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RUNNING TITLE: Mutant JAK3 and HOXA9 cooperation in leukemia

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Abstract

Leukemia is caused by the accumulation of multiple genomic lesions in hematopoietic precursor cells. However, how these events cooperate during oncogenic transformation remains poorly understood. We studied the cooperation between activated JAK3/STAT5 signaling and HOXA9 overexpression, two events identified as significantly co-occurring in T-cell acute lymphoblastic leukemia. Expression of mutant JAK3 and HOXA9 led to a rapid development of leukemia originating from multipotent or lymphoid-committed progenitors, with a significant decrease in disease latency compared to JAK3 or HOXA9 alone. Integrated RNA-seq, ChIP-seq and ATAC-seq revealed that STAT5 and HOXA9 have co-occupancy across the genome resulting in enhanced STAT5 transcriptional activity and ectopic activation of Fos/Jun (AP-1). Our data suggest that oncogenic transcription factors such as HOXA9 provide a fertile ground for specific signaling pathways to thrive, explaining why JAK/STAT pathway mutations accumulate in HOXA9 expressing cells.

Significance

The mechanism of oncogene cooperation in cancer development remains poorly characterized. In this study, we model the cooperation between activated JAK-STAT signaling and ectopic HOXA9 expression during T-cell leukemia development. We identify a direct cooperation between STAT5 and HOXA9 at the transcriptional level and identify PIM1 kinase as a possible drug target in mutant JAK-STAT/HOXA9 positive leukemia cases.
Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disease, which arises from the malignant transformation of developing T-cell progenitors. The best characterized genetic defects in T-ALL include inactivation of cell cycle regulators (p16/p15), overexpression of transcription factors (TLX1, TLX3, TAL1, HOXA), mutations that activate the NOTCH1 and PI3K signaling cascades, and hyperactivation of signaling pathways by mutant kinases such as JAK3 mutants or the NUP214-ABL1 fusion (1-3). In T-ALL there are an average of 10-20 damaging genomic lesions per case (4-6). These mutations target critical cellular pathways including lymphoid development, cell cycle regulation, tumor suppression, lymphoid signaling and drug responsiveness (6,7). Recent studies using next-generation sequencing have identified mutations in the IL7R/JAK3/STAT5 pathway in 28% of T-ALL cases with JAK3 tyrosine kinase mutations being the most frequent mutations found in up to 16% of T-ALL cases (4,5,8,9).

In normal T-cell biology, the JAK3 and JAK1 kinases are essential components of the heterodimeric interleukin-7 (IL7) receptor complex. JAK3 binds the common gamma chain (IL2RG) and JAK1 binds the IL7Ralpha chain (10). When the receptor complex is bound by its ligand IL7, there is phosphorylation of both JAK1 and JAK3. This leads to the recruitment and phosphorylation of STAT5 that then translocates to the cell nucleus to regulate gene expression. The binding of IL7 to its receptor is essential for the survival and proliferation of double-negative thymocytes and is also important in the homeostasis, differentiation and activity of mature T-cells (11,12). The JAK3 mutations found in T-ALL patients are able to circumvent this tightly controlled signaling process and activate STAT5 in the absence of cytokine, transform cells in vitro and drive the development of T-ALL in vivo using a murine bone marrow transplant leukemia model (13).

Whilst the validation of JAK3 mutations as a bona fide "driver" mutation using a one-oncogene murine model is significant, from a clinical perspective, patients have additional mutations that may cooperate for malignant cell transformation. For example, it has been shown that BCR-ABL and NUP98-HOXA9 can cooperate synergistically in acute myeloid leukemia and modeling this cooperation identified compounds that selectively target leukemic stem cells (14,15). More recently,
the existence of multiple "mini-driver" mutations has also been suggested that might substitute for a major driver change (16) and therefore given the high number of genetic changes within T-ALL, it is likely that leukemia development and progression requires synergistic modulation of downstream signaling pathways by cooperating oncogenic lesions in a polygenic manner.

Some cooperating lesions in T-ALL have already been identified and include Arf inactivation in combination with Lmo2 overexpression significantly accelerating the development of the T-cell malignancies and acquisition of Notch1 mutations (17). Similarly work in our laboratory found that loss of the phosphatase PTPN2 enhanced the kinase activity of the NUP214-ABL1 fusion or JAK1 mutations, thereby sensitizing cells to leukemogenic transformation (18,19). However, the vast majority of co-occurring lesions and their ability to cooperate in driving tumourigenesis still require functional validation.

In the current work, we set about identifying cooperating oncogenes in the context of a JAK3 mutant driven leukemia and functionally validating these in vivo using a two-oncogene model. We found that T-ALL cases with JAK3 mutations are often HOXA+, specifically expressing high levels of HOXA9 and together can drive an aggressive leukemia using mouse models.

Results

**JAK3 mutations are significantly associated with HOXA+ T-ALL cases**

Based on results of large scale sequencing studies in T-ALL (4,5,8,9,20), it is becoming clear that some mutations frequently co-occur, while others are mutually exclusive. We analyzed genetic data for 155 T-ALL cases (Vicente dataset) (5) in which IL7R/JAK/STAT5 mutations occur in 28% of the cases and found more frequently in HOXA+, immature and TLX1/3 positive cases. Focusing specifically on JAK3 mutations alone, these account for the most frequent type of mutation found in 16% of the cases (5). Upon analyzing different T-ALL subgroups, these JAK3 mutations were found to significantly associate with those T-ALL cases designated as HOXA+ (p value = 0.018) and
negatively associate with those designated as TAL1/LMO2 positive (p value = 0.002) (Fig. 1B). The designation of HOXA+ included cases with chromosomal rearrangements known to either directly or indirectly up-regulate genes within the HOXA cluster (i.e. CALM-AF10, SET-NUP214, NUP98-RAP1GDS and TCRB-HOXA) (5,21,22). Analysis of a second independent cohort of T-ALL cases (Liu dataset) (9) showed 22% of the patients had IL7R/JAK/STAT5 mutations (data included mutations within JAK1, JAK3, IL7R, PTPN2, FLT3, STAT5B, and SH2B3) (Fig. 1A). These mutations were enriched within the HOXA+, TLX1/3+ and LMO2/LYL1+ cases. Furthermore, focusing specifically on JAK3 mutant cases, again revealed a significant positive association with HOXA+ and negative association with TAL1/TAL2/LMO2 cases (Fig. 1B). Moreover, associated gene expression data showed HOXA+ cases have overall higher JAK3 expression levels compared to the majority of other T-ALL cases (Fig. 1C). Activation of the HOXA cluster in T-ALL typically results in upregulation of both HOXA9 and HOXA10. Analysis of primary T-ALL samples (Supplementary Table S1) revealed strong HOXA9 expression in 3 of 5 JAK3 mutant cases, that was up to 10-fold higher when compared to HOXA10 and HOXA11 (Fig. 1D), suggesting that HOXA9 is the most important upregulated gene of the HOXA cluster in T-ALL.

