MicroRNA-101 expression is associated with JAK2V617F activity and regulates JAK2/STAT5 signaling.

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Philadelphia negative myeloproliferative neoplasms (MPNs) are clonal hematological diseases characterized by excessive production of mature blood cells. Exome sequencing of patient samples have showed a relatively low degree genomic complexity for these diseases[1]. The majority of MPN patients carry somatic mutations in the JAK2 gene, with the JAK2V617F missense mutation being the most common in polycythemia vera (PV, 95%) and essential thrombocythemia (ET, 60%) [2].

MicroRNAs (miRNAs) act as post-transcriptional regulators of gene expression, regulate signaling, and can diffuse from one cell to another through the extracellular space or the bloodstream. However, although they have been implicated in the pathophysiology of multiple hematological malignancies, their role in MPNs and the effect of JAK2V617F mutation on miRNA expression has not been elucidated [3].

Here we used the erythroblastic leukemia cell line HEL, which carries the activating JAK2V617F mutation, to globally determine differences in miRNA expression after JAK2 inactivation. We profiled miRNAs expression by Next Generation Sequencing for microRNAs (miRNA-seq) in HEL cells (Figure 1a and Supplementary Figure S1) treated for 16 hours with 3μM TG101209, which inhibits JAK2 signaling (Figure 1d). We detected 143 miRNAs exhibiting statistically significant changes (Benjamini-Hochberg adjusted $p \leq 0.01$) in expression after JAK2 inhibition (Supplementary Table S1).

miR-101 is encoded by two loci, one adjacent to the JAK2 locus; little is known about its function in myeloid cells [4], so we focused our attention on this miRNA. We validated miR-101 down-regulation by RT-qPCR in HEL cells using two additional JAK2 inhibitors, TG101348 and AT9283 (Supplementary Figure S1a and S2b). Next, we compared miR-101 expression level in three hematopoietic cell lines, K562, UKE-1 and HEL, which carry 0, 2 and ≈9 copies of the JAK2V671F allele respectively, and found that its expression correlates with the extent of the JAK2V617F mutation (Supplementary Figure S2c). This result led us to hypothesize that JAK2V617F-positive

MPN patients exhibit increased miR-101 levels. To test this, we cultured and individually genotyped single cell-derived BFU-E colonies from JAK2V617F–positive ET and PV patients and pools of 20 colonies were analysed[5] (Supplementary Table S2). We found an increased miR-101 expression in JAK2V617F-positive BFU-e colonies compared to WT in both ET and PV patients (Figure 1b left panel). Next, we measured miR-101 levels in the serum of 14 JAK2V617F-positive ET and PV patients (Supplementary Table S2), and observed higher levels of circulating miR-101 in patients compared to controls (Figure 1b right panel, p=0.0072). The level of circulating miR-101 does not correlate with diagnosis (ET *vs* PV p=0.32) or JAK2V617F-homozygosity (Supplementary Figure S1c and S1d). These observations suggest that miR-101 expression is increased by JAK2V617F in ET and PV patients' erythroid cells and it is released into the blood stream.

Two different loci encode miR-101 primary transcript (pri-miR-101) in the human genome. While miR-101-1 is intergenic, miR-101-2 is located within the last intron of the ribosomal protein gene RCL1, immediately upstream of JAK2 (Supplementary Figure S2d). Both precursors give rise to the same mature miRNA. Thus, we asked whether miR-101 expression from both loci was equally affected by mutant JAK2, and measured the expression of both primary transcripts by qPCR. Interestingly, we observed increased pri-miR-101-1 but not pri-miR-101-2 levels after JAK2 inhibition (Figure 1c). Increased primary transcript levels might be the result of a reduced Microprocessor-mediated pri-miRNA processing upon inhibition of JAK2 activity. Importantly, the activity of the Microprocessor complex (composed of Drosha and DGCR8) is finely regulated by the helicases DDX5 (p68) and DDX17 (p72), which interact directly with Drosha/DGCR8 *in vivo* and facilitate cleavage of specific subsets of pri-miRNAs [6,7]. Therefore, we measured Drosha, DDX5 and DDX17 protein levels after treating HEL cells with JAK2 inhibitor TG101209 and found a decrease in DDX5 and DDX17 expression (Figure 1d), not paralleled by a lower mRNA expression for DDX5 or DDX17

(Supplementary Figure S2e). Thus, we speculate that JAK2 activity might enhance pri-miR-101-1 processing through increased activity of the Microprocessor by stabilizing DDX5 and DDX17 helicases. The specific effect on pri-miR-101-1 might be due to the presence of a conserved CNNC motif on its precursor (Supplementary figure S3), allowing specific enhancement of nuclear processing [8].

