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Oncogenic Cooperation Between IL7R-JAK-STAT Pathway Mutations

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Interleukin-7 (IL7) receptor (IL7R)-mediated signaling through the JAK-STAT pathway is essential for hematopoiesis, as well as for the survival, proliferation and activity of mature T and B lymphocytes.[1] The IL7R-JAK-STAT signaling pathway is frequently mutated in T-cell acute lymphoblastic leukemia (T-ALL), an aggressive hematological malignancy caused by the accumulation of multiple genetic alterations.[2–5] In about 30% of patients with T-ALL, constitutive activation of the IL7R-JAK-STAT signaling pathway is the result of activating mutations in *IL7R* (encoding for IL7Rα), Janus kinase 1 (*JAK1*), *JAK3*, signal transduced and activator of transcription 5B (*STAT5B*) or of loss-of-function alterations in negative regulators of the pathway, such as the protein tyrosine phosphatase nonreceptor type 2 (*PTPN2*) or the large GTPase dynamin 2 (*DNM2*).[3,4]

Interestingly, a subset of T-ALL cases harbors 2 or more IL7R-JAK-STAT signaling pathway mutations (Supplemental Digital Figure S1, http://links.lww.com/HS/A192).[3–7] However, whether these mutations co-occur just by chance or whether they cooperate in driving T-cell transformation and T-ALL development remains to be elucidated. Here, we investigated if mutant IL7Rα can cooperate with mutant JAK1, mutant JAK3, or inactivation of PTPN2 or DNM2 in driving cytokine-independent growth of the murine IL3-dependent pro-B cell line Ba/F3 (Ba/F3) or primary mouse hematopoietic stem/progenitor cells (HSPCs) harvested from C57BL/6 mice.

The IL7R is a heterodimer consisting of IL7Rα (encoded by the *IL7R* gene), exclusively expressed on lymphoid cells, and the common gamma chain (γc, encoded by the *IL2RG* gene), which is expressed on most hematopoietic cell types. IL7R signaling is initiated when binding of IL7 induces heterodimerization of and conformational changes in IL7Rα and γc, resulting in activation of the protein tyrosine kinases JAK1 and JAK3 which are associated with IL7Rα and γc, respectively. Subsequently, the activated kinases phosphorylate downstream effector molecules, including STAT5. Upon phosphorylation of tyrosine residue Y694, STAT5 homodimerizes and translocates to the nucleus where it activates the expression of STAT5 target genes, which are known to be involved in cell survival and cell cycle progression.[8]

We first assessed the possible cooperative effect between mutant IL7Rα and the JAK kinases JAK1 or JAK3 by transducing Ba/F3 cells or primary HSPCs with retroviral vectors expressing wild-type IL7Rα or IL7Rα p.L243_T244insSRCL (IL7Rα^{SRCL}) (MSCV-IRES-mTAG2BFP) alone or together with JAK1 p.A634D (JAK1^{A634D}) or JAK3 p.M511I (JAK3^{M511I}) (MSCV-IRES-GFP) as described previously.[9] This resulted in double-transduced, single-transduced, and nontransduced cells, which could be distinguished by fluorescent protein expression as double-positive (BFP$^+$/GFP$^+$), single-positive (BFP$^+$ or GFP$^+$), and double-negative cell populations, respectively. We cultured these cells for up to 3 weeks in the absence of IL3, and cell number and fluorescent protein-positive cells were measured on a MACSQuant VYB (Miltenyi) and analyzed using FlowJo software (Tree Star).