*JAK3 mutations and HOXA9 co-expression transforms hematopoietic stem and progenitor cells and leads to rapid leukemia development in vivo using a bone marrow transplant model*

The most common JAK3 mutation found within T-ALL cases is the M511I mutation just upstream of the pseudokinase domain (5,9,13). Therefore, we next sought to determine whether mutant JAK3 signaling (using the JAK3(M511I) mutant) can cooperate with HOXA9 in the transformation of hematopoietic stem/progenitor cells (HSPCs) and leukemia development. Two separate retroviral vectors containing JAK3(M511I) (also expressing GFP) or HOXA9 (also expressing mCHERRY) were used for the co-transduction of murine HSPCs ex vivo and yielded a mixture of non-transduced, single transduced or double transduced cells as assessed by GFP and mCHERRY fluorescence (Fig. 2A). HSPCs transduced with both JAK3(M511I) and HOXA9 rapidly transformed to cytokine
independent growth and expanded over 20 days in the absence all cytokines. All non-transduced and single transduced cells were either out competed and/or did not grow (Fig. 2B). The various JAK3 mutations activate downstream STAT5 signaling with the majority also requiring binding to a functional cytokine receptor complex (13,23). Constitutively active STAT5B(N642H) mutations have also been identified and functionally characterized in T-ALL (24,25). Therefore, the same experimental set up was repeated using STAT5B(N642H) and HOXA9 retroviral expression constructs. Again, only the double transduced STAT5B(N642H) and HOXA9 cells expanded in the absence of all cytokines, phenocopying the JAK3(M511I) and HOXA9 result (Fig. 2C). Taken together, these results show that oncogenic JAK3/STAT5 activation is necessary and sufficient for cooperation with HOXA9 in ex vivo HSPC cell transformation.

Given this evident cooperation in HSPC transformation ex vivo, we next investigated the cooperation between JAK3(M511I) and HOXA9 during in vivo leukemia development. Mouse HSPCs were isolated and transduced with retroviral vectors containing JAK3(M511I) or HOXA9 or a mixture of both and then injected into sub-lethally irradiated syngeneic recipient mice (BALB/c or C57BL/6). JAK3(M511I)+HOXA9 recipient BALB/c mice rapidly developed a leukemia characterized by a rapid increase in peripheral white blood cell (WBC) counts in excess of 10,000 cells/mm³ within 30 days compared to JAK3(M511I) only (Fig. 2D). For each recipient, the double transduced JAK3(M511I) and HOXA9 leukemic clone outcompeted all single transduced clones (Fig. 2E). This rapid leukemia development resulted in significantly decreased disease free survival (DFS) (Median DFS = 25 days, p-value < 0.0001) when compared to JAK3 (M511I) alone (Median DFS = 126.5 days) (Fig. 2F). In contrast, clones co-expressing TAL1 and JAK3(M511I) were counter selected in vivo with mice succumbing to a JAK3(M511I) only disease (Fig. 2F, Supplementary Fig. S1) and reconciled with JAK3 mutations negatively associating with TAL1 in T-ALL (Fig. 1A). Notably, when the same experimental approach was used for HOXA10 together with JAK3(M511), there was only a moderate and less profound cooperative effect compared to HOXA9 (Median DFS = 100 days) (Supplementary Fig. S1).
To negate any mouse strain specific effects, the same experimental set up was repeated on a C57BL/6 background. Similar to BALB/c, recipient C57BL/6 mice receiving co-transduced JAK3(M511I)/HOXA9 had a rapid increase in WBC compared to JAK3(M511I) or HOXA9 alone (Fig. 2G) and in all cases the double transduced clone was the major clone at end stage (Fig. 2H). This resulted in a significant decrease in DFS (Median DFS = 40 days) compared to JAK3(M511I) (Median DFS = 150 days) or HOXA9 (Median DFS = 182 days) only recipient mice (Fig. 2I). Moreover, when JAK3(M511I) was substituted for the constitutive active STAT5B(N642H) and co-transduced with HOXA9, this further reduced the disease free survival (Median DFS = 12 days).

At end stage disease, the leukemia development in vivo was characterized by both myeloid and lymphoid expansion. Phenotypic FACS staining and pathology analysis of the JAK3(M511I)/HOXA9 driven leukemia revealed that leukemic mice had polyclonal expansion of cells displaying either myeloid (CD16/32+, CD11b+, Gr1+/-) or lymphoid (CD8+) lineage markers and occurred at different frequencies (Fig 3A). At end stage disease, histopathologic and immunohistochemical analysis revealed the development of a complex hematolymphoid neoplasm characterized by the co-existence of different populations of atypical cells displaying both lymphoid and myeloid differentiation. There was significant leukemic infiltration into spleen, lymph nodes and bone marrow organs by atypical lymphocytes (CD3 positive, TdT negative) and myeloid/granulocytic (Myeloperoxidase (MPO) positive) cells (Fig. 3B) (Supplementary Fig. S2). Western blot analysis confirmed expression of JAK3 and HOXA9 in vivo with robust phosphorylation of STAT5. (Fig. 3C). These cells proliferated for up to 30 days ex vivo in the absence of all cytokines with clonal selection of those clones expressing high levels HOXA9 and JAK3(M511I) as assessed by GFP and mCHERRY expression (Fig 3D,E). Moreover, sorted JAK3(M511I)/HOXA9 CD8+ T-ALL clones were able to engraft secondary recipient mice with higher frequency compared to age matched JAK3(M511I) CD8+ leukemic clones (Supplementary Fig. S3).

