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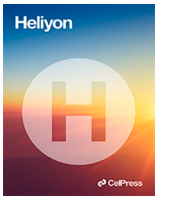

# In vitro and *in vivo* modelling of mutant JAK3/STAT5 signaling in leukemia

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ABSTRACT

Mutations within the IL7-R-JAK-STAT signaling pathway are important drivers of T-cell acute lymphoblastic leukemia (T-ALL). Here we describe the important steps required to generate retroviral particles for the stable expression of mutant JAK3 constructs that induce constitutive JAK/STAT signaling. These are subsequently used for the viral transduction of the IL-3 cytokine-dependent Ba/F3 cell line or murine hematopoietic stem and progenitor cells (HSPCs) for *in vitro* and *in vivo* modelling of cytokine-independent growth or leukemia initiation respectively.

## 1. Introduction

Mutations in the IL7-R-JAK-STAT signaling pathway are found in up to ~30 % of T-ALL patients [1,2]. Specifically, JAK3 mutations are found either within or adjacent to the pseudokinase domain (M511I, A572T, A573V, R657W, R657Q, V674A, V678 M) or directly within the kinase domain (L857Q, L875H, R925S, E1106G). The mutations cause constitutive activation of JAK3, with the concomitant phosphorylation of STAT5 and are able to drive T-ALL within an *in vivo* murine bone marrow transplant model (BMT) [3,4].

To investigate how mutant JAK3 proteins activate downstream STAT5, *in vitro* and *in vivo* model systems can be used. For *in vitro* studies, Ba/F3 cells are used to determine the ability of mutant JAK3 proteins to drive cytokine-independent growth. Ba/F3 cells, a pro-B lymphocyte cell line, depend on IL-3 for growth and proliferation and die within 24–36 h without this cytokine [5]. Ba/F3 cells are routinely used for *in vitro* assessment of gene transformative potential (i.e., the ability to drive cytokine-independent growth) because they are easily transduced with retrovirus and lentivirus [6–9]. For *in vivo* studies, the BMT model offers a rapid and cost-effective approach compared to transgenic mouse model generation to assess whether mutant JAK3 proteins can drive leukemia initiation.

In this lab protocol we demonstrate the essential steps required to achieve constitutively active expression of mutant JAK3 (M511I) in Ba/F3 cells and within an *in vivo* BMT model. We focused on JAK3 (M511I) pseudokinase domain mutation because it is the most common JAK3 mutation identified in T-ALL [3]. We cover HEK293T cell transfection and retrovirus production, Ba/F3 viral

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transduction, cytokine-independent growth and pSTAT5 staining using flow cytometry. For *in vivo* modelling, we outline isolation of lineage-depleted HSPCs, retroviral transduction, tail vein injection into sub-lethally irradiated mice and leukemia initiation and monitoring through peripheral blood analysis.

## 2. Materials and reagents

### 2.1. Retrovirus production

1. HEK293T cell line (ATCC® CRL-3216™).
2. RPMI 1640 medium (Gibco® #11875093): contains L-glutamine, phenol red and supplemented with 10 % v/v heat-inactivated fetal calf serum (FCS).
3. Trypsin 2.5 % (Gibco™ #15090046): no phenol red, diluted to a working solution of 0.25 % Trypsin and 0.02 % K3 Ethylenediaminetetraacetic acid (EDTA) in Dulbecco's Phosphate-Buffered Saline (DPBS).
4. Lipofectamine™ 2000 Transfection Reagent (ThermoFisher Scientific #11668019).
5. Opti-MEM™ Reduced Serum Medium (ThermoFisher Scientific #31985062): contains L-glutamine and phenol red.
6. EcoPac packaging vector: pCL-Eco (Addgene plasmid #12371) [10] (*see* **Note 1**).
7. MSCV-JAK3 (M511I)-IRES-GFP vector (Addgene plasmid #206290)
8. MSCV-JAK3 (wild type)-IRES-BFP (Addgene plasmid #206291)
9. 1.5 mL microcentrifuge tubes.
10. 0.45 μm low binding PES filters (Millipore Express® #SLHP033RS).
11. 10 mL syringe.
12. Sterile serological pipettes and pipette controller.
13. Polybrene (also known as hexadimethrine bromide (Sigma H9268), dissolved in nuclease free water to 8 mg/mL, and then filter-sterilized using 0.22 μm syringe filter.
14. Sterile filtered tips (Axygen® #TF-1000-R-S, TXLF-10-L-R-S, TF-200-R-S).
15. Pipettes.
16. 15 mL conical tubes (Corning® #430791).
17. RNAase- and DNAase-free 1.5 mL screw-cap tubes (SARSTEDT #72.692.405).
18. Biological safety cabinet.

### 2.2. Ba/F3 cell culture

1. Murine Ba/F3 cells (DSMZ # ACC300 or Creative-Biogene).
2. RPMI 1640 medium (Gibco® #11875093): contains L-Glutamine, phenol red, and supplemented with 10 % v/v FCS.
3. Recombinant murine Interleukin-3 (IL-3) (PEPROTECH® #213-13): reconstitute in 10 % FCS/RPMI for a 1000X stock solution of 1 μg/mL. Aliquot at 50 μL per tube and freeze at – 80 °C. For Ba/F3 culture, add IL-3 at 1:1000 dilution for a final concentration of 1 ng/mL (i.e., 1 μL per mL of culture media).
4. T25 flasks (Corning® #CLS430639).
5. Sterile serological pipettes and pipette controller.
6. Pipettes.
7. Sterile filtered tips (Axygen® #TXLF-10-L-R-S).