Ba/F3 cells single-transduced with IL7Rα^{SRCL} were able to slowly transform to cytokine-independent proliferation and outcompete non-transduced cells in the absence of IL3, whereas wild-type IL7Rα-expressing cells were not, demonstrating that IL7Rα p.L243_T244insSRCL is an activating alteration (Figure 1A, B; Supplemental Digital Figure S2, http://links.lww.com/HS/A192). Similar results were obtained by using primary HSPCs (Supplemental Digital Figure S3, http://links.lww.com/HS/A192). Co-transduction of IL7Rα^{SRCL} and the JAK1^{A634D} gain-of-function mutation considerably accelerated the transformation to cytokine-independence (Figure 1C), with IL7Rα^{SRCL}+JAK1^{A634D} double-positive cells outcompeting IL7Rα^{SRCL} single-positive cells and, to a lesser extent, JAK1^{A634D} single-positive cells (Figure 1B, E; Supplemental Digital Figure S4A, http://links.lww.com/HS/A192). Ba/F3 cells transduced with JAK1^{A634D} were also able to rapidly transform to cytokine-independent proliferation. However, once transformed, their cell proliferation rate was substantially lower compared with IL7Rα^{SRCL}+JAK1^{A634D} co-transduced cells (Figure 1C). Similar results were observed in primary HSPCs, as the percentage of IL7Rα^{SRCL}+JAK1^{A634D} double-positive cells considerably increased upon transformation to IL3-independence (Supplemental Digital Figure S5, http://links.lww.com/HS/A192). In this model, also wild-type IL7Rα was able to cooperate with JAK1^{A634D} (Supplemental Digital Figure S5, http://links.lww.com/HS/A192), illustrating the oncogenic potential of increased expression of IL7Rα, as recently demonstrated.[10]

Although cells co-expressing IL7Rα^{SRCL} and the activating mutation JAK3^{M511I} rapidly transformed to cytokine-independence (Figure 1D), IL7Rα^{SRCL} did not strongly cooperate with

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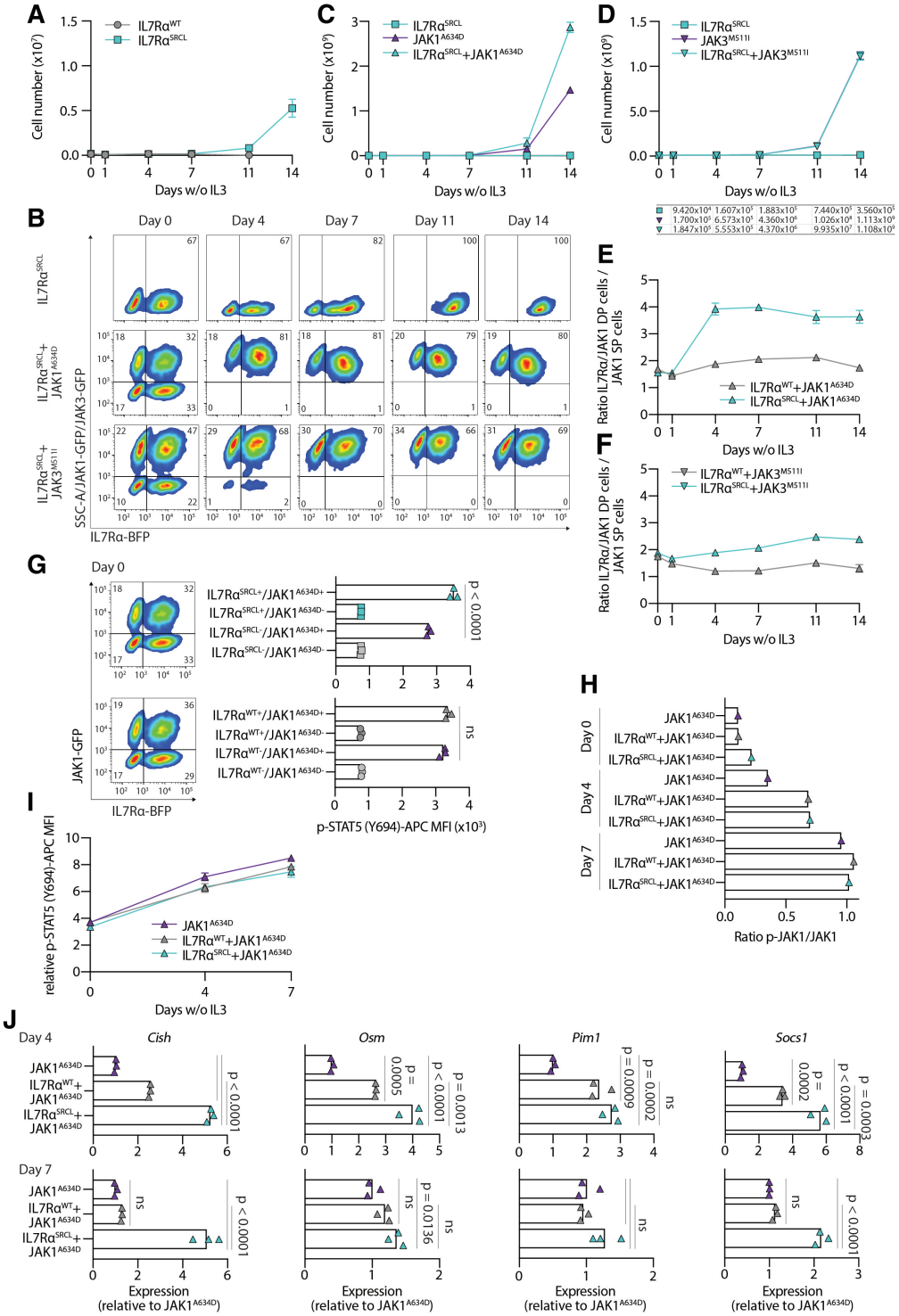