To determine whether the resulting leukemia remained dependent on activated JAK/STAT5 signaling directly, JAK3(M511I)/HOXA9 leukemic mice were treated for 20 days with the JAK3
inhibitor tofacitinib (40 mg/kg/day) (Fig. 3F). Within three days of treatment with tofacitinib, there was a significant decrease in the levels of pSTAT5 compared to vehicle treated mice (Fig 3G). After 20 days of tofacitinib treatment, there was a significant attenuation in leukemia white blood cell expansion reduced splenomegaly (Fig 3. H-J). Staining of the spleen at end stage also showed a significant reduction in the number of proliferative cells as assessed by Ki67 staining (Fig. 3K).

Tofacitinib is approved for rheumatoid arthritis and known to cause decreased numbers of neutrophils, lymphocytes and NK cells but rarely to an extent that leads to serious adverse effects in patients (26). Indeed, in our own studies, wild type mice treated at 40 mg/kg/day, showed a minor decrease in NK cell frequency but no significant alterations in CD4+ or CD8+ T-cell frequencies (Supplementary Fig. S4). Taken together, these results firstly provide strong validation that ectopic expression of HOXA9 together with JAK3/STAT5 activation are bona fide cooperating events in leukemia development. Secondly that there is a degree of transcription factor specificity for cooperation with JAK3 mutations, since JAK3 mutations do not cooperate with the oncogenic transcription factor TAL1 and thirdly, that the resulting leukemia remains dependent on activated JAK3 signaling.

A novel retroviral vector to direct transgene expression within developing lymphoid cells demonstrates cooperation of JAK3(M511I)/HOXA9 in T-ALL development

The previous data illustrate the potential of combined expression of JAK3(M511I) and HOXA9 to generate mixed myeloid/lymphoid leukemia originating from multipotent hematopoietic precursor cells. However, HOXA9 expression is often activated in T-ALL by T-cell specific enhancers that only become active in lymphoid committed progenitors (e.g. TCRB-HOXA9) (21,22). To convincingly demonstrate cooperation between JAK3(M511I) and HOXA9 during T-cell leukemia development, and to specifically model lymphoid-specific activation of HOXA9 expression as observed in human T-ALL, we developed a strategy to limit HOXA9 expression to developing lymphoid cells. We tested a previously published strategy with 3’ UTR mir223 sites (27), but in the context of
JAK3(M511I)/HOXA9, this strategy was unable to prevent overt myeloid expansion and eventual AML expansion that we speculate to indicate endogenous miR-223 saturation in the presence of high levels of transcript driven by the strong MSCV promoter (Supplementary Fig. S5).

We therefore developed a novel retroviral vector in which one or two oncogenes are initially cloned in an antisense orientation, and thus remain completely inactive. The expression of the oncogenes is only activated upon Cre-mediated inversion of the lox66/71 flanked construct (Fig. 4A). By use of hematopoietic cells isolated from cell type specific Cre mice, we could then direct expression of the oncogenes to specific hematopoietic cell types. The novel vector with JAK3(M511I)+HOXA9 was first validated in Ba/F3 cells and found to transform the cells to cytokine independent growth only when Cre-recombinase was present and did not display ectopic "leaky" expression or cell transformation in Ba/F3 wild type cells over an 8 day period (Fig. 4B). Western blot analysis confirmed the expression of JAK3 and HOXA9 in the Cre positive Ba/F3 cells and highlighted that although there was less JAK3(M511I) expression compared to constitutive expression of JAK3, both had equivalent levels of phosphor-STAT5 (Fig. 4C). The novel retroviral vector strategy was also validated in vivo using a well established NOTCH1-ICN bone marrow transplant model, but now with ICN cloned in the antisense direction between the lox66/71 sites. When the viral vector was transduced on cells derived from CD4-Cre mice the recipient mice succumbed to the anticipated CD4+/CD8+ T-ALL leukemia (Median overall survival = 43 days) (Supplementary Fig. S6).

Using this new strategy, we next tested whether combined expression of JAK3(M511I) and HOXA9 via CD2-Cre mediated activation in developing lymphoid cells (28) (Fig. 4d) could generate T-cell leukemia. Mice that received transplantation with inducible JAK3(M511I)-P2A-HOXA9 HSPCs developed T-ALL like leukemia with decreased latency when compared to JAK3(M511I)-P2A-BFP only (Median DFS = 135 days vs. 244 days). Notably, both JAK3(M511I) and JAK3(M511I)-P2A-HOXA9 mice now developed a CD8+ or CD4 CD8- T-ALL, without myeloid expansion (Fig. 4E). For all mice, CD2-mediated recombination was confirmed by PCR (Fig. 4F). These data illustrate not only that JAK3 mutants cooperate with HOXA9 to induce leukemia when expressed in common
lymphoid progenitors, but also demonstrate the utility and efficacy of this novel Cre-inducible retroviral vector for cell-type specific expression in the hematopoietic system.

*STAT5 and HOXA9 co-occupy similar genetic loci resulting in increased JAK-STAT signaling in leukemia cells*

To determine the underlying molecular mechanism driving the enhanced oncogenic potential of JAK3(M511I) in the presence of HOXA9, RNA-seq analysis was carried out on age matched sorted leukemic CD8⁺ JAK3(M511I), CD8⁺ JAK3(M511I)/HOXA9 as well as sorted normal thymic CD8+ T cells (Supplementary Table S2). The addition of HOXA9 led to skewed upregulation of genes when compared to JAK3(M511I) only T-ALL (Fig. 5A). Notably, principal component analysis revealed distinct clustering of myeloid and T-cell leukemias (Supplementary Fig. S7). The normal CD8⁺ T-cells formed a tight cluster away from the JAK3(M511I)/HOXA9 CD8⁺ T-ALL cells and in between the JAK3(M511I) only cells. Furthermore, within the myeloid cell cluster, the JAK3(M511I)/HOXA9 driven AML cells clustered separately from Meis1/Hoxa9 and MLL-AF9 AML (29) suggesting different mechanisms driving these leukemia and indeed there was no increased Meis1 expression in our leukemia suggesting the co-operation is independent of Meis1. Flt3 ITD driven leukemia (30) clustered closer to the JAK3(M511I)/HOXA9 and could in part reflect the downstream activation of STAT5 in both leukemias (Supplementary Fig. S7).