Next, we investigated the consequences of increased miR-101 on its predicted target genes. In silico search resulted in 156 overlapping genes using three algorithms (miRanda, Targetscan and PicTar). Gene Ontology (GO) and Kyoto Enciclopedia of Genes and Genomes (KEGG) pathway analysis of this gene set identified and identified JAK/STAT pathway components as putative miR-101 targets.

Suppressors of cytokine signaling (SOCS) proteins are involved in suppression of JAK2/STAT5 signaling. Early studies showed partial efficacy of these proteins on JAK2V617F mediated signaling [9], but recent work suggests SOCS proteins are capable of inhibiting JAK2V617F. Particularly, both SOCS1 and SOCS3 were shown to inhibit JAK2/STAT5 signalling in HEL cells and their overexpression correlate with decreased cell proliferation[10]. We focused our attention on SOCS2, negative regulator of JAK2 and mutant JAK2V617F[11]. Despite being rare, loss of function mutations of SOCS2 have been described in MPN patients [12]. We hypothesized that the observed miR-101 upregulation might functionally mimic the effect of SOCS2 mutation and contribute in maintenance of JAK2V617F constitutive signaling. Using reporter constructs with either WT or a mutant SOCS2-UTR, we compared the ability of miR-101 to silence luciferase. We found that miR-101 overexpression significantly reduced luciferase activity in the presence of WT but not the mutant SOCS2-UTR (Supplementary Figure S4a and S4b). In addition, overexpression of miR-101 in K562 cells resulted in a significant reduction in both SOCS2 protein and mRNA levels (Figure 2a and Supplementary Figure S4c). Other members of the SOCS family were not affected by miR-101 overexpression (Figure 2a). Moreover, SOCS2 mRNA ex-

pression was reduced in JAK2V617F-mutant relative to WT BFU-E colonies from ET and PV patients (Figure 2b)

We then tested the consequence of miR-101 inhibition in HEL cells, using a specific miR-101 Locked Nucleic Acid (LNA) antisense inhibitor. We observed significantly increased apoptosis 48 hours after transfection, increased SOCS2 protein (Figure 2c and 2d) and mRNA levels (Supplementary Figure S4d). The decrease in STAT5 phosphorylation is mediated by SOCS2 as both SOCS1 and SOCS3 showed no increase after miR-101 inhibition (Figure 2d).

Though miR-101 was shown to target the JAK2 UTR [13], JAK2 protein levels were unchanged by miR-101 inhibition in HEL cells (Figure 2d) as well as miR-101 overexpression in K562 cells (Supplementary Figure S5). This suggests JAK2-3' UTR might have a different affinity for miR-101 in different cellular contexts [14]. We conclude that miR-101 repression allows the specific increase of JAK2/STAT5 negative regulator SOCS2, which in turn inhibits STAT5 phosphorylation leading to increased apoptosis of JAK2V617F positive HEL cells.

Taken together, our data support the presence of a positive feedback loop whereby JAK2-V617F constitutive activity allows more efficient pri-miR-101 processing, increasing mature miR-101 levels in erythroid cells. In turn, this potentiates repression on SOCS2 (Supplementary Figure S6). Increased miR-101 as a consequence of JAK2V617F mutation could further enhance the constitutively active JAK2 signaling cascade. The high levels of miR-101 observed in patients JAK2V617F-positive clones suggest that this mechanism could be active in the mutant cells, and the increased level of circulating miR-101 might mediate the same effects also in the WT clone thus potentiating JAK2/STAT5 signaling in these cells.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary information is available at Leukemia's website

Figure Legends

Figure 1. JAK2V617F alters miRNAs expression a) Heatmap of the miRNAs seq shows significant change in HEL cells after TG101209 treatment. **b)** Dot plots show relative miR-101 expression, in paired WT and mutant V617F heterozygous BFU-e colonies from ET and PV patients and serum from healthy controls and JAK2V617F-positive PV and ET patients. **c)** Bargraph shows qPCR (averaged ± S.E.M.) quantification of mature miR-101 and each miR-101 precursor RNA (pri-miR) N=6. **d)** Western blot analysis of HEL cells treated with JAK2 inhibitor. 2way-Anova with Bonferroni post-test with multiple comparisons or Student t-test were used as appropriate. *p≤0.05, ** p≤0.01, *** p≤0.001

Figure 2. SOCS2 is targeted by miR-101 and downreguated in MPN JAK2V617F colonies a) Western blot analysis after miR-101 overexpression. **b)** Dot plot shows SOCS2 gene expression in paired WT and mutant V617F heterozygous BFU-e colonies from ET and PV patients. **c)** Bargraph shows percentage of apoptotic cells after LNA anti-miR-101-LNA transfection in HEL cells N=8 (averaged ± S.E.M.). **d)** Western blot analysis of HEL cells after anti-miR-101-LNA treatment. Student t-test was used. *p≤0.05,**

p≤0.01,*** p≤0.001.