Figure 1. IL7Rα^{SRCL} cooperates with JAK1^{A634D}, but not with JAK3^{M511I}, in driving Ba/F3 cell transformation via increased JAK-STAT signaling activation. (A) Growth curve of Ba/F3 cells transduced with wild type IL7Rα (IL7Rα^{WT}) or IL7Rα^{SRCL}. Data are presented as mean ± SD. (B) FACS analysis of Ba/F3 cells transduced with IL7Rα^{SRCL}, IL7Rα^{SRCL}+JAK1^{A634D} and IL7Rα^{SRCL}+JAK3^{M511I}, 0, 4, 7, 11 and 14 days after IL3 withdrawal. (C) Growth curve of Ba/F3 cells transduced with IL7Rα^{SRCL}, JAK1^{A634D}, and IL7Rα^{SRCL}+JAK1^{A634D}. Data are presented as mean ± SD. (D) Growth curve of Ba/F3 cells transduced with IL7Rα^{SRCL}, JAK3^{M511I} and IL7Rα^{SRCL}+JAK3^{M511I}. Data are presented as mean ± SD. (E) Quantification of GFP- and BFP-positive cells for Ba/F3 cells transduced with IL7Rα^{WT}+JAK1^{A634D} and IL7Rα^{SRCL}+JAK1^{A634D}, after IL3 withdrawal. Data are presented as mean ± SD. (F) Quantification of GFP- and BFP-positive cells for Ba/F3 cells transduced with IL7Rα^{WT}+JAK3^{M511I} and IL7Rα^{SRCL}+JAK3^{M511I}, after IL3 withdrawal. Data are presented as mean ± SD. (G) FACS analysis (left) and quantification of the MFI of phospho-STAT5 (Y694)-APC (right) for Ba/F3 cells transduced with IL7Rα^{WT}+JAK1^{A634D} and IL7Rα^{SRCL}+JAK1^{A634D}, 0 d (3 h) after IL3 withdrawal. Data are presented as mean ± SD. Statistical significance was calculated using one-way analysis of variance (ANOVA) with Tukey's multiple comparison correction. (H) Western blot analysis (upper) and quantification (lower) of phospho-JAK1 (Y1034/1035) and JAK1 for Ba/F3 cells transduced with JAK1^{A634D}, IL7Rα^{WT}+JAK1^{A634D}, and IL7Rα^{SRCL}+JAK1^{A634D}, 0, 4, and 7 d after IL3 withdrawal. β-actin was used as loading control. (I) Quantification of the MFI of phospho-STAT5 (Y694)-APC for Ba/F3 cells transduced with JAK1^{A634D}, IL7Rα^{WT}+JAK1^{A634D}, and IL7Rα^{SRCL}+JAK1^{A634D}, 0, 4, and 7 d after IL3 withdrawal. Data are presented as mean ± SD. (J) RT-qPCR of STAT5 target genes in Ba/F3 cells transduced with JAK1^{A634D}, IL7Rα^{WT}+JAK1^{A634D}, and IL7Rα^{SRCL}+JAK1^{A634D}, 4 and 7 d after IL3 withdrawal. Data are presented as mean ± SD. Statistical significance was calculated using ANOVA with Tukey's multiple comparison correction. ANOVA = one-way analysis of variance; MFI = mean fluorescence intensity; ns = not significant.