ChIP-seq analysis of CD8⁺ JAK3(M511I)/HOXA9 leukemia showed significant co-occupancy for both STAT5 and HOXA9 across the genome. Regions co-bound by STAT5 and HOXA9 were associated with H3K27Ac and H3K4me3 marks indicating co-localization at proximal promoters rather than distal enhancers (Fig. 5B,C). Comparison of the STAT5 signal in JAK3(M511I) vs. JAK3(M511I)/HOXA9 showed strong overlap suggesting that HOXA9 does not significantly reposition STAT5 to de novo sites (Supplementary Fig. S8). Specific analysis of canonical STAT5 target genes illustrated the strong co-occupancy of both STAT5 and HOXA9. For example, Pim1 kinase a known target gene for both STAT5 (31) and HOXA9(32) showed co-occupancy of the same
DNA region for both STAT5 and HOXA9. Similarly, Bcl2, Osm, Cish and Il7r also showed co-occupancy by STAT5 and HOXA9 (Fig. 5D, Supplementary Fig. S8). Analysis of relevant human T-ALL samples also showed strong co-occupancy of HOXA9 with STAT5 peaks at known STAT5 regulated genes including BCL6, MYC, SOCS and PIM1 (Supplementary Fig. S9).

This strong STAT5/HOXA9 co-localisation prompted the question whether a direct protein-protein interaction occurs between STAT5 and HOXA9. Indeed, a direct interaction between HOXA9 and STAT5 is suggested based on available mass spectrometry data (33). However, we were unable to confirm a robust protein-protein interaction between HOXA9 and STAT5 using co-immunoprecipitation, nor in an independent assay using a recombinant strepII-tagged HOXA9. Only a weak interaction with the constitutively active STAT5B(N642H) mutant was observed but not with wild-type STAT5 (Supplementary Fig. S10).

Global analysis of the regions with co-binding of HOXA9 and STAT5 found an association with diminished levels of repressive H3K27me3 marks at upregulated genes and concomitantly associated with increased levels of active H3K27Ac marks (Fig. 5E). Integrating the ChIP-seq analysis with the RNA-seq differentially expressed genes also revealed STAT5 and HOXA9 loading was higher in upregulated genes compared to down-regulated or unchanged genes (Fig. 5F) illustrating that the presence of HOXA9 leads to increased gene activation. Indeed, GSEA pathway analysis comparing CD8+ JAK3(M511I)-T-ALL vs. CD8+ T-cells and CD8+ JAK3(M511I)/HOXA9 vs. CD8+ JAK3(M511I) found a significant and increasingly positive enrichment for JAK/STAT signaling (Fig. 5G, Supplementary Table S3). These data show that ectopic expression of HOXA9 enhances STAT5 transcriptional activity in CD8+ leukemic cells rather than inducing a de novo gene expression program complimentary to STAT5.

PIM1 kinase is over expressed in a number of different cancers and is an emerging cancer drug target (34,35). Consistent with our RNA-seq and ChIP-seq data, we confirmed increased PIM1 expression in JAK3(M511I)/HOXA9 leukemic mouse samples by qPCR (Fig. 6A). Analysis of primary human T-ALL patient samples showed the highest levels of PIM1 expression in HOXA+
cases and those cases with IL7R/JAK3/JAK1 mutations (Fig. 6B). Furthermore, PIM1 RNA and protein levels were rapidly downregulated in a HOXA9+ PDX sample with active JAK-STAT signaling after treatment with the JAK1 inhibitor ruxolitinib (Fig. 6C). This provided further evidence for a functional signaling network of PIM1 regulation downstream of JAK-STAT signaling. Exploiting this observation, we next sought to determine whether dual inhibition of PIM1 and JAK1 would be beneficial in JAK/STAT mutant T-ALL samples. Here we observed a synergistic response in JAK3 mutant T-ALL samples when treated with a JAK kinase inhibitor (ruxolitinib) in combination with a PIM1 inhibitor (AZD1208) within ex vivo cell culture and a significant attenuation in leukemia burden in vivo using a patient derived xenograft sample (Fig. 6D,E). Taken together, these data suggest that the ectopic expression of HOXA9 co-binds with STAT5 to enhance the JAK-STAT and targeting of both PIM1 and JAK1 provides a strong therapeutic benefit.

Chromatin accessibility by ATAC-seq analysis reveals a role for AP-1 activation

To further investigate and identify additional factors and mechanisms underlying the JAK3/HOXA9 cooperation, chromatin accessibility was assessed at a global level using ATAC-seq comparing JAK3(M511I) to JAK3(M511I)/HOXA9 leukemia. This revealed significant changes of chromatin architecture with appearing peaks associated with upregulated gene expression and loading of STAT5 and HOXA9 (Fig. 7A,B). Surprisingly, many upregulated sites were neither bound by STAT5 or HOXA9. We interpreted this finding as activation by secondary events downstream of activated STAT5/HOXA9 signaling. Motif scanning using iCis-target revealed these peaks were highly enriched for Fos and Jun motifs (Fig. 7C). RNA-seq data further confirmed increased levels of members of the AP-1 complex including Fos and Jun (Fig. 7D) and increased expression of AP-1 target genes (Fig. 7E). Whilst some of these target genes such as FasL were bound by STAT5 and HOXA9, many others such as App, Klf4 and Csf1 had very weak or no binding (Fig. 7F) and represent examples of genes activated by AP-1 independent of STAT5.
Discussion

The development of T-cell acute lymphoblastic leukemia (T-ALL) is a step-wise process via the accumulation of somatic mutations (36). Whilst we and others have identified and confirmed various important driver mutations in T-ALL, this has often been in the context of a single oncogene, either within transgenic (e.g. TLX1 (37)) or bone marrow transplant mouse models (e.g. JAK3(M511I) (13)). However, patients present with multiple mutations at diagnosis and we therefore hypothesized that mutations that significantly associate with one another are potentially cooperating events in driving T-ALL. In a recent analysis, significant associations were found between mutations in the IL7R-JAK signaling pathway and epigenetic regulators (5). Here we extend this analysis and find that those cases with JAK3 mutations are significantly associated with cases designated HOXA+. In particular, we show that HOXA9 expression levels are significantly elevated compared to either HOXA10 or HOXA11 in HOXA+ cases and co-expression of HOXA9 and JAK3(M511I) leads to the rapid development of leukemia in vivo.