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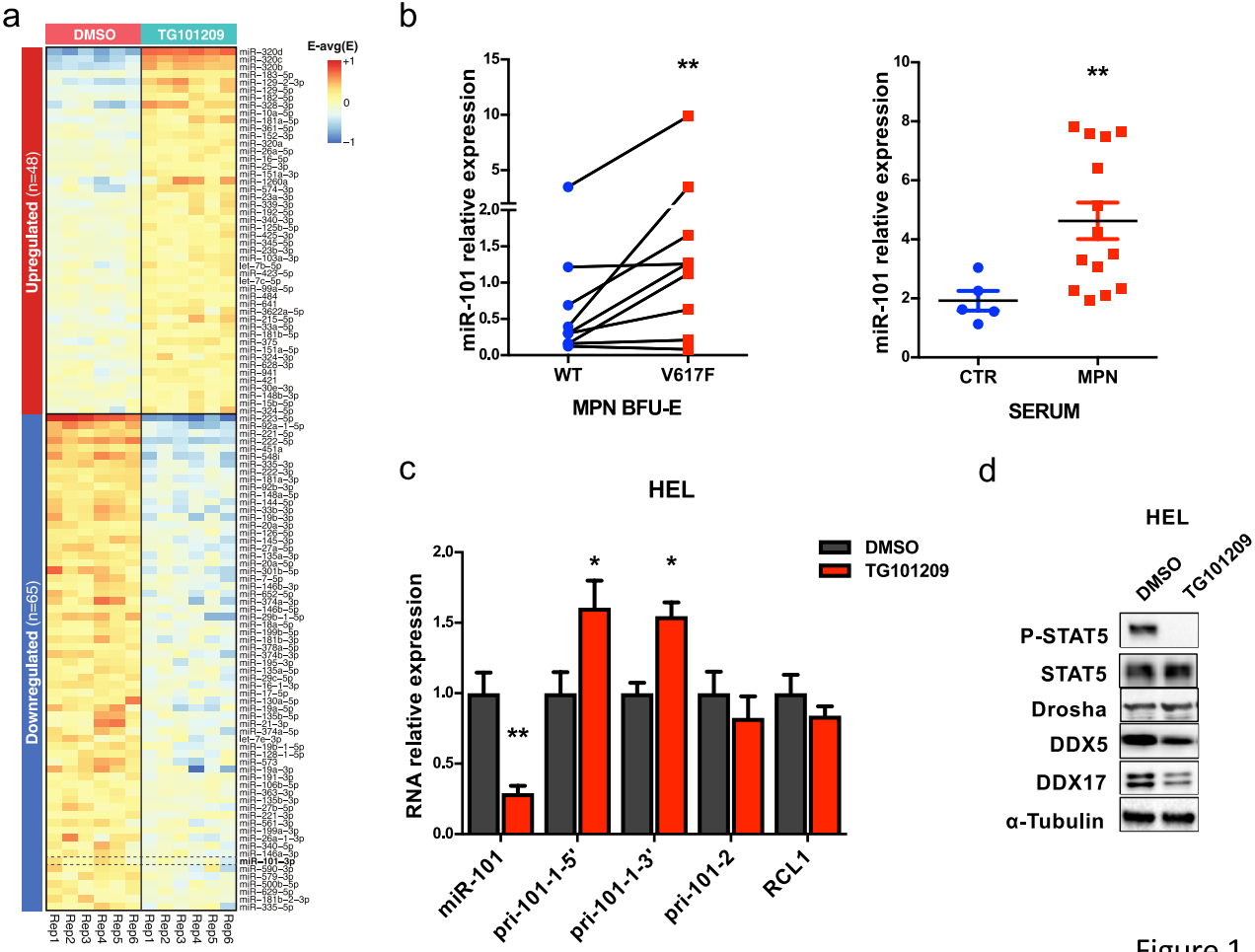
a
DMSO
TG101209
E-avg(E)
+1
0
−1
Upregulated (n=48)
Downregulated (n=65)
Rep1
Rep2
Rep3
Rep4
Rep5
Rep6
b
miR-101 relative expression
**
WT
V617F
MPN BFU-E
CTR
MPN
SERUM
c
HEL
DMSO
TG101209
RNA relative expression
*
**
miR-101
pri-101-1-5'
pri-101-1-3'
pri-101-2
RCL1
d
HEL
DMSO
TG101209
P-STAT5
STAT5
Drosha
DDX5
DDX17
α-Tubulin