### 2.3. Ba/F3 retrovirus transduction

1. 6-well non-tissue culture treated plate (Nunc™ #150239).
2. Polybrene stock (8 mg/mL), as per *section 2.1*.
3. Filtered tips and pipettes.
4. 10 % FCS/RPMI, as per *section 2.2*.
5. Murine IL-3 (PEPROTECH® #213-13), as per *section 2.2*.
6. Swing bucket centrifuge with plate holder.
7. Trypan Blue 0.4 % for cell counting (Sigma-Aldrich #T8154-100 ML) (*see* **Note 2**).
8. Cell counter or hemocytometer.
9. Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco™ #14190144).
10. 5 mL polystyrene flow cytometry tubes (Falcon® #352008).
11. Flow cytometer with the required detection filters and lasers suitable for detection of fluorescent proteins (e.g., GFP).

### 2.4. Analysis of pSTAT5 downstream of JAK3 (M511I) using flow cytometry

1. DPBS (Room Temperature).
2. 16 % paraformaldehyde, electron microscopy (EM) grade.
3. Fume hood.

4. Staining buffer (2 % bovine serum in DPBS) (*see* **Note 3**).
5. Ice-cold 90 % methanol (Sigma-Aldrich #34860), stored for at least 2 h at – 20 °C, or overnight.
6. Alexa Fluor® 647 Mouse Anti-STAT5 (pY694), clone 47 (BD Phosflow™ #612599).
7. Alexa Fluor® 647 Mouse IgG1 κ Isotype control, clone MOPC-21 (BD Phosflow™ #557783).
8. Tofacitinib (CP-690550) Citrate (Selleckchem #S5001): dissolved in Dimethyl sulfoxide (DMSO), aliquoted and stored at – 80 °C at 100 mM stock solution.
9. Serological pipettes and pipette controller.
10. 15 mL conical tubes (Corning® #430791).
11. Tissue culture-treated 6-well plate (Costar® #3506).
12. 5 mL polystyrene flow cytometry tubes (Falcon® #352008).
13. FlowJo™ Software (version 10 onwards, Ashland, OR: Becton, Dickinson and Company; 2019).
14. Vortex mixer.

### 2.5. Bone marrow collection

1. BALB/c (or C57BL/6) mice. Donor mice (males) and syngeneic recipient mice (females) from 6 weeks to 10 weeks old (*see* **Note 4**).
2. Surgical scissors and forceps.
3. Mortar and pestle (autoclaved).
4. 50 mL conical tubes (Corning™ #430829).
5. 40 μm strainers (Greiner EASYSTRAINER #542040).
6. 0.45 μm low binding PES filters (Millipore Express® #SLHP033RS).
7. RPMI 1640 medium (Gibco™ #11875093): contains L-glutamine and phenol red, supplemented with 20 % FCS.
8. Cold DPBS ($mg^{2+}$ and $Ca^{2+}$ free, Gibco™ #14190144).
9. Swing bucket centrifuge.
10. Sterile transfer pipette.

### 2.6. Isolation of lineage-negative HSPCs and viral transduction

1. EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit (negative selection) (STEMCELL Technologies #19856).
2. EasySep™ Magnet (STEMCELL Technologies #18000).
3. DPBS ($Ca^{+}$ and $Mg^{+}$ free) (Gibco™ #14190144).
4. Sterile 0.5 mM EDTA, pH 8.0 (Life Technologies™ Ultrapure™ #15575-038).
5. Sterile polystyrene round bottom tubes 12 × 75 mm (5 mL) (e.g., Falcon® #352235).
6. 0.45 μm low binding PES filters (Millipore Express® #SLHP033RS).
7. Cell counter.
8. Trypan Blue 0.4 % for cell counting (Sigma-Aldrich #T8154-100 ML).
9. 15 mL conical tubes (Corning® #430791).
10. Murine Cytokines: IL-3 and IL-6 (PEPROTECH® #213-13 and #216-16, respectively). Recombinant Murine Stem Cell Factor (SCF) (PEPROTECH® #250-03): dilute in 10 % FCS/RPMI media to final concentration of 10 ng/μL (IL-3), 10 ng/μL (IL-6) and 50 ng/μL (SCF). Aliquot as 50 μL in screw cap tubes (SARSTEDT #72.692.405), and store at – 80 °C freezer) (*see* **Note 5**).
11. Penicillin/streptomycin (Gibco™ #15140-122).
12. Incubator (37 °C, 5 % $CO_2$).
13. 6-well non-tissue culture treated plate (Nunc™ #150239).
19. Polybrene 8 mg/mL (as previously described in *section 2.1*).
14. A flow cytometer with the required detection filters and lasers suitable for detection of fluorescent proteins (e.g., GFP).
15. 1 mL insulin syringes with 27G needles attached (Terumo® #51907).
16. Swing bucket centrifuge with plate holder.
17. Calibrated irradiator.

### 2.7. Mice monitoring and leukemia burden measurement

1. Scale (to weigh mice in grams).
2. 23G needles (BD precisionGlide™ needle #302008) for vein blood collection.
3. Capillary blood collection tube with EDTA (Microvette® #20.1341.100).
4. Automatic blood cell counter.
5. Flow cytometer with necessary lasers and filters.
6. Fluorochrome-conjugated antibodies.
7. Cold staining buffer (2 % bovine serum in DPBS).
8. 5 mL polystyrene flow cytometry tubes (Falcon® #352008).
9. Tips and pipettes.

10. Cell counter.
11. Trypan blue 0.4 % (Sigma-Aldrich #T8154-100 ML).
12. Red blood cells (RBCs) lysis buffer (eBioscience™ 10X RBC Lysis Buffer #00-4300-54): diluted 1:10 in Milli-Q water prior to use.
13. FlowJo™ Software (version 10.7.1 onwards, Ashland, OR: Becton, Dickinson and Company; 2019).