In our initial mouse model, the developing leukemia in vivo was characterized by overt myeloid and lymphoid expansion due to the constitutive expression of both JAK3(M511I) and HOXA9 within the HSPCs. Traditionally, the modeling of T-cell leukemia in mice and the role on oncogenes has often used transgenic mice using tissue specific promoters to limit the expression within developing lymphoid cells (e.g. CD2-Lmo2 (38), Lck-TLX1 (37)). However, generating transgenic knock-in mice remains technically laborious and time consuming. To this end, we developed a novel retroviral expression strategy for lineage specific expression using the bone marrow transplant system that is compatible with the numerous well characterized Cre-mice now available, and we demonstrated the efficacy of this novel retroviral vector to rapidly generate mouse T-ALL models. Moreover, this system allows a more physiological comparison of cellular competition between wild type and transgene expressing cells during differentiation and potential leukemia development.

From a mechanistic perspective, the integrated ChIP-seq and RNA-seq of the JAK3(M511I)/HOXA9 driven leukemias revealed that HOXA9 expression leads to a significant amplification of the STAT5
transcriptional output, and to addiction of the leukemia cells to STAT5 activation. This reconfirms STAT5 as a major central player in T-ALL pathogenesis and identifies STAT5 target genes (such as PIM1) as potential targets for therapy. PIM1 is a constitutive serine threonine kinase over expressed in a number of cancers and regulates a number of different oncogenic processes including cell survival and apoptosis making it an attractive target for inhibition (39). Indeed, PIM1 inhibition has been shown to be effective in a triple negative breast cancer model in vivo (40), and more recently also in T-ALL cell lines and human T-cell leukemia virus type 1 (HTLV-1)–associated adult T-cell leukemia and T-cell lymphoma (ATL) (41,42). Secondly, HOXA9 through co-binding with STAT5 also leads to the upregulation of Fos and Jun and AP-1 target genes. Members of the AP-1 complex are over expressed in numerous lymphoid malignancies including T-cell leukemia and has been recently reviewed (43) and therefore could further contribute to the enhanced leukemogenesis. The addition of HOXA9 with JAK3 mutations thereby upregulates the AP-1 complex at the transcriptional levels directly rather than the canonical AP-1 activation by the upstream MAPK-signaling pathway.

HOXA9 has a well established role in both hematopoiesis and leukemia. Hoxa9 loss of function studies in mice show deficits in committed myeloid, erythroid and B-cell progenitors and perturbations in T-cell development (44,45). Conversely, over expression of HOXA9 in transgenic mice using lymphoid specific regulatory elements results in mild CD8 expansion (46). Early evidence for HOXA9 in the development of leukemia was the identification of recurrent translocation t(7;11) producing the NUP98-HOXA9 fusion (47) with subsequent studies showing MLL-fusions and NPM1 also resulting in HOXA9 over expression (48). Early studies of ectopic Hoxa9 expression in mice showed weak leukemogenic potential with either AML developing after a long latency using retroviral transduction/transplantation (49,50) or a T-cell malignancy (preT LBL) using a transgenic approach (51). Our bone marrow transplant studies presented here showed high leukemia penetrance for MSCV-based HOXA9 expression and reconciles with a recent study using a similar retroviral strategy (52) highlighting the role of promotor strength and viral titers in dictating leukemia latency.
Nevertheless, what is now well understood is that the ability for HOXA9 to drive rapid leukemia development and cellular transformation is dependent on the expression of different cofactors. Most of these co-factors belong to the “three loop amino-acid-loop extension” (TALE) homeobox family (48). Early work over a decade ago showed an essential role for Meis1 as a co-factor of Hoxa9 driven AML in mice (49,53) and since this time many additional HOXA9 interacting factors have been identified including STAT5 in AML (33,48). Indeed, STAT5B activation is important in AML and loss of STAT5B decreases the leukemia-initiating cell frequency in Hoxa9/MN1-induced leukemias (54). In our current work, we find a striking growth advantage and cellular transformation of HSPC cells transduced with JAK3(M511I) and HOXA9 that is phenocopied by constitutive active STAT5B(N642H) co-expressed with HOXA9. Moreover, in our BMT model we see the rapid development of both AML and T-ALL clones in vivo showing that sustained STAT5 activation and HOXA9 expression are bona-fide cooperative events in driving leukemia development in both lineages. Significantly, the levels of Meis1 expression are very low in our STAT5/HOXA9 driven leukemias and together with the PCA analysis segregating Hoxa9/Meis1 AML away from both JAK3(M511I)/HOXA9 AML and T-ALL suggest that Meis1 is not important in our JAK3(M511I)/HOXA9 leukemia models. Furthermore, we showed that the resulting JAK3(M511I)/HOXA9 driven leukemia were sensitive to tofacitinib suggesting these leukemias remain dependent on the STAT5 signaling and is not a purely HOXA9 driven leukemia. Taken together, our data identify STAT5 as a novel co-factor of HOXA9 in T-ALL.

Interestingly, transformed progenitor cells expressing Hoxa9 and Meis1a but not hoxa9 induced the expression of Flt3 and Il7R (50), both of which activate STAT5 downstream suggesting that our leukemia model in part bypasses the requirement of Meis1 via direct activation of STAT5. The link between HOX cluster activation and activated STAT signaling is also observed in acute megakaryoblastic leukemia with HOXr cells also carrying an activating mutation of the MPL receptor driving the phosphorylation of STAT5 (55). Similarly, MLL intragenic abnormalities in AML upregulate HOXA9 and also correlated with FLT3 mutations that activate STAT5 (56).
In conclusion, we show that HOXA9 and JAK3/STAT5 are bona fide cooperating factors in driving leukemia development and that the cooperation is situated at the transcriptional level where STAT5 and HOXA9 co-occupy similar genomic regions. Furthermore, our results have not only significance for T-ALL but also for other leukemias where activated STAT5 would potentially cooperate with HOXA9.

Methods

Expression plasmids and Retrovirus production

The MSCV-JAK3 (M511I)-IRES-GFP retroviral vector is as previously described (13). The human HOXA9 cDNA was synthesized by Genscript and cloned into the MSCV-IRES-mCHERRY vector. The lox66/71-IRES GFP vector was generated by cloning in the lox66/71 sites directly into the multiple cloning site of MSCV-GFP retroviral vector. Retrovirus production using 293T cells and retroviral transduction of Ba/F3 cells and hematopoietic lineage negative cells were performed as previously described (13,57).