JAK3^{M511I} to drive this transformation. IL7Rα^{SRCL}+JAK3^{M511I} double-positive cells were not able to outcompete JAK3^{M511I} single-positive cells, as the percentage of IL7Rα^{SRCL}+JAK3^{M511I} double positive cells and the percentage of JAK3^{M511I} single-positive cells increased at similar rates upon transformation (Figure 1B, F; Supplemental Digital Figure S4B, http://links.lww.com/HS/A192). Moreover, the IL3-independent proliferation rate of IL7Rα^{SRCL}+JAK3^{M511I} co-transduced cells was similar to that of JAK3^{M511I} transduced cells (Figure 1D). These results show that cells transformed by IL7Rα^{SRCL}+JAK3^{M511I} did not proliferate substantially faster compared with JAK3^{M511I} transformed cells.

The fact that an oncogenic cooperative effect was observed between mutant IL7Rα and mutant JAK1, but not mutant JAK3, could be explained by the type of *IL7R* mutation. The p.L243_T244insSRCL alteration introduces an unpaired cysteine in the extracellular-juxtamembrane domain of IL7Rα. Such unpaired cysteine residues promote the formation of de novo intermolecular disulfide bonds between 2 mutant IL7Rα chains, resulting in constitutive IL7Rα homodimerization and phosphorylation and activation of JAK1, which is associated with IL7Rα, and STAT5, independent of IL7, γc or JAK3.[11–13] Moreover, JAK1 was demonstrated to be more important than JAK3 for IL7R-JAK-STAT signaling pathway activation.[14]

To assess if transformation to IL3-independent proliferation was the result of increased IL7R-JAK-STAT signaling activation, we analyzed phospho-JAK1 and phospho-STAT5 levels by Western blot and/or flow cytometry and determined STAT5 target gene expression by quantitative real time (qRT)-PCR. At day 0, 3 hours after IL3 withdrawal, STAT5 phosphorylation was significantly increased in the double-positive population compared with the single-positive populations and double-negative population of cells co-transduced with IL7Rα^{SRCL}+JAK1^{A634D} but not with wild-type IL7Rα+JAK1^{A634D} (Figure 1G). Upon transformation, phospho-JAK1 (Figure 1H; Supplemental Digital Figure S6; http://links.lww.com/HS/A192) and phospho-STAT5 (Figure 1I) levels substantially increased, and 4 days after IL3 withdrawal, JAK1 phosphorylation was higher in IL7Rα^{SRCL}+JAK1^{A634D} co-transduced cells compared with JAK1^{A634D} transduced cells, but not wild-type IL7Rα+JAK1^{A634D} co-transduced cells (Figure 1H).

The increased phospho-JAK1 levels in wild-type IL7Rα+JAK1^{A634D} co-transduced cells compared with cells transduced with JAK1^{A634D} could be explained by increased phosphorylation of JAK1 and activation of downstream signaling upon co-expression of IL7Rα, as the transforming ability of JAK1^{A634D} was shown to be dependent on binding to an alpha chain.[15] Indeed, although no differences were observed in phospho-STAT5 levels, STAT5 target gene expression was significantly higher in wild-type IL7Rα+JAK1^{A634D} co-transduced cells compared with cells transduced with JAK1^{A634D} (Figure 1J). In addition, cells co-transduced with IL7Rα^{SRCL}+JAK1^{A634D} showed higher expression of STAT5 target genes compared with JAK1^{A634D} transduced cells and cells co-transduced with wild-type IL7Rα+JAK1^{A634D} (Figure 1J). Together, these results show that activating mutations in IL7Rα and JAK1 cooperate in driving cell transformation via increased activation of JAK-STAT signaling.

Next, we examined whether inactivation of *Ptpn2* or *Dnm2* could cooperate with IL7Rα^{SRCL} in transforming Ba/F3 cells. To this end, we used Ba/F3 cells stably expressing Cas9, allowing CRISPR genome editing, as described previously.[16] We co-transduced the cells with a retroviral vector (MSCV-IRES-mTAG2BFP) expressing wild-type IL7Rα or IL7Rα^{SRCL} and a retroviral vector (pMxs-U6-GFP; Cell Biolabs) expressing a CRISPR guide RNA (gRNA) targeting exons of the murine *Ptpn2* or *Dnm2* genes. The crispor.tefor.net design tool was used to find gRNAs with minimal off-target effects.