## 3. Method

### 3.1. Production of retrovirus encoding JAK3 (M511I) in HEK293T cells

Note: The following procedure is optimized for the transfection of 1 construct per T75 cell culture flask.

1. Maintain HEK293T cells in RPMI medium with 10 % FCS in T75 flask.
2. Day 1 (morning):
   1. If HEK293T are confluent, trypsinize the cells by adding 2 mL of 1X trypsin solution per T75 flask and incubate for 2 min at 37 °C. Add 2 mL (equal volume) of media to neutralize the trypsin, collect cell suspension and centrifuge at 250×*g*, and discard the supernatant (see **Note 6**).
   2. Resuspend the cells in media to achieve 70–80 % confluency by the next day (24 h), by seeding ~ 140,000 cells/cm$^2$ in 15–20 mL of 10 % FCS/RPMI.
2. Day 2 (afternoon):
   1. Upon 70–80 % HEK293T cell confluency, replace the existing media with 12 mL of 10 % FCS/RPMI by holding the flask vertically bending it slightly either to the left or the right, then pipette out the media from the corner of the flask (*see* **Note 7**).
   2. For each flask, prepare the transfection solution according to Table 1 by mixing the required volume of Opti-MEM™ media with the transfection reagent in a 1.5 mL microcentrifuge tube. Invert the tube 5 times and incubate for 5 min at RT (*see* **Note 8**).
   3. In a separate 1.5 mL microcentrifuge tube, mix 10 μg of the DNA construct e.g., MSCV-JAK3 (M511I)-IRES-GFP [3,4] with 10 μg of EcoPac (pCL-Eco).
   4. Mix the content of both 1.5 mL microcentrifuge tubes (EcoPac/construct mix and the transfection solution) and incubate for 20 min at RT.
   5. Using the 1000 μL filtered tip and pipette, and while the flask is sitting horizontally inside the biological cabinet, gently insert the pipette inside the flask and distribute the transfection mixture on top of the HEK293T cells dropwise across the whole flask.
   6. Return the flask to the incubator. Incubate at 37 °C, 5 % $CO_2$ for 16 h.
3. **Day 3 (morning):** Carefully replace the existing media with 5–8 mL of 10 % FCS/RPMI, as described previously in *Day 2, step 1* to avoid dislodging the cells. Make sure that the media entirely covers the cells. Incubate the cells at 37 °C, 5 % $CO_2$ for a further 24 h.
4. **Day 4 (afternoon)**:
   1. Collect the virus-containing supernatant by holding the flask vertically tilted slightly either to the left or right using a serological pipette and immediately place in a 15 mL conical tube.
   2. Spin down at 250×*g*, for 5 min at RT to remove any floating HEK293T cells.
   3. Filter the supernatant through a 0.45 μm PES filter using the 10 mL syringe into a 15 mL conical tube.
   4. Aliquot the virus into 1.5 mL screw-cap tubes (1 mL/tube) and freeze the virus aliquots at – 80 °C (*see* **Note 9**).

### 3.2. Modelling of JAK3 (M511I) mutation in vitro

#### 3.2.1. Retrovirus transduction on Ba/F3 cells

1. Pre-warm a centrifuge with a swing out rotor capable of holding a 6-well cell culture plate to 30 °C for spinfection (*see* **Note 10**).
2. Ensure Ba/F3 cells are healthy and growing in exponential phase in 10 % FCS/RPMI supplemented with 1 ng/mL IL-3. *Warning*: cells will not survive in the absence of IL-3!
3. Count Ba/F3 cells using 0.4 % trypan blue and place 1 × 10$^6$ cells in a 15 mL conical tube.
4. Centrifuge the cells at 250×*g* for 5 min at RT. Discard the supernatant and resuspend the cells gently in 1 mL 10 % FCS/RPMI.
5. Place 1 mL of cells into a 6-well non-tissue culture treated plate.

**Table 1**
Transfection reagent ratios per T75 flask

| Transfection reagent | Lipofectamine™ 2000 | Genejuice® |
|---|---|---|
| **Supplier/catalogue #** | ThermoFisher Scientific # 11668019 | Sigma-Aldrich #70967 |
| **Opti-MEM™ (Reduced serum medium)** | 1000 μL | 600 μL |
| **Transfection reagent volume** | 47 μL | 36 μL |
| **Mutant JAK3 construct e.g., MSCV-JAK3(M511I)-IRES-GFP** [3,4] | 10 μg | 10 μg |
| **pCL-Eco (plasmid)** | 10 μg | 10 μg |

6. Thaw 1 mL of virus aliquot (from *section 3.1*) on ice.
7. Add 2 μL of 8 mg/mL polybrene to the virus-containing supernatant. For each milliliter of final volume, add 1 μL (total volume = 2 μL/well) for a final concentration of 8 μg/mL) (*see* **Note 11**).
8. Add 1 mL virus/polybrene mix to the 1 mL Ba/F3 cells in the plate in *Step 5*.
9. Add 2 μL of the 1000X murine IL-3 cytokine to the 2 mL cell/virus mix (final concentration = 1 ng/mL).
10. Place 2 mL of cells/viral mix into a single well of the 6-well non-tissue culture treated plate (*see* **Note 12**).
11. Centrifuge the 6-well plate at 2500 rpm for 90 min at 30 °C (*see* **Note 13**).
12. Incubate at 37 °C, 5 % $CO_2$ for 16–24 h.
13. After incubation, collect 500 μL of the cell suspension directly from the plate into a 5 mL polystyrene tube and wash cells twice with 4 mL of DPBS, centrifuge at 250×*g* for 5 min and discard supernatant.
14. After the final wash, resuspend the cells in 300 μL of DPBS.
15. Run the sample on a flow cytometer with blue laser (488 nm) settings, filter 530/30, to determine the level of GFP fluorescence. A successful transduction and virus production should lead to >50 % GFP + cells (*see* Fig. 1A).
16. For the remaining JAK3 (M511I) transduced Ba/F3 cells, collect in 15 mL conical tube, spin down at 250×*g* and discard supernatant. Resuspend the cells in 5 mL 10 % FCS/RPMI and culture in T25 flask without the addition of IL-3 cytokine.