RNA extractions and qPCR

RNA was extracted from tissue and cells using either the illustra RNAspin Mini Kit (GE Healthcare Life Sciences) or the Maxwell 16 LEV Simply RNA purification kit (Promega) as per manufacturers instructions. cDNA synthesis was carried out using GoScript (Promega) and real time quantitative performed using the GoTaq qPCR master mix (Promega) with the ViiA7 Real Time PCR system (Applied Biosystem). Quality control, primer efficiency and data analysis was carried out using qbase+ software (Biogazelle). All gene expression was normalized using two housekeeping reference genes. Primers used for qPCR are listed in Supplementary Table S4.

RNA-Sequencing

The single-end RNA-sequencing data was first cleaned (i.e. removal of adapters and low quality parts) with the fastq-mcf software after which a quality control was performed with FastQC. The
reads were then mapped to the Mus Musculus (mm10) genome with Tophat2. To identify the gene expression HTSeq-count was used to count the number of reads per gene. These read count numbers were then normalized to the sample size. Differential gene expression analysis was performed with the R-package DESeq2 (Supplementary Table S2). Pathway enrichment and other geneset enrichment analyses were done with GSEA. GSEA datasets were downloaded from: http://download.baderlab.org/EM_Genesets/ The data is accessible through GEO Series accession number GSE109653 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109653).

**Western blotting**

Cells were lysed in cold lysis buffer containing 5 mM Na3VO4 and protease inhibitors (Complete EDTA -free, Roche). The proteins were separated on NuPAGE NOVEX Bis-Tris 4%-12% gels (Life Technologies) and transferred to PVDF membranes. Subsequent Western blot analysis was performed using antibodies directed against phospho-JAK1/JAK3 (SC-16773), STAT5 (SC-1081), JAK1 (Millipore 05-1154), phospho-STAT5, (Cell Signaling #9359), HOXA9 (Millipore 07-178) and beta-actin (Sigma A1978). Anti-phospho-JAK1 antibody was used to detect both phosphorylated JAK1 and JAK3. Western blot detection was performed with secondary antibodies conjugated with horseradish peroxidase (GE Healthcare). Bands were visualized using a cooled charge-coupled device camera system (ImageQuant LAS-4000, GE Health Care).

**Murine bone marrow transplantation**

Male C57BL/6 or BALB/c mice were purchased from Charles River Laboratories. Male mice were sacrificed and bone marrow cells were harvested from femur and tibia. Lineage negative cells were enriched (STEMCELL Technologies) and cultured overnight in RPMI with 20% FCS with IL3 (10ng/mL, Peprotech), IL6 (10ng/mL, Peprotech), SCF (50ng/mL, Peprotech), and penicillin-streptomycin. The following day, 1x10^6 cells were transduced by spinoculation (90 min at 2500rpm) with viral supernatant and 8 μg/mL polybrene. The following day, the cells were washed in PBS and injected (1x10^6 cells/0.3 mL) into the lateral tail vein of sub-lethally irradiated (5 Gy) syngeneic female recipient mice. Mice were housed in IVC cages and monitored daily. End point
analysis for disease free survival was defined at WBC >30,000 cells/mm³. In vivo treatment of mice with tofacitinib was carried out as previously described (13).

**Ex vivo cell culture transformation assay**

The Lin- cells were spin infected with two retroviral constructs leading to lineage negative cells with all four combinations – non-transduced, single transduced JAK3(M511I) only, HOXA9 only, and double transduced JAK3(M511I)/HOXA9. The day after transduction, the cells were split into media containing only 20% FBS/RPMI, removing the SCF, IL6 and IL3 that was present. The cells were then left to grow in this cytokine free media and split when appropriate. There was no sorting of cells prior to cytokine withdrawal.

**In vivo and ex vivo treatment of patient derived xenograft (PDX) samples**

PDX samples were transplanted in 8 week old NSG mice through tail vain injection. Human leukemic cell expansion was monitored through human CD45 staining on blood samples. Single cells were isolated from the spleen, which at time of sacrifice contained > 85% human CD45+ cells. Spleen cells were seeded in 96-well plate (5 x 10⁵ cells/well) and incubated with vehicle (DMSO) or inhibitor. Cell viability was assessed 48 hours using ATP-Lite. CompuSyn was used to calculate the combination index (CI). For in vivo treatment studies, the XC65 was transduced overnight with lentivirus pCH-SFFV-eGFP-P2A-fLuc. The GFP positive cells were then sorted using the S3 Sorter (Bio-Rad) and re-transplanted back into recipient NSG mice. Upon confirmation that XC65 was greater than >95% GFP positive, leukemic cells were isolated from the spleen and reinjected into a larger cohort of NSG mice for acute 7-day in vivo treatment. Ruxolitinib (HY-50858, MedChem Express) was dissolved in 0.5% methylcellulose and AZD1208 dissolved in 50% PEG400/0.5% methylcellulose and both were administered by oral gavage.

**Flow cytometry analyses**

Single-cell suspensions were prepared from peripheral blood, bone marrow, spleen, thymus and lymph nodes. Cells were analyzed on either a FACS Canto flow cytometer (BD Bio-sciences) or
MACSQuant Vyb (Miltenyi) with the following antibodies: CD3e, CD8a, CD45, CD4, CD45R, CD44, CD25, CD11b, Gr1/Ly6G, CD127, CD117 and TCRbeta. These antibodies were conjugated to phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein Cyanine5.5 (PerCP-Cy5), VioBlue, PE-Vio770, or APC-Vio770. For intracellular phospho-STAT5 staining, cells were first fixed in a 1:1 ration with IC fix buffer (Invitrogen #00-8222-49) and then permeabilized in ice cold 100% methanol. Staining was then carried out in PBS/2% FCS with 1:20 dilution pSTAT5-APC (eBioscience #4334964) or IgG1 kappa isotype (eBioscience #17-4714-41) control. Data were analyzed with the FlowJo software (Tree Star).

