

## LYMPHOID NEOPLASIA

## Mutant JAK3 signaling is increased by loss of wild-type JAK3 or by acquisition of secondary JAK3 mutations in T-ALL

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## KEY POINTS

- One-third of T-ALL cases with JAK3 mutation harbor 2 JAK3 mutations.
- Double JAK3 mutants show stronger signaling than single JAK3 mutants.

**The Janus kinase 3 (JAK3) tyrosine kinase is mutated in 10% to 16% of T-cell acute lymphoblastic leukemia (T-ALL) cases. JAK3 mutants induce constitutive JAK/STAT signaling and cause leukemia when expressed in the bone marrow cells of mice. Surprisingly, we observed that one third of JAK3-mutant T-ALL cases harbor 2 JAK3 mutations, some of which are monoallelic and others that are biallelic. Our data suggest that wild-type JAK3 competes with mutant JAK3 (M511I) for binding to the common  $\gamma$  chain and thereby suppresses its oncogenic potential. We demonstrate that JAK3 (M511I) can increase its limited oncogenic potential through the acquisition of an additional mutation in the mutant JAK3 allele. These double JAK3 mutants show increased STAT5 activation and increased potential to transform primary mouse pro-T cells to interleukin-7-independent growth and**

**were not affected by wild-type JAK3 expression. These data extend our insight into the oncogenic properties of JAK3 mutations and provide an explanation of why progression of JAK3-mutant T-ALL cases can be associated with the accumulation of additional JAK3 mutations. (*Blood*. 2018;131(4):421-425)**

## Introduction

Janus kinase 3 (JAK3) is a nonreceptor tyrosine kinase that interacts with the common  $\gamma$  chain (IL2RG) of cytokine receptor complexes.<sup>1</sup> JAK1 kinase is also present within the cytokine receptor complex and is bound to the complementary  $\alpha$  or  $\beta$  chain receptor.<sup>1</sup> Sequencing studies have identified recurrent mutations in both JAK1 and JAK3 in several hematological malignancies.<sup>2-10</sup> We described the importance of JAK3 mutations in the development of T-cell acute lymphoblastic leukemia (T-ALL) and showed that a majority of JAK3 mutants require both binding to the common  $\gamma$  chain and presence of JAK1 for their transforming capacities.<sup>11-13</sup> However, the JAK3 L857Q/P and L875H kinase domain mutants are exceptions and do not require the common  $\gamma$  chain or JAK1 for their transforming capacity.<sup>11,12</sup> Between 10% and 16% of patients with T-ALL carry at least 1 mutation in JAK3.<sup>2,9,10,14-16</sup> A more detailed analysis presented here shows that up to a third of these patient cases harbor a second JAK3 mutation, and we investigated the biological significance of having multiple JAK3 mutations.

