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ACUTE MYELOID LEUKEMIA

# Cre recombinase promotes leukemogenesis in the presence of both homozygous and heterozygous *FLT3*-ITD

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**TO THE EDITOR:**

Cre recombinase murine models allow the expression of mutated genes in a cell type-specific manner or via an inducible mechanism and has revolutionized biomedical research. However, these models may be associated with some caveats, such as off-target effects and lack of fidelity. In the issue 4 of LEUKEMIA in 2023, Straube et al., described an unexpected observation where Cre expression alone was able to drive early acute myeloid leukemia (AML) in the context of *FLT3*-ITD/ITD (homozygous) [1]. Herein, we found that expression of Cre recombinase induced early AML in the presence of homozygous *FLT3*-ITD in different models, but not in the presence of the *Kit* D814V mutation (murine homolog of human *KIT* D816V mutation). Moreover, Cre recombinase also promoted leukemogenesis in the presence of heterozygous *FLT3*-ITD.

To identify cooperating partners for the *FLT3*-ITD in the development of AML, we analyzed the activation of ≥ 42 receptor tyrosine kinases in primary samples from AML patients using a phospho-kinase antibody array. The phosphorylated kinases, Macrophage colony stimulating factor receptor (MCSFR) and Fibroblast growth factor receptor 2 (FGFR2) were detected in 78% and 31% of AML patients ($n = 90$), respectively (Fig. 1A and data not shown). The expression of MCSFR on blasts was confirmed in all analyzed primary samples with phosphorylated kinases ($n = 32$) by flow cytometric analysis (Fig. 1B). In a separate cohort, we detected MCSFR expression almost in all primary samples from AML patients ($n = 125$) by flow cytometric analysis (data not shown). Importantly, MCSFR was identified as a therapeutic target in AML leukemia [2]. Moreover, MCSFR is crucial for leukemic stem cells (LSC) potential induced by the MOZ-TIF2 fusion [3]. Interestingly, FGFR2 has been suggested to be important for leukemic-regenerating cells (LRCs) that are induced by chemotherapy and responsible for disease relapse [4].

To test whether MCSFR or FGFR2 is important for LSC potential induced by *FLT3*-ITD, we crossed *FLT3*-ITD knock-in mice with *Mcsfr*$^{flox}$ and *Mxl-Cre, and Fgfr2*$^{flox}$ and *Mxl-Cre*, to generate ITD/ITD; *Mcsfr*$^{flox}$; *Mxl-Cre* (homozygous *FLT3*-ITD) and ITD/ITD; *Fgfr2*$^{flox}$; *Mxl-Cre* mice. ITD/ITD; *Mxl-Cre* mice were used as a control. To our surprise, the ITD/ITD; *Mcsfr*$^{flox}$; *Mxl-Cre*, ITD/ITD; *Fgfr2*$^{flox/o}$; *Mxl-Cre* and ITD/ITD; *Mxl-Cre* mice developed an early aggressive AML approximately 34 days after birth ($n = 16$, Table 1, Fig. 1C–E), whereas the median survival of the ITD/ITD mice was 431 days ($p < 0.0001$). The animals demonstrated a very high degree of leukocytosis (white blood cell (WBC): 477.9 ± 181.2/µl, $n = 14$ vs. control mice: 5.8 ± 1.4/µl, $n = 7$; Fig. 1D). Such high leukocytosis with WBC > 400,000/µl was not previously observed in any of our murine models including >200 animals with acute leukemia [5, 6]. Diseased mice had pronounced splenomegaly (705 ± 215 mg, $n = 15$ vs. control: 179 ± 21 mg, $n = 7$) and hepatomegaly was observed in the majority of diseased mice (1783 ± 550 mg, $n = 15$ vs control: 1262 ± 230 mg, $n = 7$). In contrast, *Kit* D814V mutation, murine homolog of *KIT* D816V, which is a very common mutation found in patients with systemic mastocytosis and in some AML patients, did not cooperate with Cre to induce early AML (Table 1).

Although ITD/o; *Mcsfr*$^{flox}$; *Mxl-Cre* and ITD/o; *Fgfr2*$^{flox}$; *Mxl-Cre* (ITD/o = heterozygous *FLT3*-ITD) mice did not develop early AML, a diagnosis of AML was made in all analyzed mice ($n = 12$) at the endpoint analysis (Fig. 1F, G). Moreover, these mice had much shorter survival than mice with ITD/o alone (279 vs. 783 days, $p < 0.0001$, Fig. 1H). A similar observation with shorter survival for mice carrying ITD/o and Cre was also made by others (personal communications by Florian H. Heidel) [7]. In another study, all mice ($n = 9$) transplanted with bone marrow (BM) cells from ITD/o; *Fgfr2*$^{flox/flox}$; *Mxl-Cre* or ITD/o; *Mxl-Cre* mice developed AML around 7 months after transplantation, while only chronic myelomonocytic leukemia (CMML) was observed in diseased mice ($n = 3$) transplanted with ITD/o BM cells approximately 14 months after transplantation (Supplementary Fig. 2). Importantly, the median survival of mice transplanted with ITD/ITD ($n = 3$), ITD/ITD; *p53*$^{+/-}$ [6], and ITD/ITD; *p53*$^{-/-}$ [6] BM cells was around 6, 5, and 5 months, respectively. Taken together, Cre recombinase promotes leukemogenesis of both homozygous and heterozygous *FLT3*-ITD.