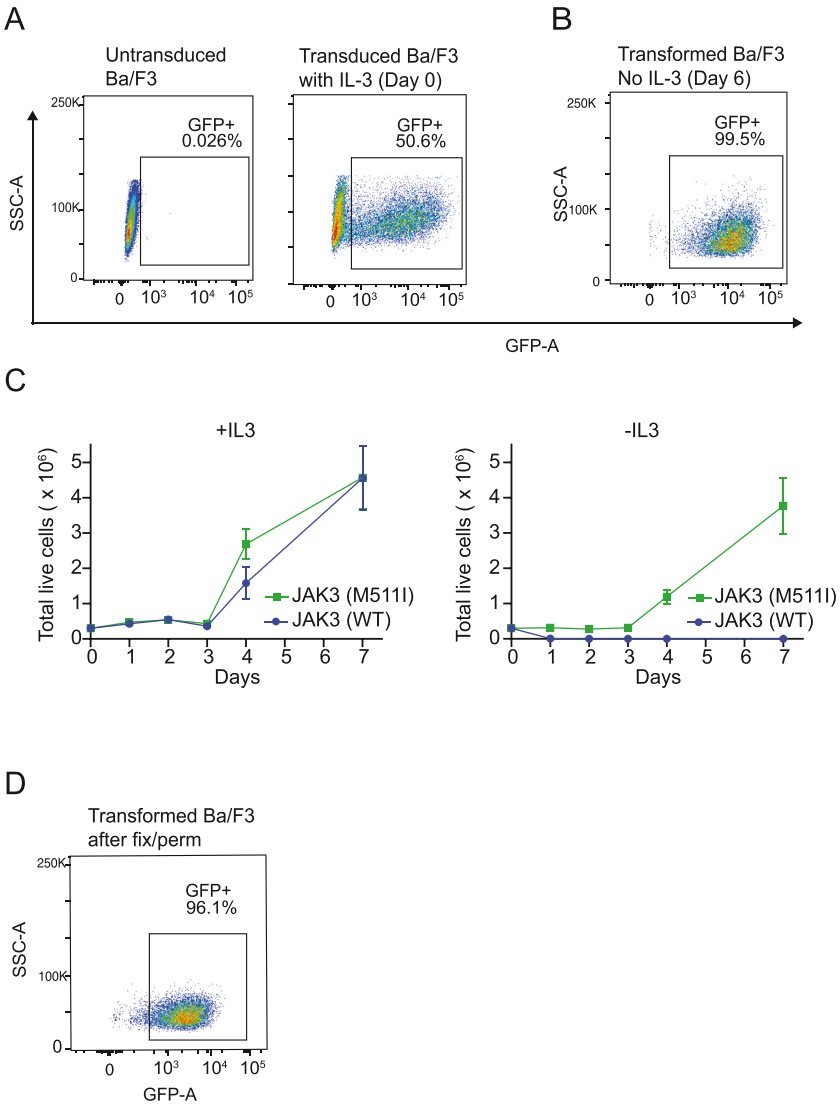


**Fig. 1. Ba/F3 cell transformation with JAK3 (M511I) retroviral particles to cytokine-independent cell line detected using flow cytometry.** (A) Transduction efficiency of Ba/F3 cells after incubation with JAK3 (M511I) retrovirus particles for 16–24 h in the presence of IL-3 in 10 % FCS/RPMI (Day 0). (B) Ba/F3 cells analyzed at day 6 after transformation to IL-3-independent cells. (C) Growth kinetics for JAK3 (M511I) and JAK3 Wildtype (WT) transduced Ba/F3 cells in the presence and absence of IL-3 cytokine. (D) GFP fluorescence in transformed Ba/F3 cells after fixation with ~1.5 % paraformaldehyde and permeabilization with 90 % methanol.

17. After 3–4 days, the Ba/F3 cells will be ~100 % GFP + through cytokine-independent growth due to the expression of the JAK3 (M511I). Check the %GFP + cells using flow cytometry (*see* Fig. 1B). Only activating JAK3 mutations and not JAK3 wild type (WT) will result in cytokine-independent growth (*see* Fig. 1C). Similarly different JAK3 mutations (or other activated mutant kinases) can have different growth kinetics to drive cytokine-independent growth [3]. It is also possible to test cytokine-independent growth through direct competition of different constructs within the same well (*see* Fig. 2) making this cell model system an excellent way to assess whether kinase mutations are activating.