**ChIPmentation, ChIP-seq and CUT&RUN ChIP-Seq**

ChIPmentation ChIP-seq on mouse leukemic samples was carried out as described with modifications (58). ALL-SIL cells were originally sourced from DSMZ, Germany in 2004 and authenticated using STR analysis and PCR for the NUP214-ABL1 fusion in 2017. Briefly, 20-40 million cells were washed in PBS and cross-linked with 1% formaldehyde for 10 min at room temperature and then quenched by addition of glycine (125 mM final concentration). For Nuclei isolation, cells were resuspended in 1X RSB buffer (10 mM Tris pH7.4, 10 mM NaCl, 3 mM MgCl2) and left on ice for 10 min to swell. Cells were collected by centrifugation and resuspended in RSBG40 buffer (10 mM Tris pH7.4, 10 mM NaCl, 3 mM MgCl2, 10% glycerol, 0.5% NP40) with 1/10 v/v of 10% detergent (3.3% w/v sodium deoxycholate, 6.6% v/v Tween-40). Nuclei were collected by centrifugation and resuspended in L3B+ buffer (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-Lauroylsarcosine, 0.2% SDS). Chromatin was fragmented to 200-400 bp using 20 cycles (30 sec on, 30 sec off, High Setting) using the Bioruptor (Diagenode). Chromatin immunoprecipitation was carried out overnight in the presence antibodies (H3K27me3 07-449 Millipore or H3K27me3 Cat#39155 Active Motif Lot#06517018; H3K4me3 Cat#39159 Active Motif Lot#15617005, H3K4me1 Cat#39297 Active Motif Lot#01714002 Active Motif, H3K27Ac Cat#4729 Lot#GR251958-1 or H3K27Ac Cat#39134 Active Motif Lot#20017009): STAT5 Cell Signaling Cat#8363 Lot9; HOXA9 Sigma Atlas Antibodies
Cat#HPA061982 Lot#86503) coupled to magnetic protein A/G beads (Millipore). Tagmentation and library preparation was carried out as described (http://www.medical-epigenomics.org/papers/schmidl2015/). DNA was purified using triple-sided SPRI bead cleanup using 1.0X; 0.65X; 0.9X ratios (Agencourt AMPure Beads, Beckman Coulter) and analyzed by Illumina Hiseq 2000 (Illumina, San Diego, CA, USA). Raw sequencing data were mapped to the human reference genome (GRCh37/h19) using Bowtie. Peak calling was performed using MACS 1.4. A custom Python script was used to generate the centered heatmaps. To find the enriched motifs in the peaks we made use of i-CisTarget (https://gbiomed.kuleuven.be/apps/lcb/i-cisTarget/). For the patient sample XE89, CUT&RUN ChIP seq was carried out as described(59) using HOXA9 Sigma Atlas Antibodies Cat#HPA061982 Lot#86503.

Ethical aspects

Animal experiments were approved by the Ethical committee on animal experimentation of KU Leuven. Human leukemia samples were collected at the University Hospital Leuven (UZ Leuven) after approval by the ethical committee. Informed consent was obtained from all patients or their parents, according to the Declaration of Helsinki.

Acknowledgements

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REFERENCES


Figure Legends

Figure 1. JAK3 mutations and ectopic expression HOXA9 are significantly associated in clinical T-ALL cases. (A) Frequency of IL7R-JAK-STAT mutations in 155 T-ALL cases (Vicente Dataset) and of 264 T-ALL cases (Liu Dataset) within different defined transcription factor subgroups. Dotted line denotes average % of IL7R-JAK-STAT mutations across entire data set. (B) Association between JAK3 mutations only and defined transcription factor subgroups within the Vicente and Liu datasets. (C) RNA-seq FPKM levels for JAK3 and HOXA9 in the Liu dataset. (D) Real time qPCR of primary T-ALL samples for HOXA9, HOXA10 and HOXA11.

Figure 2. Co-expression of JAK3(M511I) and HOXA9 transforms hematopoietic progenitor cells and leads to rapid leukemia development in vivo. (A) Schematic of the two retroviral expression vectors used in the transduction of murine hematopoietic stem progenitor cells (HSPCs) for the ex vivo transformation assay and bone marrow transplant model. Dual transduction results in single, double and non-transduced cells for direct clonal competition analyses ex vivo and in vivo. (B) Cell proliferation of HSPCs transduced with both JAK3(M511I) and HOXA9 over 20 days in the absence all cytokines with matching FACS profiles. (C) Cell proliferation of HSPCs transduced with both STAT5(N642H) and HOXA9 over 20 days in the absence all cytokines with matching FACS profiles. (D) White Blood Cell counts (WBC) in recipient BALB/C mice that received lin- cells transduced with JAK3(M511I) and HOXA9 retrovirus or JAK3(M511I) only retrovirus. (E) Clonal analysis by FACS for GFP and mCHERRY reporters representing JAK3(M511I) and HOXA9 respectively at injection start and end-stage. (F) Kaplan-Meier disease free survival (DFS) for JAK3(M511I)/HOXA9 mice (Median DFS = 25 days, p-value < 0.0001), JAK3 (M511I) alone (Median DFS = 126.5 days); JAK3(M511I)/TAL1 (Median DFS = 144.5 days). DFS determined by WBC <30,000 cells/mm³. (G,H) Reciprocal experiments in C57BL/6 mice co-expressing JAK3(M511I) and HOXA9 for WBC and clonal analysis by FACS for GFP and mCHERRY reporters.
representing JAK3(M511I) and HOXA9 respectively at injection start and end-stage. (I) Kaplan-Meier disease free survival for STAT5B(N642H)/HOXA9 (Median DFS = 12 days); JAK3(M511I)/HOXA9 (Median DFS = 40 days); JAK3(M511I) (Median DFS = 150 days) and HOXA9 (Median DFS = 182 days) only recipient C57BL/6 mice.

**Figure 3.** Constitutive expression of JAK3(M511I) and HOXA9 leads to development of both AML and T-ALL in vivo. (A) Representative peripheral blood FACS of expanding leukemic clones expressing JAK3(M511I) and HOXA9. (B) Histopathology and for MPO and CD3 staining of bone marrow, spleen and lymph nodes (Scale bar Bone marrow = 40 μM, Spleen and lymph nodes = 80 μM). (C) Western blot analysis spleen and bone marrow leukemic cells. (D,E) Ex vivo growth assay of leukemia clones and matching FACS with JAK3(M511I) and HOXA9 levels measured by GFP and mCHERRY expression respectively. (F) Schematic of in vivo treatment of primary bone marrow transplant recipients. Mice were treated daily for 20 days from day 14 post-transplantation with either 40 mg/kg/day tofacitinib (oral gavage) or vehicle control. (G) Phospho-flow cytometry of pSTAT5 levels in JAK3(M511I)/HOXA9 leukemic cells in peripheral blood after three days Tofacitinib treatment compared to vehicle control. (H) Change in white blood cell count before and after the treatment. (I) Total white blood cell counts at day 35. (J) Spleen weights of tofacitinib vs. vehicle treated mice at day 35. (K) Ki67 staining of spleens.

