Supplemental information: Targeting compensatory MEK/ERK activation increases JAK inhibitor efficacy in myeloproliferative neoplasms

1. Supplemental methods

Serum inhibitor levels. Determination of inhibitor concentrations was performed in serum of Jak2V617F and MPLW515L mice treated with ruxolitinib, binimetinib or both inhibitors for 4 weeks. Blood was sampled 4 hours post-last dose, serum preparation, and storage of serum at -80°C until analysis. Ruxolitinib serum levels were determined by UPLC/MS-MS as described previously(1). Binimetinib serum levels were determined analogously including the subtle modification of 70 μl supernatant being transferred into a fresh vial and diluted with 60 μl water before further separation by UPLC. The precursor to product ion transition were 441.2 m/z→164.7 for binimetinib and 457.1 m/z → 361.0 for its internal standard LEF158. The limit of quantification (LOQ) was set to 4 ng/ml.

Spleen sonography. For in vivo studies in Jak2V617F and MPLW515L mice treated by ruxolitinib, binimetinib or combined inhibitors followed by a 4-week observation period for tumor recurrence, spleen size was evaluated at the end of treatment and at the end of the observation period. To enable sequential spleen size evaluation, sonography was performed on a Vevo 2100 imaging system (VisualSonics) after the left flank was shaved. The spleen was imaged using a MS-250 scanhead operating at 21MHz. The spleen area was measured using the abdominal package of the Vevo2100 workstation software as described(2). Anesthesia was induced with 4% and maintained at 1.5% isoflurane at 37°C body temperature throughout the procedure and animals were observed until full recovery from anesthesia.
2. Supplemental references


### 3. Supplemental tables

#### Supp. Table 1. List of primers for qRT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
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<tr>
<td><strong>Mouse Pdgfa</strong></td>
<td>FW: 5'-GAGATACCCCGGGAGTTGAT-3'&lt;br&gt;REV: 5'-CTTGCTCTCAAGGCATCCTC-3'</td>
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<td><strong>Human PDGFRA</strong></td>
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<td><strong>Human GAPDH</strong></td>
<td>FW: 5'-GGGAAGCTTGTCATCAATGGA-3'&lt;br&gt;REV: 5'-TCTCGCTCTGGGAAGATGGT-3'</td>
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4. Supplemental figure legends

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Supp.Figure 6. Combined JAK/MEK inhibition is tolerable in the Jak2V617F and in the MPLW515L mouse models. A. Body weight course over a 4-week treatment period in the Jak2V617F model does not indicate an adverse effect of combined ruxolitinib/binimetinib (n=5/group). B. Body weight after 4 weeks of treatment in the Jak2V617F model shows similar weights among treatment groups (n=5/group, one-way ANOVA with p≤0.05 considered significant). C. Histopathological analysis of kidney cortical and medullar regions after 4 weeks of treatment in the Jak2V617F model does not indicate nephrotoxic effects of JAK2, MEK or combined inhibitor treatment. D. Body weight course over a 4-week treatment period in the MPLW515L model does not indicate an adverse effect of combined ruxolitinib/binimetinib (n=5-10/group). E. Body weight after 4 weeks of treatment (or at time of sacrifice for vehicle-treated mice) in the MPLW515L model shows similar weights among treatment groups with slightly higher weights upon ruxolitinib treatment (n=5/group, student’s t-test with p≤0.05 considered significant). F. Histopathological analysis of kidney cortical and medullar regions after 4 weeks of treatment in the MPLW515L model does not indicate nephrotoxic effects. Quantitative results are shown as mean ± SD.