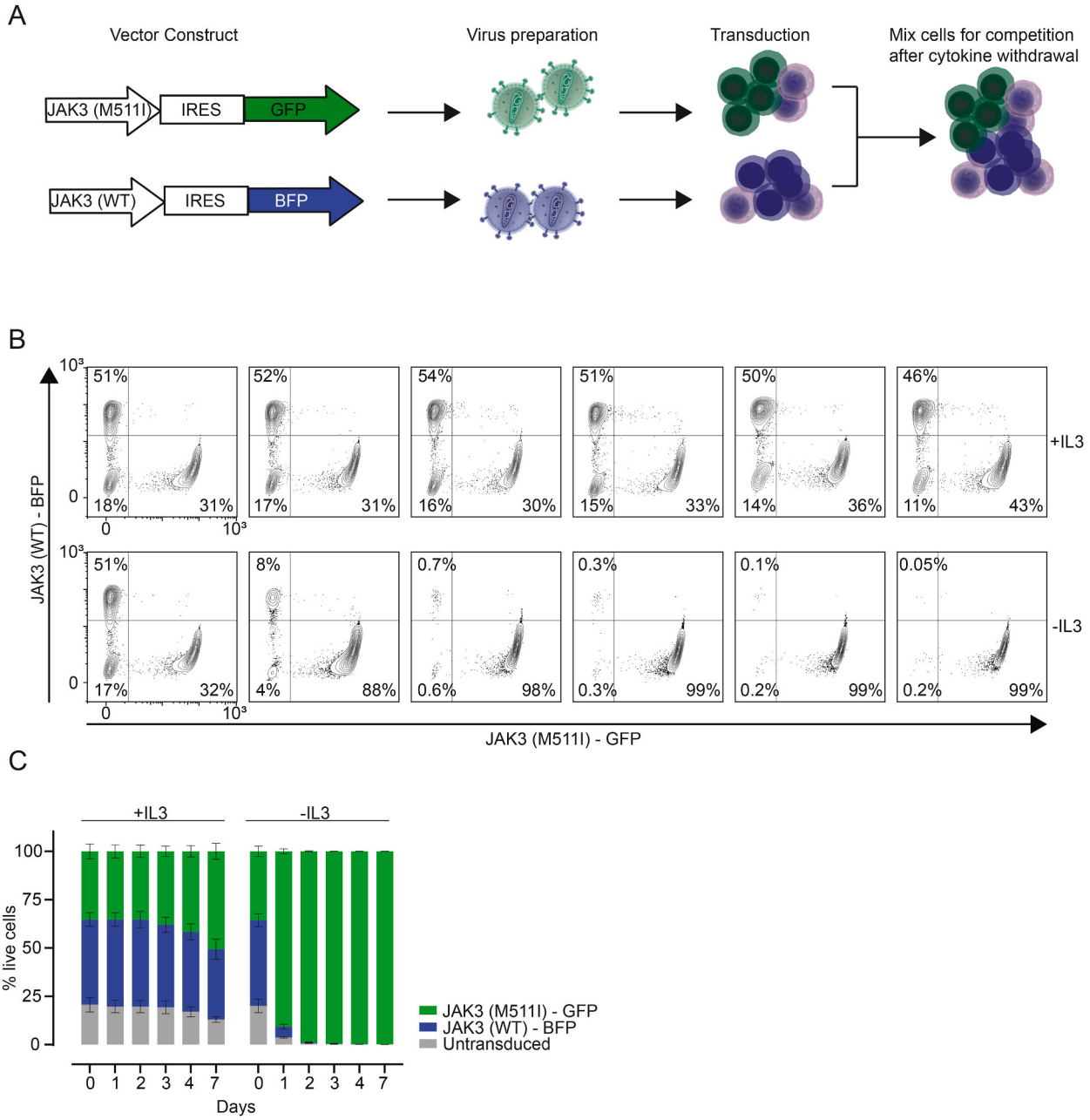


**Fig. 2. Competitive growth assay to assess cytokine-independent growth**. (A) Schematic outline illustrating the generation of transduced Ba/F3 cells that are then mixed together for subsequent IL-3 withdrawal and assessment for growth kinetics. (B) Representative flow cytometry analysis of Ba/F3 cells showing proportion of JAK3 (M511I) cells marked by GFP expression, JAK3 wildtype (WT) cells marked by BFP expression over time after IL-3 withdrawal. (C) Quantification of the separate cell populations over time in the presence or absence of IL-3.

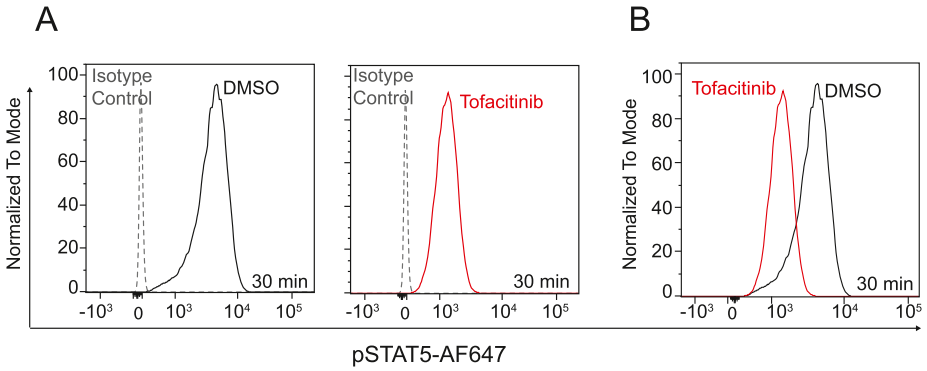


**Fig. 3. Staining of pSTAT5 downstream of constitutively active JAK3(M511I) detected by flow cytometry**. (A) pSTAT5 staining in tofacitinib- or DMSO- treated cells *vs.* isotype controls showing specific antibody binding. (B) Overlay of pSTAT5 fluorescence in DMSO- *vs.* tofacitinib-treated cells showing reduction of STAT5 phosphorylation after 30 min incubation with JAK3 selective inhibitor, tofacitinib.