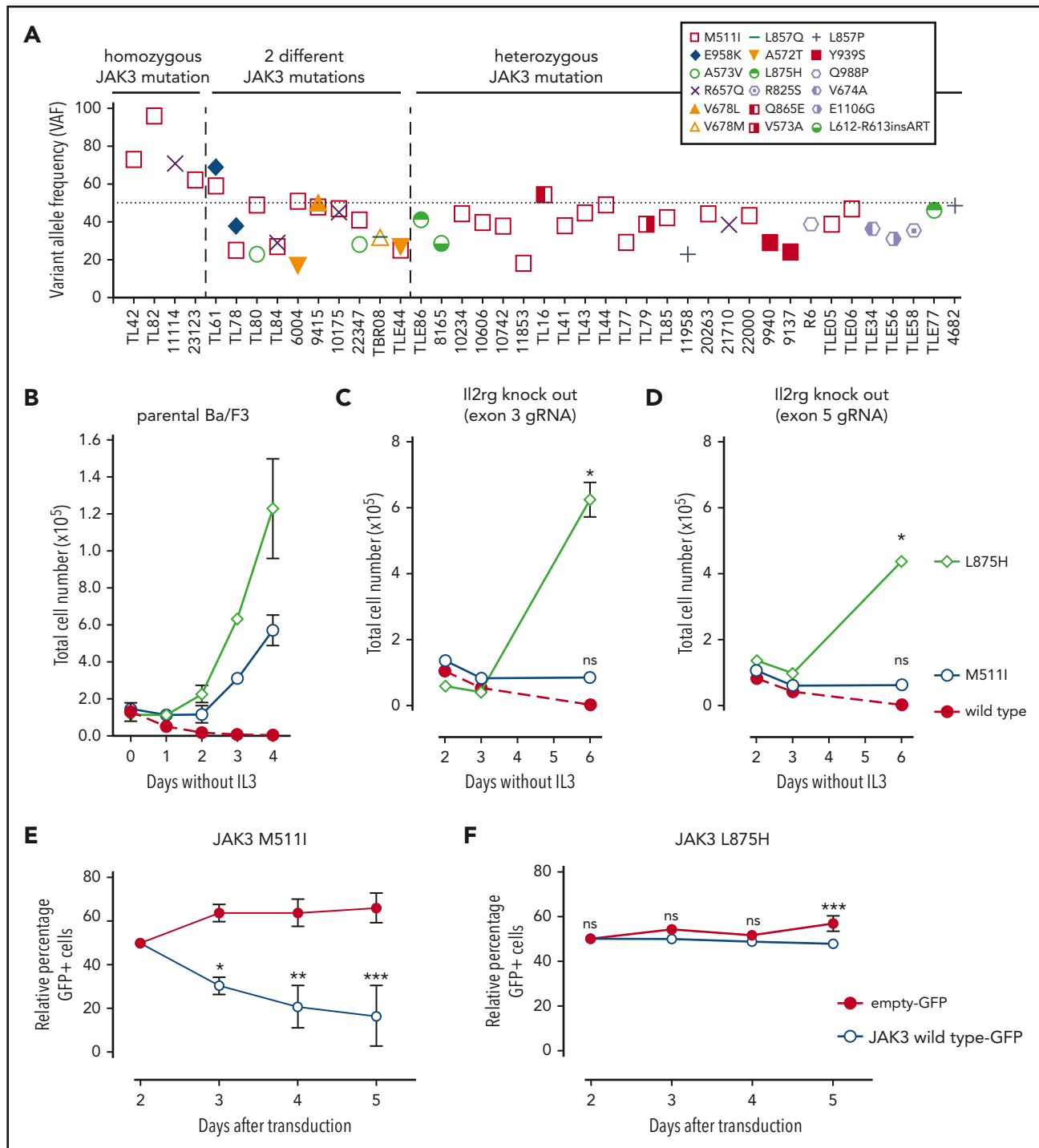
## Materials and methods

The JAK3 genomic sequence was amplified by polymerase chain reaction and subjected to PacBio long-read sequencing (Pacific Biosciences). 293T and Ba/F3 cells were cultured in

RPMI1640 medium supplemented with 10% fetal bovine serum. Ba/F3 cell medium was supplemented with interleukin-3 (IL-3; 10 ng/mL). Pro-T-cell cultures were established as described previously.<sup>17</sup> Retroviral vector production and transduction of Ba/F3 and pro-T cells were performed as described.<sup>11</sup> Cell growth was analyzed using a Guava easyCyte Flow Cytometer (Merck Millipore). For phospho flow, cells were fixed with Inside Fix and permeabilization buffer (Macs Miltenyi) and stained with an allophycocyanin-labeled antibody against phospho-STAT5 (eBioscience). Cells were analyzed using a FACSVerse cell analyzer (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star). This study was approved by the ethics committees of the institutes involved, and informed consent was obtained from the participants. Samples and clinical data were stored in accordance with the Declaration of Helsinki.