Although co-transduction with IL7Rα^{SRCL}+Ptpn2 gRNA did not accelerate the transformation to IL3-independent proliferation, the proliferation rate of co-transduced Ba/F3 cells was substantially higher in comparison with IL7Rα^{SRCL} expressing cells, as measured by cell number and EdU incorporation (Figure 2A; Supplemental Digital Figures S7 and S8, http://links.lww.com/HS/A192). Indeed, for co-transduced cells, IL7Rα^{SRCL}+Ptpn2 gRNA double-positive cells fully outcompeted IL7Rα^{SRCL} single-positive cells, as well as Ptpn2 gRNA single-positive cells (Figure 2B, C; Supplemental Digital Figure S9, http://links.lww.com/HS/A192), illustrating a strong cooperation between activation of IL7Rα and inactivation of Ptpn2 in Ba/F3 cells, and similar results were observed in primary HSPCs (Supplemental Digital Figure S9, http://links.lww.com/HS/A192). Ptpn2 protein levels were substantially reduced in transformed IL7Rα^{SRCL}+Ptpn2 gRNA double-positive Ba/F3 cells (Figure 2D). The cooperation was due to increased activation of the JAK-STAT signaling pathway, as phosphorylation of JAK1 and STAT5 (Figure# 2E, F; Supplemental Digital Figure S11, http://links.lww.com/HS/A192) as well as STAT5 target gene expression (Figure 2G) were significantly increased in cells co-transduced with IL7Rα^{SRCL}+Ptpn2 gRNA compared with IL7Rα^{SRCL} transduced cells. As expected, wild-type IL7Rα + Ptpn2 gRNA did not cooperate in driving transformation to cytokine-independent proliferation of Ba/F3 cells nor of primary HSPCs (Supplemental Digital Figures S7, S9, and S10, http://links.lww.com/HS/A192).

Similar results were obtained for activation of IL7Rα and inactivation of Dnm2. IL7Rα^{SRCL}+Dnm2 gRNA co-transduced cells showed a considerably higher IL3-independent proliferation rate cells compared with IL7Rα^{SRCL} transduced cells (Figure 2H; Supplemental Digital Figure S12, http://links.lww.com/HS/A192), with IL7Rα^{SRCL}+Dnm2 gRNA double-positive cells outcompeting IL7Rα^{SRCL} single-positive cells and Dnm2 gRNA single-positive cells (Figure 2I, J; Supplemental Digital Figure S13, http://links.lww.com/HS/A192). Transformed IL7Rα^{SRCL}+Dnm2 gRNA double-positive cells showed substantially reduced Dnm2 protein levels (Figure 2K). The considerable increase in phospho-JAK1 and phospho-STAT5 levels Figure 2L; Supplemental Digital Figure S14, http://links.lww.com/HS/A192) as well as in STAT5 target gene expression (Figure 2M) in IL7Rα^{SRCL}+Dnm2 gRNA co-transduced cells compared with IL7Rα^{SRCL} transduced cells demonstrated that mutant IL7Rα also cooperates with inactivation of DNM2 via increased JAK-STAT signaling activation. Again, wild-type IL7Rα and Dnm2 gRNA did not cooperate in driving transformation to cytokine-independent proliferation (Supplemental Digital Figures S12 and Sl3; http://links.lww.com/HS/A192).

DNM2 is involved in clathrin-dependent endocytosis, a process that regulates, amongst others, cell surface expression levels of receptors.[17] Although we expected that the increased JAK-STAT signaling activation in transformed IL7Rα^{SRCL}+Dnm2 gRNA co-transduced Ba/F3 cells was the result of increased IL7Rα^{SRCL} levels due to decreased IL7Rα^{SRCL} endocytosis, we did not observe this (Supplemental Digital Figure S15, http://links.lww.com/HS/A192). Using an Lmo2^{Tg}Dnm2^{V265G} transgenic mouse model, Tremblay et al[17] showed that, when co-occurring with overexpression of *LMO2*, inactivation of *Dnm2* blocked the internalization of IL7R, resulting in higher IL7R cell surface expression levels, increased activation of the IL7R-JAK-STAT signaling pathway and accelerated development of T-ALL. However, in the absence of LMO2 overexpression, the defective endocytic process did not result in higher IL7R cell surface expression levels nor in increased IL7R-JAK-STAT signaling pathway activation.[17] In addition, Henriques et al[18] demonstrated that IL7R internalization upon stimulation with IL7 is essential for activation of downstream signaling, whereas in the absence of IL7, blocking endocytosis did not have any effect. These results suggest that the oncogenic effect of loss-of-function alterations in *DNM2* is dependent on both cell intrinsic