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A. Peripheral blood counts of C57BL/6 mice at 2 and 4 weeks of treatment with binimetinib 30 mg/kg, ruxolitinib 60 mg/kg or combined ruxolitinib/binimetinib remained within the normal range. B. Total bone marrow (BM) cell number was reduced by ruxolitinib monotherapy and not further aggravated by combined ruxolitinib/binimetinib. C. Spleen weight was slightly reduced by ruxolitinib monotherapy, which was not further aggravated by combined ruxolitinib/binimetinib. D. Histology of BM and spleen revealed intact organ architectures and no difference between treatment groups. E. Liver weight was not significantly altered in any treatment group. F. Histopathological analyses of liver, lung and kidney showed no signs of toxicity for ruxolitinib or binimetinib monotherapies or for the combination. G-H. Body weight course during the 4-week treatment period as well as body weights at the end of treatment were similar among treatment groups. N=5-9/group (1 vehicle-treated sample lost in B). Results are shown as mean ± SD and were analyzed by one-way ANOVA with p≤0.05 considered significant.

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**Supp.Figure 10. Combined JAK2/MEK inhibition impacts on Jak2V617F-induced expression patterns.** A-B. Combined JAK2/MEK inhibition showed superior reduction of soluble factors in bone marrow (BM) interstitial fluid and serum (n=3-5/group). Results were analyzed by one-way ANOVA with p≤0.05 considered significant and shown as mean ± SD. C. Principal component analysis (PCA) of paired Jak2V617F BM and spleen samples showed that ERK target expression was primarily affected by the type of treatment and not as much by the site assessed (n=4-5/group).
**Supp.Figure 11. Combined JAK2/MEK inhibition provides superior therapeutic efficacy in a MPLW515L MPN mouse model.**

A. A single oral dose of binimetinib at 30 mg/kg consistently inhibited ERK phosphorylation in MPLW515L mouse splenocytes. B. Inhibition of ERK phosphorylation by binimetinib at 30 mg/kg is sustained at 1 and 2 weeks of treatment as well as with combined JAK2/MEK inhibition for 2 weeks in MPLW515L mouse splenocytes. C-F. Combined JAK2/MEK inhibition was superior to ruxolitinib as a single agent in reduction of splenomegaly, hepatomegaly, leukocytosis and thrombocytosis over 4 weeks of treatment in the MPLW515L model. Results from 2 weeks treatment are shown in Fig. 6. Results are from recipients of MPLW515L transduced bone marrow (n=5/group). G-H. Binimetinib as a single agent was not effective in reducing splenomegaly or leukocytosis in the MPLW515L model upon 1-2 weeks of treatment. Results are from recipients of MPLW515L transduced bone marrow (n=3-4/group). Results are shown as mean ± SD and were analyzed by student’s t-test with p≤0.05 considered significant.

**Supp.Figure 12. Combined JAK2/MEK inhibition with ruxolitinib and selumetinib is similarly effective in a MPLW515L MPN mouse model.** Combined JAK2/MEK inhibition with ruxolitinib 90 mg/kg and selumetinib (AZD6244) at 25 mg/kg showed a trend for improved effects as compared to ruxolitinib alone regarding reduction of splenomegaly (A), hepatomegaly (B) and leukocytosis (C), while ERK phosphorylation was completely inhibited (D) in MPLW515L mouse splenocytes. Effects of combined treatment were not as evident due to low mouse numbers and the higher dose of ruxolitinib. Results are from recipients of MPLW515L transduced bone marrow, which were treated for 2 weeks (n=2-3/group). Quantitative results were analyzed by student’s t-test with p≤0.05 considered significant and shown as mean ± SD.