**Figure 4.** Novel retroviral strategy to restrict JAK3(M511I)/HOXA9 expression to developing lymphoid cells results in significantly decreased leukemia latency. (A) Schematic of new retroviral vector incorporating anti-parallel lox66/71 sites for unidirectional inversion in the presence of Cre-recombinase. (B,C) Cell proliferation of Ba/F3 or Ba/F3_Cre recombinase cells transduced with the retroviral constructs encoding JAK3(M511I) or JAK3(M511I)_P2A_HOXA9 in between the lox66/71 sites. (C) Western blot analysis of the transduced Ba/F3. (D) Model of improved BMT utilizing Cre-donor mice in combination with novel retroviral vector. (E) Kaplan-
Meier disease free survival cure for mice with hCD2-iCre driven inversion of JAK3(M511I) and HOXA9 and the cell surface phenotype frequency for the resulting leukemia. (DFS = WBC <30,000 cells/mm$^3$). (F) Genomic PCR detection of the inversion of the retroviral construct.

Figure 5. HOXA9 and STAT5 co-occupy similar genomic regions and increases JAK/STAT signaling. (A) Volcano plot of differential gene expression analysis of age matched CD8+ T-ALL JAK3(M511I)/HOXA9 vs. JAK3(M511I) only leukemia cells. (B) JAK3(M511I)/HOXA9 leukemia ChIP-seq density heat maps centered on STAT5 signal alongside HOXA9 and associated H3K27Ac, H3K27me3, H3K4me3 and H3K4me1 histone marks. (C) Frequency of STAT5/HOXA9 co-occupied DNA regions with promoters (H3K4me3+ve) and Enhancers (H3K27Ac+ve/H3K4me3-ve). (D) Individual ChIP-seq tracks for canonical STAT5 target genes Pim1 and Bcl2. (E) GSEA analysis of RNA-seq differentially expressed genes for JAK3(M511I)/HOXA9 vs. JAK3(M511I) and loss of the repressive H3K27me3 chromatin marks or gain in H3K27ac activation mark. (F) Transcription factor loading of STAT5 and HOXA9 in upregulated, downregulated or genes that do not change. (G) Heat map and GSEA pathway of JAK/STAT signaling pathway genes in CD8+ control T-cell vs. JAK3(M511I) vs. JAK3(M511I)/HOXA9 leukemia.

Figure 6. PIM1 expression in increased in JAK3(M511I)/HOXA9 leukemia (A) qPCR analysis of Pim1 levels in normal CD8+ T-cells, CD8+ JAK3(M511I) and CD8+ JAK3(M511I)/HOXA9 cells (B) Analysis of clinical T-ALL samples for PIM1 expression levels and those cases with JAK3/IL7R/STAT mutations (Red dots). (C) Analysis of PIM1 RNA and protein expression in HOXA9 positive PDX samples ex vivo after 1 μM Ruxolitinib over 6 hours. (D) Combinatorial treatment with small molecular inhibitors against JAK kinases (Ruxolitinib) and PIM1 kinase (AZD1208) in DND41(IL7R mutant) and JAK3 mutant PDX samples. (E) In vivo combination treatment with Ruxolitinib and AZD1208 (7 days) of the JAK3 mutant XC65 PDX sample.
Figure 7. ATAC-seq reveals role for AP-1 complex in JAK3(M511I)/HOXA9 driven leukemia.

(A) GSEA results showing appearing and disappearing ATAC-seq peaks correlated to up-regulated and down-regulated genes. (B) HOXA9 and STAT5 bound genomic regions relative to appearing and disappearing ATAC-seq peaks. (C) iCis-Target results for DNA-binding motifs within the appearing ATAC-seq peaks. (D) Relative expression of members of the AP-1 complex including Fos, Jun, Jund and FosL2. (E) Relative expression of AP-1 target genes in normal CD8+ cells and JAK3(M511I) or JAK3(M511I)/HOXA9 leukemias. (F) ChiP-seq analysis tracks for AP-1 targets such as FasL (bound by STAT5 and HOXA9) and Csf1, App and Klf4 not bound by STAT5 or HOXA9 (all track heights =0-100).
FIGURE 1

A. JAK3 wild type (n=130) vs. JAK3 mutant (n=25)

HOXA+ p = 0.018
TAL1/LMO2+ p = 0.002

B. IL7R-JAK-STAT5 pathway mutation (Vicente dataset)

C. IL7R-JAK-STAT5 pathway mutation (Liu dataset)

D. HOXA+ p = 0.006
TAL1/LMO2+ p = 0.0047

FIGURE 1
FIGURE 3
**FIGURE 4**

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Dataset: CD8+ vs. JAK3(M511I)
Geneset: JAK-STAT SIGNALING PATHWAY
KEGG HSA04630

NES: 1.525
p-value: 0.007
FDR q-value: 0.135

Dataset: JAK3(M511I) vs. JAK3(M511I)+HOXA9
Geneset: JAK-STAT SIGNALING PATHWAY
KEGG HSA04630

NES: 1.659
p-value: 0.018
FDR q-value: 0.098

FIGURE 5
FIGURE 6
FIGURE 7

A. Dataset: JAK3(M511I)/HOXA9 vs. JAK3(M511I)
Geneset: Appearing ATAC-seq peaks
NES: 1.83
p-value <0.001
FDR q-value <0.001

B. Dataset: JAK3(M511I)/HOXA9 vs. JAK3(M511I)
Geneset: Disappearing ATAC-seq peaks
NES: -1.69
p-value <0.001
FDR q-value <0.001

C. Fosl2 (NES = 6.34) Jun (NES = 6.29)

D. Normalized Counts

E. AP1 target genes

F. 5 kb 50 kb

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HOXA9 cooperates with activated JAK/STAT signaling to drive leukemia development.

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