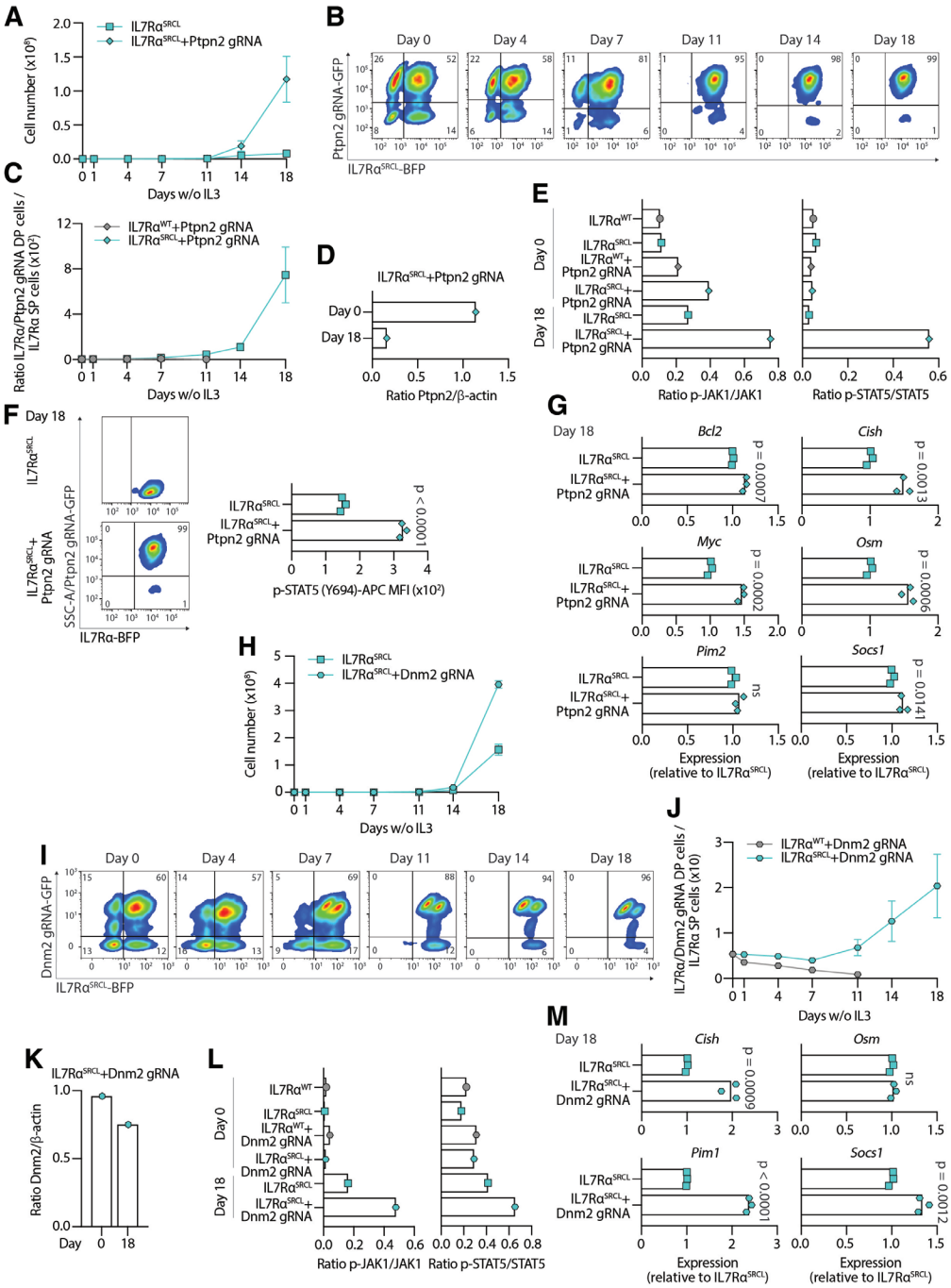

Figure 2. IL7Rα^{SRCL} cooperates with inactivation of PTPN2 and DNM2 in driving Ba/F3 cell transformation via increased JAK-STAT signaling activation. (A) Growth curve of Cas9-expressing Ba/F3 cells transduced with IL7Rα^{SRCL} and IL7Rα^{SRCL}+Ptpn2 gRNA. Data are presented as mean ± SD. (B) FACS analysis of Ba/F3 cells transduced with IL7Rα^{SRCL}+Ptpn2 gRNA, 0, 4, 7, 11, 14, and 18 d after IL3 withdrawal. (C) Quantification of GFP- and BFP-positive cells for Ba/F3 cells transduced with IL7Rα^{WT}+Ptpn2 gRNA and IL7Rα^{SRCL}+Ptpn2 gRNA after IL3 withdrawal. Data are presented as mean ± SD. (D) Western blot quantification of Ptpn2 for Ba/F3 cells transduced with IL7Rα^{SRCL}+Ptpn2 gRNA, 0 and 18 d after IL3 withdrawal. β-actin was used as loading control. (E) Western blot quantification of phospho-JAK1 (Y1034/1035) and JAK1 (left) and phospho-STAT5 (Y694) and STAT5 (right) for Ba/F3 cells transduced with wild-type IL7Rα, IL7Rα^{SRCL}, IL7Rα^{WT}+Ptpn2 gRNA, and IL7Rα^{SRCL}+Ptpn2 gRNA, 0 and 18 d after IL3 withdrawal. β-actin was used as loading control. (F) FACS analysis (left) and quantification of the MFI of phospho-STAT5 (Y694)-APC (right) for Ba/F3 cells transduced with IL7Rα^{SRCL} and IL7Rα^{SRCL}+Ptpn2 gRNA, 18 d after IL3 withdrawal. Data are presented as mean ± SD. Statistical significance was calculated using ANOVA with Tukey's multiple comparison correction. (G) RT-qPCR of STAT5 target genes in Ba/F3 cells transduced with IL7Rα^{SRCL} and IL7Rα^{SRCL}+Ptpn2 gRNA, 18 d after IL3 withdrawal. Data are presented as mean ± SD. Statistical significance was calculated using ANOVA with Tukey's multiple comparison correction. (H) Growth curve of Cas9-expressing Ba/F3 cells transduced with IL7Rα^{SRCL} and IL7Rα^{SRCL}+Dnm2 gRNA. Data are presented as mean ± SD. (I) FACS analysis of Ba/F3 cells transduced with IL7Rα^{SRCL}+Dnm2 gRNA, 0, 4, 7, 11, 14, and 18 d after IL3 