## Results and discussion

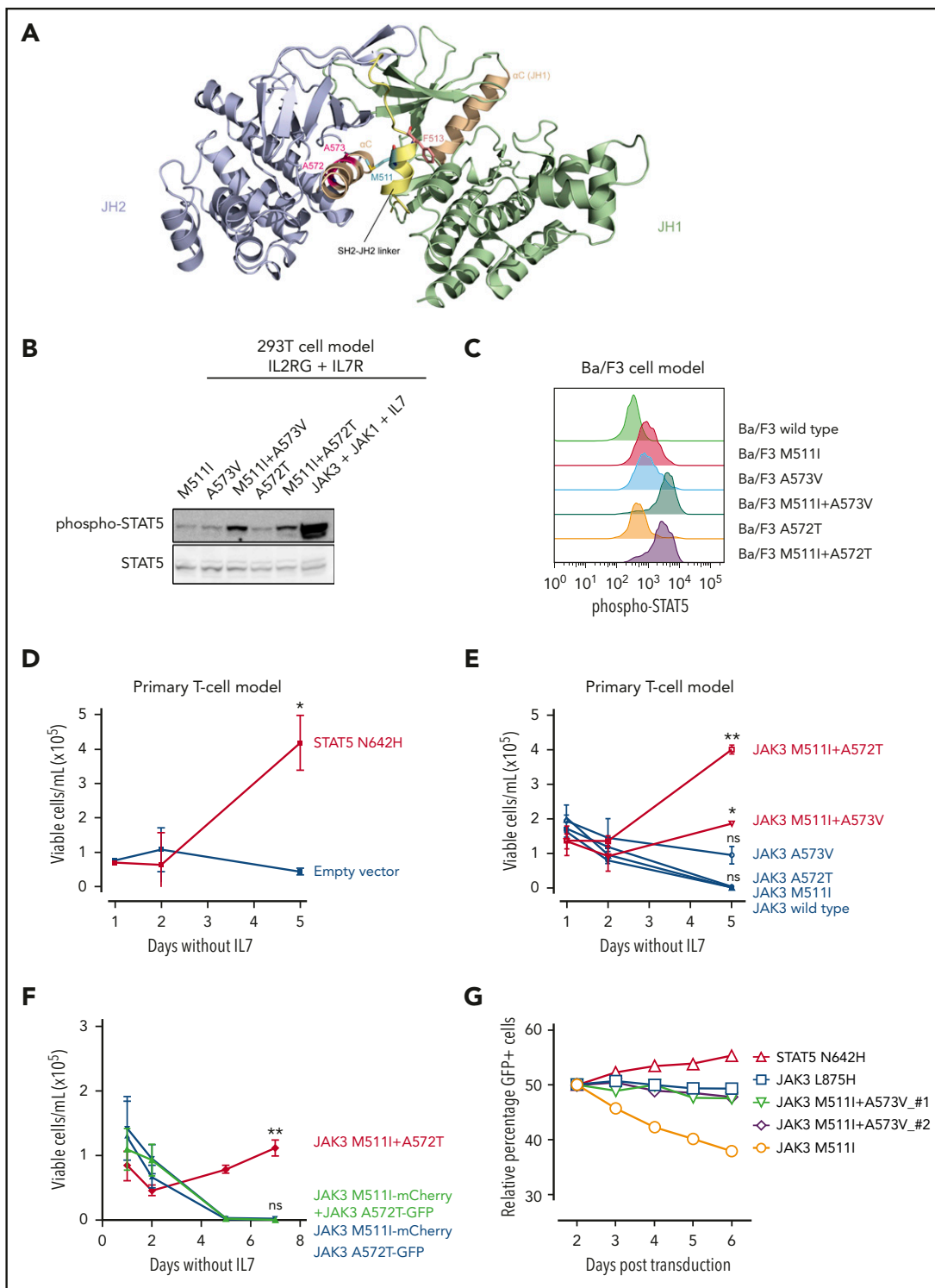
JAK3 (M511I) is the most frequent JAK3 mutation in T-ALL, but other mutations have also been described.<sup>2,9,10,14-16</sup> Detailed analysis of our next-generation sequencing data<sup>9,10,18</sup> revealed that 14 of 41 JAK3-mutant T-ALLs have either a homozygous JAK3 mutation or 2 different JAK3 mutations (Figure 1A). In a recently published exome sequencing study, 3 of 20 JAK3-mutant T-ALL patient cases were reported to harbor 2 JAK3 mutations.<sup>14</sup>



**Figure 1. JAK3 mutation data in T-ALL and investigation of JAK3 as a competitive factor for JAK3-mutant signaling.** (A) Graph shows variant allele frequency (VAF) of JAK3 mutations in patients with T-ALL; 34% of JAK3-mutant patient cases either have a second JAK3 mutation or a homozygous JAK3 mutation. (B-D) Proliferation curves of Ba/F3 cells expressing JAK3 (M511I), JAK3 (L875H), or JAK3 wild type in the absence of cytokines: parental Ba/F3 cells (B), Ba/F3 cells lacking common  $\gamma$  chain (guide RNA [gRNA] targeting exon 3 of Il2rg) (C), and Ba/F3 cells lacking common  $\gamma$  chain (gRNA targeting exon 5 of Il2rg) (D). Significance was calculated compared with wild-type control using the Kruskal-Wallis test and Dunn's multiple comparisons correction. (E-F) Graph showing relative percentage of Ba/F3 cells expressing wild-type JAK3 or empty vector in cells transformed by JAK3 M511I (E) or JAK3 L875Q (F). Competition between wild-type JAK3 and mutant JAK3 results in the disappearance of wild-type JAK3-expressing cells, which is observed only in panel E. Experiment was performed in biological triplicate. Standard error of the mean is shown. Significance was calculated using 1-way analysis of variance and the Bonferroni correction. \*\*\* $P \leq .001$ , \*\* $P \leq .01$ , \* $P \leq .05$ , not significant [ns]  $P \geq .05$ . GFP, green fluorescent protein.