**Table 2**
Transplant media preparation

| Item | Stock concentration | Supplier/Cat# | Final Concentration | Top up media |
|---|---|---|---|---|
| Murine IL-3 | (10 ng/μL) | PEPROTECH® #213-13 | 10 ng/mL | 50 mL of 20 % FCS/RPMI |
| Murine IL-6 | (10 ng/μL) | PEPROTECH® #216-16 | 10 ng/mL | |
| Murine SCF | (50 ng/μL) | PEPROTECH® #250-03 | 50 ng/mL | |
| Penicillin/Streptomycin | 10000 unit/mL Penicillin 10000 μg/mL strep | Gibco #15140-122 | 100 unit/mL (500 μL) | |

**Table 3**
Cocktail staining of GFP + cells for myeloid, B- and T-cells markers

| Antibody | Fluorochrome | Clone | Recommended[a] dilution |
|---|---|---|---|
| CD11b | PE | M1/70 | 1:500 |
| CD8 | APC ef780(Cy7) | 53–6.7 | 1:100 |
| CD4 | PerCP | RM4-5 | 1:500 |
| Gr1 | APC | RB6-8C5 | 1:500–1:1000 |
| CD19 | PE-Cy7 | ID3 | 1:250 |

[a] Use the recommended dilution as a guide only, all antibodies should be titrated to ensure high signal: noise ratio.

### *3.2.2. Analysis of pSTAT5 downstream of JAK3 (M511I) in transformed Ba/F3 cells*

1. Maintain the cytokine-independent JAK3 (M511I) transduced Ba/F3 cells (described in *section 3.2.1)* in 10 % FCS/RPMI only. After confirming ~100 % GFP+, collect 1 × $10^6$ cells/sample in a 15 mL conical tube.
2. Centrifuge the cells at 250×*g*, for 5 min at RT and discard supernatant.
3. In a 6-well tissue-culture treated plate, resuspend the cells in 1 mL 10 % FCS/RPMI with 1–1.5 μM tofacitinib (JAK3 selective inhibitor). Similarly, add 1 × $10^6$ cells/mL to a second well (vehicle control) (i.e., add equal volume of DMSO).
4. Incubate at 37 °C, 5 % $CO_2$ for 30 min.

#### *3.2.2.1. Fixation.*

1. Collect the cells from the 6-well plate into 15 mL conical tubes. Centrifuge the cells at 250×*g*, for 5 min at RT and discard supernatant. Resuspend the cells in 1 mL of RT DPBS.

2. In a fume hood, add 100 μL of 16 % paraformaldehyde for each 1 mL of cell suspension and mix gently (final concentration = 1.5 %).

3. Allow the cells to be fixed for 10 min at RT.

4. Wash the cells with 5 mL RT DPBS, and centrifuge at 250×*g* for 5 min at RT. Carefully remove and discard all supernatant using a pipette.

*3.2.2.2. Permeabilization.*

1. Slowly resuspend the pellet in 1 mL of ice-cold 90 % methanol, while gently vortexing.

2. Store overnight (or at least for 2 h) at – 20 °C (*see* **Note 14**).

*3.2.2.3. pSTAT5 Immunostaining.*

1. When ready to stain, wash the cells with 10 mL staining buffer by spinning the cells at 250×*g* for 5 min, then remove the supernatant.

2. Resuspend the cells in 50 μL/tube staining buffer containing a fluorochrome-conjugated anti-mouse pSTAT5 antibody, e.g., Alexa flour® 647 at 1:5, or the isotype control for both DMSO- and tofacitinib-treated cells (*see* **Note 15**).

3. Incubate the cells for 1 h at RT in the dark.

4. Wash the cells twice with 1 mL staining buffer, centrifuge at 250×*g* for 5 min, and resuspend in 150 μL of staining buffer.

5. Run the samples on a flow cytometer within 30 min of staining, using the red laser (640 nm) settings, filter 670/14, to determine pSTAT5-AF647 fluorescence. GFP fluorescence can also be detected after fix/perm using the settings in *section 3.2.1* (*see* Fig. 1D).

6. Analyse the pSTAT5 status in tofacitinib- or DMSO- treated cells *vs.* isotype controls, to confirm anti-pSTAT5 antibody specificity (*see* Fig. 3A). pSTAT5 staining in DMSO- *vs.* tofacitinib-treated cells can be overlayed (*see* Fig. 3B). Flow cytometry results were analyzed using Flowjo software version 10.7.

### *3.3. Modelling of JAK3 (M511I) mutation in vivo*

#### ***3.3.1. Day 1:** isolation of bone marrow (BM) lineage-negative cells*

1. Sacrifice donor mice according to your approved ethics protocol (e.g., $CO_2$ asphyxiation followed by cervical dislocation).
2. Dissect out both hind legs to isolate femur and tibia. Remove as much muscle as possible and place the bone in DPBS (*see* **Note 16**).
3. In a biosafety cabinet, isolate the BM cells by placing muscle-free leg bones in an autoclaved mortar and pestle. Add 500 μL of 20 % FCS/RPMI media and slowly crush the bones.
4. Using a P1000 pipette or transfer pipette, collect the cell suspension and filter it through a 40 μm strainer placed in a 50 mL conical tube. Add another 500 μL of media to the bones and crush again, collect and filter through the strainer. Repeat until bone fragments are "white" in color.
5. Collect the filtered BM cells by centrifugation (250×*g*, 5 min, 4 °C).
6. As per manufacturer's instructions for the EasySep™ mouse hematopoietic progenitor isolation kit (negative selection), prepare the recommended media (2 % FCS, 1 mM EDTA, and DPBS) (*see* **Note 17**).
7. After centrifugation, tip off the supernatant and resuspend the cells with 5 mL of recommended media.
8. Count the cells using trypan blue and a cell counter or manually using a hemocytometer.
9. Adjust the cell concentration to $1 \times 10^8$ cells/mL in the recommended media.
10. Isolate the BM lineage-negative cells as per manufacturer's instruction for the EasySep™ mouse hematopoietic progenitor isolation kit.
11. Prepare 50 mL of transplant medium as described in Table 2.
12. After lineage depletion, resuspend lineage-negative cells in the transplant medium at a concentration of 0.75–1 x $10^6$ cells/mL (*see* **Note 18**).
13. Place 2 mL of cell suspension into each well of a 6-well non-tissue culture treated plate, starting at the center of the well then distribute evenly across the well by gently swirling the plate with the lid on. Ideally you will have ~12 million cells across 6 wells.
14. Incubate cells at 37 °C, 5 % $CO_2$ in the cell culture incubator overnight (~16 h).