withdrawal. (J) Quantification of GFP- and BFP-positive cells for Ba/F3 cells transduced with IL7Rα^{WT}+Dnm2 gRNA and IL7Rα^{SRCL}+Dnm2 gRNA after IL3 withdrawal. Data are presented as mean ± SD. (K) Western blot quantification of Dnm2 for Ba/F3 cells transduced with IL7Rα^{SRCL}+Dnm2 gRNA, 0 and 18 d after IL3 withdrawal. β-actin was used as loading control. (L) Western blot quantification of phospho-JAK1 (Y1034/1035) and JAK1 (left) and phospho-STAT5 (Y694) and STAT5 (right) for Ba/F3 cells transduced with wild-type IL7Rα, IL7Rα^{SRCL}, IL7Rα^{WT}+Dnm2 gRNA, and IL7Rα^{SRCL}+Dnm2 gRNA, 0 and 18 d after IL3 withdrawal. β-actin was used as loading control. (M) RT-qPCR of STAT5 target genes in Ba/F3 cells transduced with IL7Rα^{SRCL} and IL7Rα^{SRCL}+Dnm2 gRNA, 18 d after IL3 withdrawal. Data are presented as mean ± SD. Statistical significance was calculated using ANOVA with Tukey's multiple comparison correction.
ANOVA = one-way analysis of variance; MFI = mean fluorescence intensity; ns = not significant.

factors, such as co-occurring genetic alterations, and external stimuli, such as cytokines. Since we used the Ba/F3 pro-B cell line for our studies, we cannot exclude that this would be different in a T-cell context.

In conclusion, T-ALL cells with an IL7R-JAK-STAT pathway mutation seem to have high genomic instability that allows them to accumulate an increasing number of mutations in the JAK-STAT pathway during their development and progression. We show that such genetic alterations in the IL7R-JAK-STAT signaling pathway are able to cooperate in driving hematopoietic cell transformation. These results provide an explanation why developing T-ALL cells accumulate multiple mutations in the same signaling pathway, since these combinations of mutations lead to increased JAK-STAT signaling.

Disclosures

The authors have no conflicts of interest to disclose.

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