We questioned why the acquisition of a homozygous mutation in JAK3 would be beneficial. JAK3 normally binds to the common  $\gamma$  chain of cytokine receptors,<sup>1</sup> and most JAK3 mutants require

binding to the common  $\gamma$  chain to activate downstream signaling.<sup>11,12</sup> In T-ALL cells with heterozygous JAK3 mutation, there could be competition between mutant and wild-type JAK3



**Figure 2. Double JAK3 mutants show enhanced signaling and transformation potential.** (A) Crystal structure of JAK3 JH1 (kinase domain; Protein Data Bank [PDB]: 1YVJ) and a model of JAK3 JH2 (pseudokinase domain) obtained by SWISS-MODEL server using JAK1 JH2 (PDB: 4L00) as a template. The 2 domains were assembled together by superimposition of the TYK2 JH1-JH2. (B) Western blot analysis of whole-cell lysates after reconstitution of the IL-7 receptor signaling complex in 293T cells. The 293T cells were transiently transfected with the constructs as indicated. (C) Phospho-flow analysis of phospho-STAT5 in Ba/F3 cells expressing single or double JAK3 mutants after 3 hours without IL-3 stimulation. Results are compared with Ba/F3 wild-type cells after 3 hours without IL-3 stimulation. (D-F) Growth of primary mouse pro-T cells in the absence of IL-7. (D) STAT5 (N642H) confers IL-7-independent growth. Significance was calculated compared with empty vector control using the Mann-Whitney test. (E) Analysis of single or double JAK3 mutants; only double mutants confer IL-7-independent growth to pro-T cells. Significance was calculated compared with wild-type control using the Kruskal-Wallis test and Dunn's multiple comparisons correction. (F) Comparison of proliferation of pro-T cells transduced with 2 JAK3-mutant constructs or 1 double JAK3-mutant construct; only the JAK3 double mutant confers IL-7-independent growth. Significance was calculated compared with JAK3 A572T using the Kruskal-Wallis test and Dunn's multiple comparisons correction. (G) Graph showing relative percentage of Ba/F3 cells expressing wild-type JAK3 (green fluorescent protein positive [GFP<sup>+</sup>]) in cells transformed by STAT5 (N642H), JAK3 (L875H), JAK3 (M511I+A573V), or JAK3 (M511I). Competition between wild-type JAK3 and mutant JAK3 results in the disappearance of wild-type JAK3-expressing cells, observed only in the case of JAK3 (M511I). \*\* $P \leq .01$ , \* $P \leq .05$ .

for binding to the same receptor. Biallelic mutation of JAK3, which is associated with loss of wild-type JAK3, would then be beneficial for the leukemia cells.

To verify if wild-type JAK3 can compete with mutant JAK3, we coexpressed wild-type JAK3 with JAK3 (M511I) or JAK3 (L875H) in Ba/F3 cells. These JAK3 mutants represent 2 types of JAK3 mutants: JAK3 (M511I) is dependent on binding to the common  $\gamma$  chain, whereas the JAK3 (L875H) mutant is not. Using CRISPR/Cas9 editing, we showed that in Ba/F3 cells, JAK3 (M511I) also requires binding to the common  $\gamma$  chain, whereas the JAK3 (L875H) mutant could easily transform in the absence of the common  $\gamma$  chain (Figure 1B-D). Next, we used wild-type Ba/F3 cells transformed by JAK3 (M511I) or JAK3 (L875H) to determine the effect of JAK3 coexpression. Ba/F3 cells express low levels of Jak3; therefore, expression of JAK3 from the same viral vector as the mutant JAK3 constructs would result in nearly equal expression levels of wild-type and mutant JAK3. Ba/F3 cells coexpressing wild-type JAK3 and JAK3 (M511I) had a significant disadvantage in cell growth compared with cells only expressing JAK3 (M511I; Figure 1E). In contrast, cells that coexpressed wild-type JAK3 and JAK3 (L875H) showed equivalent cell growth compared with cells only expressing JAK3 (L875H; Figure 1C). Taken together, these data indicate that JAK3 wild type acts as a suppressive factor for JAK3 mutants that are dependent on the receptor complex.