At the molecular level, chromatin profiling revealed the existence of a poised enhancer in intron 15 of *Flt3* in *FLT3*-ITD/o mouse hematopoietic stem/progenitor cells but not in wildtype counterparts, which were marked by increased chromatin accessibility, enrichment of H3K4me1, and lower levels of H3K27ac (Fig. 1I). Cre expressions resulted in a fully active enhancer mapping at intron 15 of *Flt3* and increased the expression of *Flt3* gene (Supplementary Fig. 3A, B), suggesting that Cre-mediated recombination may facilitate chromatin activation. Recently, we described that the *FLT3*-ITD mutation alone can remodel the chromatin landscape to prime the development of full-blown leukemia in cooperation with other mutations [8]. A possible causative mechanism may involve Cre cleavage of genomic sites activated by *FLT3*-ITD. We focused on 3786 genomic regions that gained chromatin accessibility in the presence of *FLT3*-ITD [8], and then scanned for three loxP motif patterns to identify pseudo loxP sites [9] (Fig. 1J). Interestingly, we identified two pseudo-loxP sites mapping to the *FLT3*-ITD open chromatin region (Fig. 1K). Whether Cre enhances leukemogenesis of *FLT3*-ITD through these pseudo-loxP sites needs to be determined. In ongoing studies we wish to understand the underlying molecular mechanism for the development of AML by Cre and FLT3-ITD in more details.

In summary, our data not only confirm the cooperation between Cre and *FLT3*-ITD homozygous in the induction of AML

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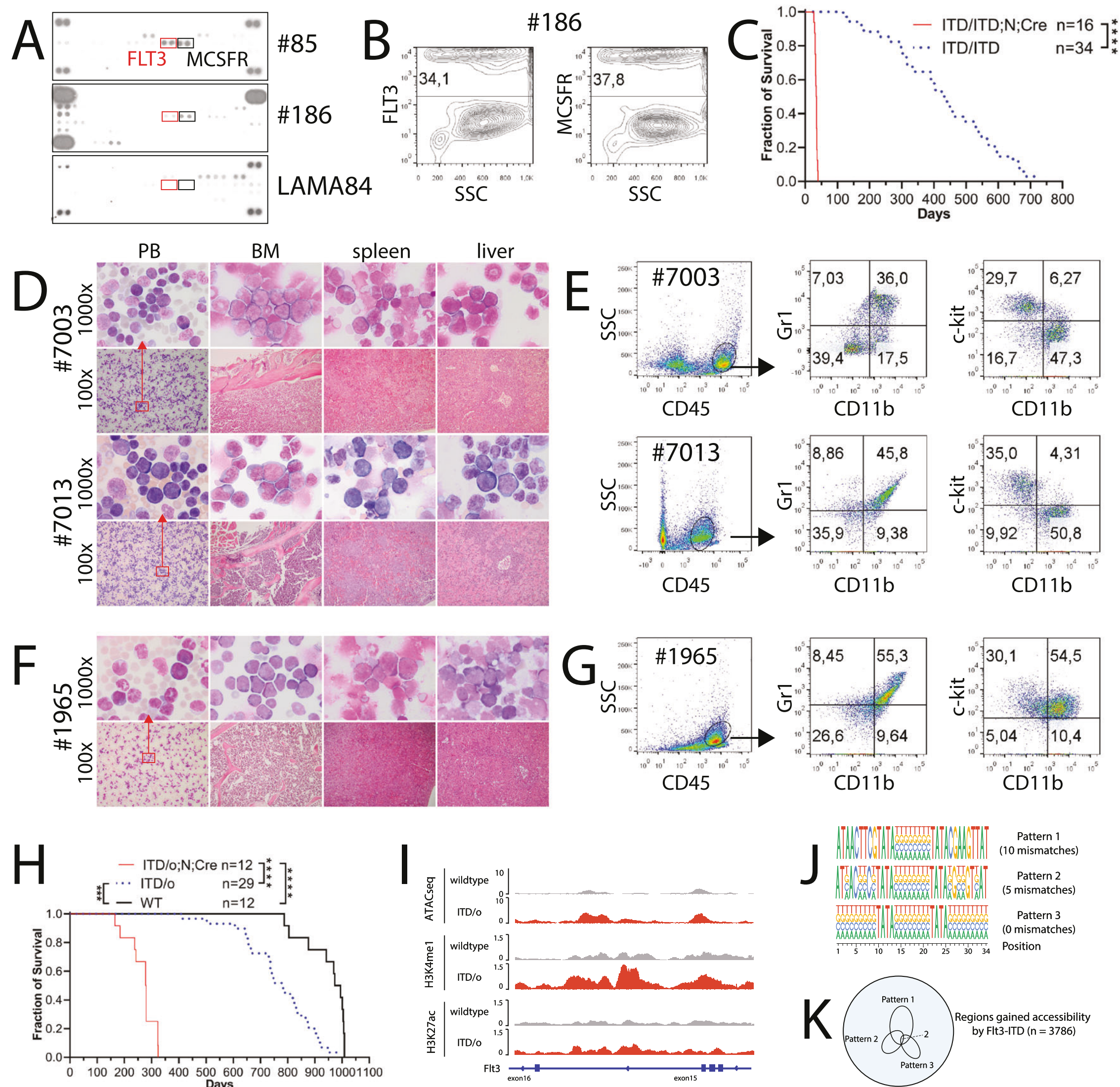