#### ***3.3.2. Day 2:** viral transduction of lineage-negative cells*

1. Pre-warm a centrifuge with a swing-out rotor capable of holding a 6-well cell culture plate to 30 °C for spinfection (*see* **Note 10**).

2. Thaw the 6 mL of JAK3 (M511I) retrovirus vials on ice (1 mL/vial).
3. Collect the cells from each well of the 6-well plate and pool together in a 15 mL conical tube, centrifuge at 250×*g* for 5 min at RT, then discard supernatant.
4. Resuspend the cell pellet in 6 mL stored transplant medium as described above, then count the cells.
5. Adjust the cell concentration to $5 \times 10^5$ cells/mL.
6. Seed the cells in a 6-well non-tissue culture treated plate at 2 mL per well starting from the middle of the well then distribute across the well by swirling the plate (lid on).
7. Add 18 μL of polybrene (stock concentration = 8 mg/mL) to 6 mL of JAK3 (M511I) retrovirus. For each milliliter of final volume, add 1 μL (total volume = 3 μL/well, for a final concentration of 8 μg/mL) (*see* **Note 11**).
8. Add 1 mL virus/polybrene mixture to the cells in each well, and spinfect by centrifuging the plate at 2500 rpm, at 30 °C for 90 min (*see* **Note 13**).
9. Return the plate to the incubator and incubate at 37 °C, 5 % $CO_2$ for a further 4–6 h.
10. Replace media by collecting all cells from the different wells into a 15 mL conical tube and centrifuge at 250×*g* for 5 min at RT, discard supernatant and resuspend the cells in transplant medium (12 mL for $1 \times 10^6$ cells/mL). Return the cells to the same 6-well non-tissue culture treated plate at 2 mL per well.
11. Incubate overnight at 37 °C, 5 % $CO_2$.
12. Irradiate recipient mice; mice total body irradiation with sub-lethal irradiation of 5 Gy. (*see* **Note 19**)

#### *3.3.3. **Day 3:** injection of transduced cells*

1. Collect and pool all the cells into a 15 mL tube through centrifugation at 250×*g* for 5 min at RT. Wash the cells 2-3 X using sterile DPBS. Discard supernatant.
2. Resuspend the cells after the final wash to 10 mL DPBS. Count the cells using trypan blue to determine the total number of isolated lineage-negative cells.
3. Centrifuge the cells and resuspend in DPBS adjusting the concentration to $5 \times 10^6$ cells/mL for $1 \times 10^6$ cells/200 μL/mouse (*see* **Note 20**).
4. Any excess cells over the 6 million cells required for 6 mice injections, should be used to measure the transduction efficiency of the cells (%GFP+) using flow cytometry as described in *section 3.2.1*. This can be achieved by running 150–200 μL of cell suspension in a 5 mL polystyrene tube on the flow cytometer. This will confirm cell transduction and should ideally range between 30 and 50 % GFP positivity. (*see* **Note 21**).
5. Optional: sort cells on a flow sorter (FACS) to increase %GFP + cells for injection if necessary.
6. Tail vein injection:

I. Prep the 27G insulin syringes by pulling the plunger in and out 2-3X.
II. Make sure the cell suspension is equilibrated to RT to avoid mice hypothermia.
III. Warm the recipient mice using a heat lamp (30 cm distance from mice) to increase vasodilation of the tail vein (*see* **Note 22**)
IV. Fill syringes with 200 μL cell suspension and remove any air bubbles (*see* **Note 23**).
V. Inject cell suspension in tail vein of the irradiated recipient mice (*see* **Note 24**).

#### *3.3.4. BMT: monitoring leukemia burden*

1. **Week 1:** collect a blood sample via vein puncture using a 23G needle in an EDTA treated tube [11]. Typically, 30–50 μL is sufficient for downstream analysis (*see* **Note 25**).
2. Measure white blood cell (WBC) count by automatic blood cell counter (typically this will require 8–20 μL depending on the cell counter) (*see* **Note 26** and **27**).
3. Bleeding and monitoring the mice weights should occur on a weekly/fortnightly basis.
4. %GFP + cells will increase and result in a $CD8^+$ T-ALL *in vivo* with a mean latency of approximately 150 days.
5. Upon end stage (when mice become moribund or ethical end data determined a priori such as WBC >30,000 per uL), leukemic blast cells can be harvested from primary and secondary lymphoid organs.
6. Single cell suspension can be treated *ex vivo* with 0.5–1 μM tofacitinib for 30–90 min and pSTAT5 phospho-flow cytometry undertaken as per *section 3.2.2* above and as illustrated in de Bock et al. [4], or lysed and processed for Western blot analysis as illustrated by Degryse et al. [3].

##### *3.3.4.1. Quick blood preparation guide for monitoring transduced JAK3 (M511I) cells using flow cytometry.*

1. After blood collection into an EDTA tube (20–50 μL), lyse the RBCs using 300 μL 1X lysis buffer in a 5 mL polystyrene tube (*see* **Note 28**).

2. Leave at RT for 4–6 min until the solution turns clear red (*see* **Note 29**).

3. Add 3 mL of staining buffer, and spin at 250×*g* for 5 min.

4. Discard the supernatant and wash again with staining buffer (2 washes total). If the RBC lysis was inefficient, repeat steps 1–3 (*see* **Note 30**).