To determine whether T-ALL patient cases with 2 different JAK3 mutations had monoallelic or biallelic mutation of JAK3, PacBio long-read sequencing was performed on samples from individuals TLE44 (M511I+A572T) and 22347 (M511I+A573V; Figure 1A). We observed that 16% to 34% of the reads contained both mutations, respectively; 3% to 9% of the reads contained M511I only; and <1% of the reads contained the A572T or A573V mutation (on a total of 913 and 8182 high-quality reads; data not shown). These data indicate that the leukemia cells acquired the M511I mutation first and that the A572T or A573V mutation was acquired later on the same allele.

It has been shown that single M511I, A572T, or A573V mutations lead to activation of JAK3 kinase activity.<sup>5,11</sup> M511I is part of the SH2-pseudokinase (JH2) linker (Figure 2A). The pseudokinase domain (JH2) interacts with the kinase domain (JH1) active site through residues from the  $\alpha$ C and N-lobe of the pseudokinase domain. In JAK3, M511I could slightly destabilize the conformation of the SH2-JH2 linker, leading to the activation of the kinase domain. The 2 alanines (A572 and A573) are found in the  $\alpha$ C helix of the pseudokinase domain (Figure 2A), which was shown to be involved in the mechanism of activation of JAK2 V617F.<sup>19</sup>

This structural information suggests that a combination of M511I and A572T or A573V mutations might be additive and lead to a stronger activation of JAK3 than each mutation alone. We compared the signaling and transforming properties of the double mutants JAK3 (M511I-A572T) and JAK3 (M511I-A573V) compared with the single mutants. When these proteins were expressed in 293T cells (together with the IL-7 receptor proteins),<sup>11</sup> STAT5 phosphorylation was significantly stronger for double JAK3 mutants compared with single JAK3 mutants (Figure 2B). Ba/F3 cells expressing double mutant JAK3 constructs also showed stronger STAT5 phosphorylation compared with single JAK3 mutants as measured by phospho flow (Figure 2C). Despite these differences in STAT5 phosphorylation, growth of Ba/F3 cells was

not different between single or double JAK3 mutant-expressing cells (data not shown).

We next tested the capability of the JAK3 mutants to drive IL-7-independent growth of primary mouse pro-T cells.<sup>17</sup> We confirmed that constitutive JAK/STAT signaling was able to confer IL-7-independent growth by expressing the STAT5B (N642H) mutant (Figure 2D). Expression of the single JAK3 mutants (M511I, A572T, or A572V) was unable to confer IL-7-independent growth (Figure 2E-F), whereas the double JAK3 mutants (M511I-A572T and M511I-A572V) were able to drive IL-7-independent growth of the pro-T cells (Figure 2E-F). Strikingly, the double JAK3 mutant required Jak1 and was sensitive to a JAK1 selective inhibitor (ruxolitinib; supplemental Figure 1, available on the *Blood* Web site) but was not affected by wild-type JAK3 overexpression (Figure 2G). These data suggest an increased binding of the double JAK3 mutant to the receptor associated with increased signaling.

Together, these data illustrate that JAK3 M511I is a relatively weak kinase that can obtain increased signaling by acquisition of an additional mutation in the pseudokinase domain. Previous reports have described the cooccurrence of JAK1 and JAK3 mutations, JAK3 and IL-7 receptor mutations, and JAK1 and PTPRC deletions, all of which act to increase JAK/STAT signaling.<sup>19-22</sup> We demonstrate that acquisition of additional JAK3 mutations is another mechanism increasing JAK/STAT signaling during T-ALL progression.

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## Authorship

Contribution: S. Degryse, S.B., C.E.d.B., E.L., M.V.B., S. Demeyer, K.J., O.G., E.G., J.S., C.J.H., and S.N.C. performed experiments and analyzed data; S. Degryse, C.E.d.B., and J.C. wrote the manuscript; and J.C. supervised the study.

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## Footnotes

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