**Fig. 1 Cre recombinase promoted leukemogenesis in the presence of *FLT3*-ITD. A** Representative antibody arrays from two patients with AML (#85 and #186). Phosphorylation of FLT3 and MCSFR was observed in patients #85 (FLT3-TKD) and #186 (FLT3-ITD), but not in LAMA84 cells. LAMA84 cells were isolated from a patient with chronic myeloid leukemia in a blast crisis. We did not observe FLT3 or MCSFR phosphorylation in any of the healthy controls analyzed ($n = 8$). Hybridization signals at the corners (three or four) served as positive controls. MCSFR and FGFR2 belong to the most phosphorylated receptor in the AML specimens in our analysis. **B** Representative flow cytometry analyses confirming expression of FLT3 and MCSFR in patient #186. **C** Survival curves of ITD/ITD; *Mcsfr$^{flox}$*; *Mxl-Cre*, ITD/ITD; *Fgfr2$^{flox/o}$*; *Mxl-Cre*, ITD/ITD; *Mxl-Cre* (3 strains together ITD/ITD;N;*Cre*), and ITD/ITD mice. The animals were not treated with polyinosinic-polycytidylic acid (polyIC), as this was scheduled fo around 6 weeks after birth. No phenotypic differences were observed between TD/ITD; *Mcsfr$^{flox}$*; *Mxl-Cre*, ITD/ITD; *Fgfr2$^{flox/o}$*; *Mxl-Cre*, and ITD/ITD; *Mxl-Cre*, especially there was no additional acceleration or delay of the disease in TD/ITD; *Mcsfr$^{flox}$*; *Mxl-Cre*, and ITD/ITD; *Fgfr2$^{flox/o}$*; *Mxl-Cre* mice. ****$p < 0.0001$. **D** Representative Pappenheim-stained blood smears and cytospins of bone marrow (BM), spleen and liver, and hematoxylin and eosin (H&E)-stained histopathology of BM, spleen, and liver from #7003 (ITD/ITD; *Mcsfr$^{flox}$*; *Mxl-Cre*) and #7013 (ITD/ITD; *Mxl-Cre* mice) mice. Note infiltration of myeloblasts in these organs and in the lung (Supplementary Fig. 1A). **E** Flow cytometry analysis of spleen samples from mice #7003 and #7013 demonstrated a population of myeloblast/immature cells with lower side scatter (SSC) and CD45$^{dim}$ expression, which were positive for CD11b, c-Kit, and Gr1, but negative for CD3 and CD19 (Supplemental Fig. 1B). **F** Representative Pappenheim-stained blood smears and cytospins of bone marrow (BM), spleen and liver, and hematoxylin and eosin (H&E)-stained BM, spleen, and liver samples from #1965 (ITD/o; *Fgfr2$^{flox/flox}$*; *Mxl-Cre*). **G** Flow cytometry analysis of the spleen sample showing a population of myeloblast/immature cells with lower side scatter (SSC) and CD45$^{dim}$ expression in sample from mouse #1965. These blasts were positive for CD11b, c-Kit, and Gr1, but negative for CD3 and CD19 (data not shown). **H** Survival curves of ITD/o; *Mcsfr$^{flox}$*; *Mxl-Cre*, ITD/o; *Fgfr2$^{flox}$*; *Mxl-Cre* (2 strains together ITD/o;N;*Cre*), ITD/o, and wildtype (WT) mice. One mouse from ITD/o; *Fgfr2$^{flox}$*; *Mxl-Cre* was treated with polyIC. ***$p < 0.001$, ****$p < 0.0001$. **I** Illustration of chromatin profiling on accessibility (ATACseq) and modification states (ChIPseq on H3K4me1 and H3K27ac) in wildtype vs. ITD/o mouse HSPCs. An *FLT3*-ITD-associated enhancer was identified in the intron 15 of *Flt3* gene in ITD/o cells, marked by a high enrichment of H3K4me1, and modest levels of ATACseq and H3K27ac. Interesting, Straube et al. reported the presence of a neomycin resistance cassette (NRC) flanked by loxP sites in intron 15 of the *Flt3* gene in *FLT3*-ITD mice, and the excision of NRC in the presence of Cre [1]. **J** Visualization of motif logos representing pseudo loxP sites using three parameters. **K** Identification of chromatin regions containing pseudo loxP sites in the context of accessibility gained by *FLT3*-ITD.