5. After centrifugation, discard the supernatant and resuspend the cells in 150–200 μL staining buffer and run on a flow cytometer (*see* **Note 31**).

6. Determine the %GFP in the peripheral blood.

7. Once WBC count >20000–25000/μL and GFP + cells are expanding, staining with T-ALL specific markers can begin (Table 3). Stain by incubating the cells at step #5 with 50 μL/sample of antibody cocktail in antibody staining buffer, incubate for 30 min at 4 °C, then wash twice and run on the flow cytometer.

## 4. Notes

1. Platinum-E (Plat-E) cell line can be alternatively used instead of the EcoPac for retrovirus production.
2. Important to filter the solution using a 0.45 μm syringe filter to remove precipitates that can hinder cell counting.
3. Bovine serum albumin or FCS can be used as an alternative to the bovine serum. Prepare fresh, filter with a 0.22 μm filter to remove precipitates and store at 4 °C. Use within 2 days to avoid bacterial growth.
4. Usually one donor mouse for each recipient mouse. However, if you take all the bones of the donor mouse (front + hind legs) this can be reduced to 1 donor mouse per 2 recipient mice. We prefer to use male donors and female recipients to minimize aggressive behavior between male mice. It also allows for the distinction between donor derived and recipient derived blood cells based on sex chromosomes.
5. Do not vortex after reconstitution and avoid freeze thaw cycles of the cytokines.
6. HEK293T cells do not always need trypsinization because they do not adhere strongly to the flask. You can often detach them by washing them down by pipetting them off, spin, then resuspend in fresh 10 % FCS RPMI media.
7. HEK293T cells do not strongly adhere to the flask, therefore removing and changing media must be performed carefully to avoid dislodging the cells.
8. We recommend that the transfection reagent is directly pipetted into media and not onto the sides of the plastic microcentrifuge tube.
9. Snap freezing in liquid nitrogen prior to long term storage is recommended.
10. Some centrifuges need to run at 1000 rpm for 20–30 min to increase the temperature to 30 °C prior to spinfection.
11. Pipette the polybrene directly into the virus-containing supernatant, avoiding touching the sides of the plastic tube.
12. We found that non-treated tissue culture plates lead to increased viral transduction compared to the treated plates which can significantly reduce efficiencies.
13. It is critical to return the plate immediately to the incubator after the 90 min centrifugation. Do not leave the plate in the centrifuge for extended periods.
14. For best pSTAT5 detection, stain the cells within one week of fixing and permeabilizing the cells.
15. Isotype control should be used at the same immunoglobulin isotype, concentration and conjugated fluorochrome as your primary antibody.
16. Kimwipes™ or other similar tissues can help provide additional grip when removing muscles/tendons from the bones. Be gentle to avoid breaking the bones.
17. We have found that syringe filtering the recommended media through a 0.45 μm filter after preparation helps remove any protein aggregates and debris in FCS and aids in isolation of the cells.
18. Keep the remaining transplant media at 4 °C for Day 2 of the procedure.
19. A sublethal irradiation renders the mouse transiently immunosuppressed and we have found is sufficient for accepting donor hematopoietic stem cells. Lethal irradiation (8–10 Gy) whilst also an option, can increase the risk of morbidity.
20. The recommended maximum volume for a tail vein injection is 1 % of body weight (e.g., a 20 g mouse can be injected with 0.2 mL).
21. It is recommended to produce an excess of transduced cells to have sufficient cells to confirm transduction efficiency; have back up cells for any volume loss during tail vein injections; and allow the possibility for sorting the cells should transduction efficiency be low (e.g., <5 %).
22. Time for optimal tail vasodilation can be variable depending on the lamp type and heating procedure.
23. Injection of air bubbles into the vein will cause death in recipient mice.
24. Begin at the end of the tail and move towards the base if unsuccessful with initial injections.
25. Alternative blood collection methods such as submandibular or retro-orbital bleeding can also be performed. Ensure consistency in the blood collection method you choose because WBC count may vary between different collection sites. Ensure animal ethics approval per method.

26. Lymphocyte counts at 1 week post injection should be significantly lower than the normal range (<5000 cells/μL) due to sub-lethal irradiation.
27. If the blood sample volume is too low for the cell counter, blood can be diluted 1:2 with DPBS prior to cell counting. Then values can be adjusted by multiplying by 2.
28. Test the pH of the lysis buffer periodically. pH 7.1–7.4 should be maintained for efficient RBCs lysing.
29. Be careful with RBCs lysis incubation time to avoid WBC lysis.
30. If any blood clots remain after lysis, filter cell suspension using a 5 mL round bottom tube fitted with a cell strainer prior to flow cytometry.
31. If multiple samples need processing simultaneously and to collect events faster on a flow cytometer, lyse the blood samples in a 96 U-bottom non-sterile plate, and use the multichannel pipette to lyse and wash the cells (180 μL lysis buffer/well). After the final wash, transfer the samples to 1.1 mL single mini cluster tubes (Axygen® #MTS-11-C-R). Run each sample on the flow cytometer by placing the whole cluster tube inside a 5 mL polystyrene tube, then load the tube as usual.

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## Availability of data and materials

All data is included in the article and supplementary materials. The JAK3 plasmids are available in Addgene: MSCV-JAK3 (M511) _IRES_GFP (#206290) and MSCV_JAK3_IRES_BFP (#20691).

## CRediT authorship contribution statement

**Sofia A. Omari:** Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Hansen J. Kosasih:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Thomas Chung:** Writing – original draft. **Charles E. de Bock:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing, Investigation, Resources, Supervision.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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