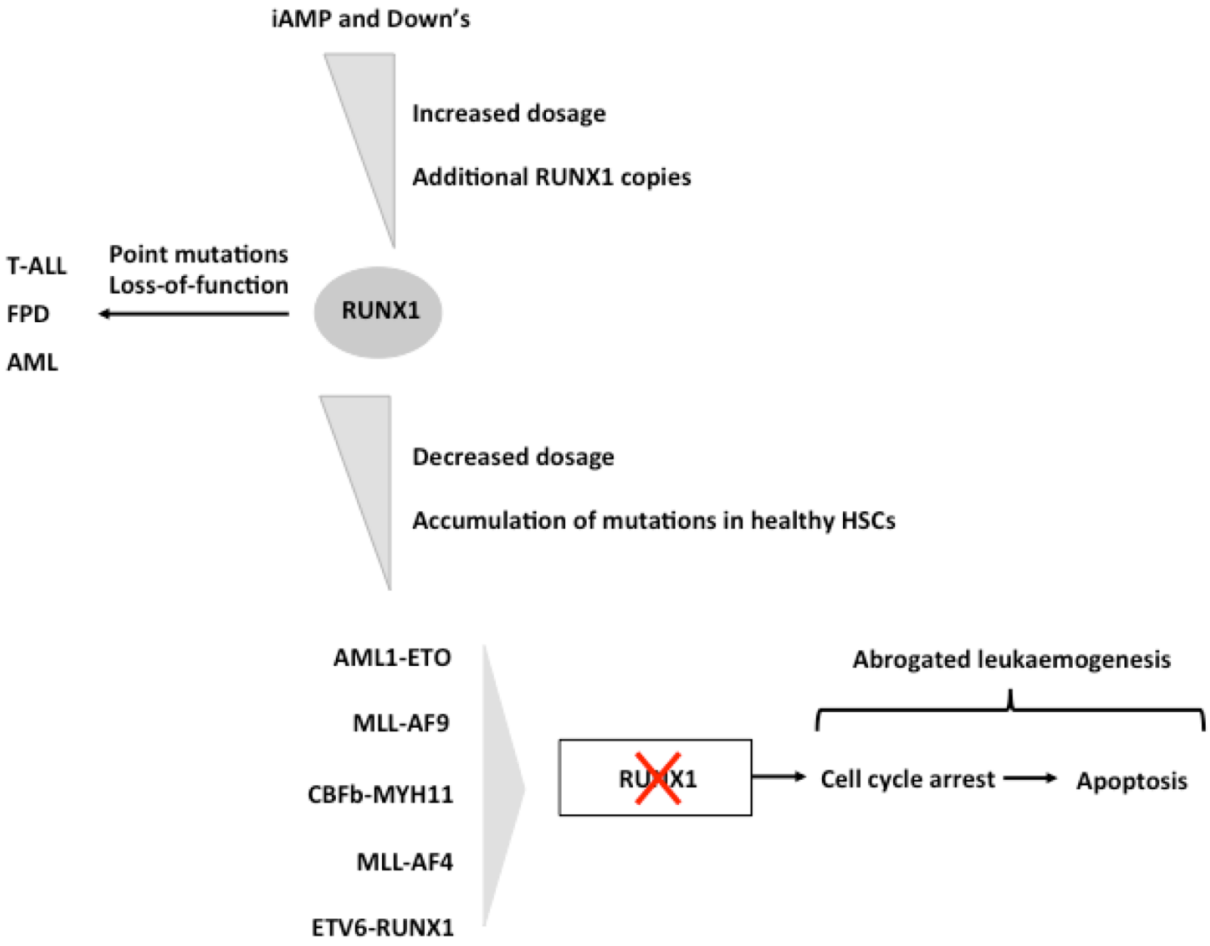
iAMP and Down's
Increased dosage
Additional RUNX1 copies
T-ALL
FPD
AML
Point mutations
Loss-of-function
RUNX1
Decreased dosage
Accumulation of mutations in healthy HSCs
AML1-ETO
MLL-AF9
CBFb-MYH11
MLL-AF4
ETV6-RUNX1
Abrogated leukaemogenesis
RUNX1
Cell cycle arrest
Apoptosis

Figure 3.