**Supp.Figure 13. Combined JAK2 and MEK inhibition by the type II JAK2 inhibitor CHZ868 and binimetinib is superior in a Jak2V617F MPN mouse model.** Combined binimetinib 15 mg/kg bid and CHZ868 15 mg/kg bid was superior to single agent therapies in regard to splenomegaly reduction (A). The elevated hematocrit was only slightly reduced (B), while reticulocytes were significantly decreased by CHZ868 and by combined treatment (C). Expanded myelo-erythroid progenitor populations were better controlled by combined CHZ868 and binimetinib than with the single agents, as shown by a representative FACS panel (D) and the quantitation (E, MP: multipotent myeloid
progenitors). Even at these low doses of 15 mg/kg, combined CHZ868 and binimetinib reduced *Jak2V617F* mutant allele burden reflected by the CD45.2/CD45 ratio of bone marrow (BM) cells (F). This reduction in *Jak2V617F* mutant allele burden was also confirmed in CD71+Ter119 erythroid BM progenitors as shown by a representative FACS panel (G) and by quantitation (H). Results are from recipients of *Jak2V617F* (CD45.2) and *WT* (CD45.1) BM treated for 2 weeks (n=3-5/group). Quantitative results were analyzed by student’s t-test with p≤0.05 considered significant and shown as median with boxes representing 25th to 75th percentiles and whiskers indicating minimum to maximum (A-C) or as mean ± SD (E-F, H).