Figure 1

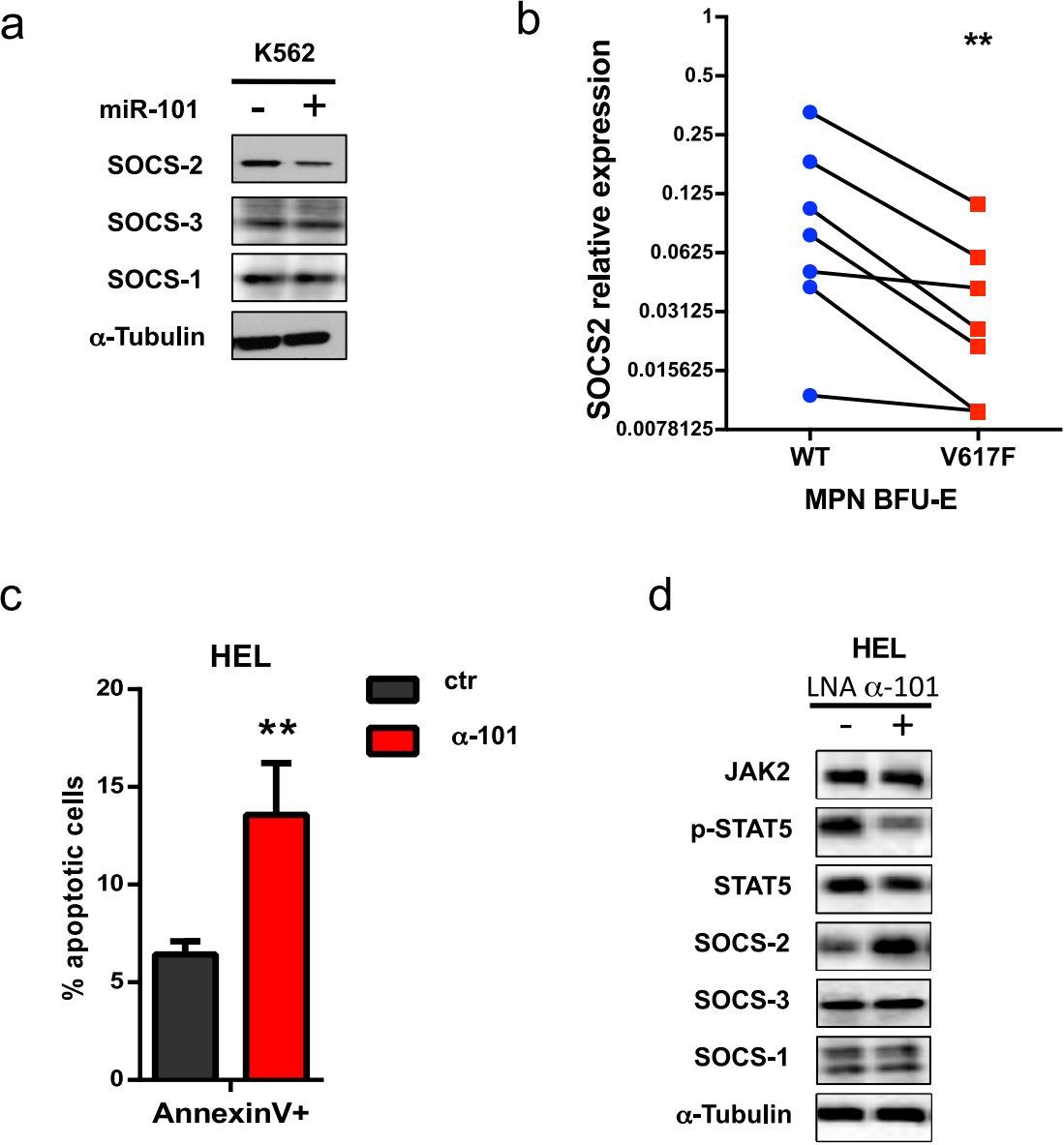
a
K562
miR-101
-
+
SOCS-2
SOCS-3
SOCS-1
α-Tubulin
b
SOCS2 relative expression
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.0078125
**
WT
V617F
MPN BFU-E
c
HEL
20
15
10
5
0
% apoptotic cells
**
ctr
α-101
AnnexinV+
d
HEL
LNA α-101
-
+
JAK2
p-STAT5
STAT5
SOCS-2
SOCS-3
SOCS-1
α-Tubulin

Figure 2