**Table 1.** Development of early AML.

| Strains | Number of diseased mice (*n*) | Latency (days) |
|---|---|---|
| ITD/ITD; *Mcsfr*$^{flox}$; *Mxl-Cre*<br>ITD/ITD; *Fgfr2*$^{flox/o}$; *Mxl-Cre* | 12 | 34 |
| ITD/ITD; *Mxl-Cre* | 4 | 34 |
| *total* | 16 | 34 |
| *Kit* D814V$^{flox}$; *Mxl- Cre* [11] | 0 (out of 35 analyzed mice) | n.a. |

*Mcsfr*$^{flox}$ heterozygote and homozygote, *Fgfr2*$^{flox/o}$ heterozygote, *n.a.* not applicable.

described by Straube et al., but also provide the first evidence of enhanced leukemogenesis of *FLT3*-ITD heterozygous by Cre. *FLT3*-ITD knock-in mice have been used to identify cooperative partners, also in the presence of Cre [10]. Our data strongly support the findings of Straube et al. and indicate the need for a careful study design and interpretation of the data when using the Cre-loxP recombination system. In our hands, ITD/ITD in the presence of Cre activity does not allow to investigate the role of *MCSFR* or *FGFR2* in the pathogenesis of *FLT3*-ITD. Whether ITD/o in the presence of Cre is suitable for testing of cooperative events remains to be determined.

Min Yang[1,2,12], Zhiyuan Ma[1,12], Chonggang Wang[1,12], Muammer Cihan Agca[1], Hongyun Liu[1], Kezhi Huang[1], Silke Glage[3], Regina Rumpel[3], Alexander Gerbaulet[4], Axel Roers[5], Xuemei Liu[6], Fatih Noyan[7], Nils von Neuhoff[8], Arnold Ganser[1], Ligen Liu[9], Haiyang Yun[10]✉ and Zhixiong Li[1,2,11]✉
*[1]Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany. [2]Institute for Hematologic Malignancies, East Hospital, Tongji University School of Medicine, Shanghai, China. [3]Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Hannover, Germany. [4]Institute for Immunology, Medical Faculty Carl Gustav Carus, TU Dresden, Dresden, Germany. [5]Institute for Immunology, University Hospital Heidelberg, Heidelberg, Germany. [6]Department of Gastroenterology, Digestive Disease Hospital, Affiliated Hospital of Zunyi Medical University, Zunyi, China. [7]Department of Gastroenterology, Hepatology, Infectious Diseases and Endocrinology, Hannover Medical School, Hannover, Germany. [8]Department of Pediatric Hematology and Oncology, University Children's Hospital Essen, University of Duisburg-Essen, Essen, Germany. [9]Department of Hematology, Shanghai Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. [10]Robert Bosch Center for Tumor Diseases, Stuttgart, Germany. [11]Department of Hematology, East Hospital, Tongji University School of Medicine, Shanghai, China. [12]These authors contributed equally: Min Yang, Zhiyuan Ma, Chonggang Wang.*
*✉email: haiyang.yun@bosch-health-campus.com; li.zhixiong@mh-hannover.de*

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## AUTHOR CONTRIBUTIONS

MY performed experiments, collected, analyzed and interpreted data, and wrote the manuscript; ZM and CW performed experiments, collected, analyzed and interpreted data; MCA, HL, KH, SG, RR, AG, AR, XL, FN, MR, NvN, AG, LL performed experiments, interpreted data and provided support; HY performed analyses including ATACseq and ChIPseq, interpreted data, and wrote the paper; ZL conceived the concept, designed the studies, performed research, collected, analyzed and interpreted data, wrote the paper, and took responsibility in the construction of the whole manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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**Correspondence** and requests for materials should be addressed to Haiyang Yun or Zhixiong Li.

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