**Supp.Figure 14. Therapeutic benefit of combined JAK2/MEK inhibition is partially maintained after treatment cessation in the *MPLW515L* model.** Mice treated for 4 weeks were followed for 4 additional weeks after treatment discontinuation (n=5-6/group, histology assessed for n=2-3/group). **A.** Survival of *MPLW515L* mice was significantly improved by combined JAK2/MEK inhibition as compared to single agent therapies also after stopping treatment. **B.** Correction of splenomegaly was significantly improved with combined JAK2/MEK inhibition followed by recurrence of splenomegaly 4 weeks after cessation of treatment as shown by sequential spleen sonography (mice deceased during treatment period included in post-treatment analysis, mice deceased during observation period included in post-cessation analysis). **C.** Correction of leukocytosis (top) and thrombocytosis (bottom) was improved by combined JAK2/MEK inhibition with a partially maintained corrective impact on white blood cell (WBC) counts 4 weeks after stopping therapy. **D.** Gross fibrosis characteristic of the *MPLW515L* model showed a residual benefit of combined JAK2/MEK inhibition after cessation of treatment (top) with a lower mean fibrosis grade according to WHO-grading (myelofibrosis grade 0-3, bottom). **E.** Extramedullary hematopoiesis of liver and lung was most potently reduced by combined JAK2/MEK inhibition after stopping therapy. **F.** Body weights were similar between the single and combined therapy groups at end of treatment as well as off-treatment observation periods (mice alive at end of period included). **G.** Histopathological analysis of kidney tissue did not indicate nephrotoxic effects. Quantitative results were analyzed by one-way ANOVA with p≤0.05 considered significant and shown as mean ±SD (B, D, F) and ±SEM (C).
Supp. Figure 15. Therapeutic benefit of combined JAK2/MEK inhibition is partially maintained after treatment cessation in the Jak2V617F model. A. Splenomegaly showed improved correction upon combined JAK2/MEK inhibition and recurred upon cessation of treatment with a residual benefit most pronounced for the combination at 4 weeks off-therapy. B-D. Red cell parameters including hematocrit, hemoglobin and reticulocytes rebounded 4 weeks after cessation of treatment with residual benefit on reticulocyte counts most pronounced with combined JAK2/MEK inhibitor therapy. E. Ter119^CD71^- erythroid bone marrow (BM) progenitors also showed a partial, maintained reduction after stopping treatment as compared to vehicle-treated Jak2V617F mice. F. Fibrosis correction according to WHO-grading (myelofibrosis grade 0-3) in BM and spleen by combined treatment was still detectable 4 weeks after cessation of treatment as reflected by Gomori reticulin stain. G-H. Body weights 4 weeks after stopping treatment were similar between treatment groups and kidney histopathological analysis was normal, suggesting no late toxic effect of combined JAK/MEK inhibitor treatment. N=4-6/group. Quantitative results were analyzed by one-way ANOVA with p≤0.05 considered significant and shown as mean ±SD.
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Supp.Figure 9. Superior efficacy of combined JAK2/MEK inhibition in Jak2V617F primary mice. To study therapy-induced changes in the expression patterns downstream of Jak2V617F, we treated primary mice for 9 days and the therapeutic benefit of combined ruxolitinib and binimetinib was also evident. Combined JAK2/MEK inhibition by ruxolitinib 60 mg/kg bid and binimetinib 30 mg/kg bid corrected the disease phenotype to a greater extent than the respective monotherapies including splenomegaly (A), polyclonality with increased hematocrit (B), hemoglobin (C), and reticulocytes (D–E) as well as elevated white blood cell count (F). N=5 per group. Results were analyzed by one-way ANOVA with p≤0.05 considered significant and shown as median with boxes representing 25th to 75th percentiles and whiskers indicating minimum to maximum values.
Supp. Figure 10. Combined JAK2/MEK inhibition impacts on Jak2V617F-induced expression patterns. A-B. Combined JAK2/MEK inhibition showed superior reduction of soluble factors in bone marrow (BM) interstitial fluid and serum (n=3-5/group). Results were analyzed by one-way ANOVA with *p≤0.05* considered significant and shown as mean ± SD. C. Principal component analysis (PCA) of paired Jak2V617F BM and spleen samples showed that ERK target expression was primarily affected by the type of treatment and not as much by the site assessed (n=4-5/group).
Supp.Figure 11. Combined JAK2/MEK inhibition provides superior therapeutic efficacy in a MPLW515L MPN mouse model. A. A single oral dose of binimetinib at 30 mg/kg consistently inhibited ERK phosphorylation in MPLW515L mouse splenocytes. B. Inhibition of ERK phosphorylation by binimetinib at 30 mg/kg is sustained at 1 and 2 weeks of treatment as well as with combined JAK2/MEK inhibition for 2 weeks in MPLW515L mouse splenocytes. C-F. Combined JAK2/MEK inhibition was superior to ruxolitinib as a single agent in reduction of splenomegaly, hepatomegaly, leukocytosis and thrombocytosis over 4 weeks of treatment in the MPLW515L model. Results from 2 weeks of treatment are shown in Fig. 6. Results are from recipients of MPLW515L transduced bone marrow (n=5/group). G-H. Binimetinib as a single agent was not effective in reducing splenomegaly or leukocytosis in the MPLW515L model upon 1-2 weeks of treatment. Results are from recipients of MPLW515L transduced bone marrow (n=3-4/group). Results are shown as mean ± SD and were analyzed by student’s t-test with p<0.05 considered significant.
Supp.Figure 12. Combined JAK2/MEK inhibition with ruxolitinib and selumetinib is similarly effective in a MPLW515L MPN mouse model. Combined JAK2/MEK inhibition with ruxolitinib 90 mg/kg and selumetinib (AZD6244) at 25 mg/kg showed a trend for improved effects as compared to ruxolitinib alone regarding reduction of splenomegaly (A), hepatomegaly (B) and leukocytosis (C), while ERK phosphorylation was completely inhibited (D) in MPLW515L mouse splenocytes. Effects of combined treatment were not as evident due to low mouse numbers and the higher dose of ruxolitinib. Results are from recipients of MPLW515L transduced bone marrow, which were treated for 2 weeks (n=2-3/group). Quantitative results were analyzed by student’s t-test with p<0.05 considered significant and shown as mean ± SD.
Supp. Figure 13. Combined JAK2 and MEK inhibition by the type II JAK2 inhibitor CHZ868 and binimetinib is superior in a Jak2V617F MPN mouse model. Combined binimetinib 15mg/kg bid and CHZ868 15mg/kg bid was superior to single agent therapies in regard to splenomegaly reduction (A). The elevated hematocrit was only slightly reduced (B), while reticulocytes were significantly decreased by CHZ868 and by combined treatment (C). Expanded myelo-erythroid progenitor populations were better controlled by combined CHZ868 and binimetinib than with the single agents, as shown by a representative FACS panel (D) and the quantitation (E, MP: multipotent myeloid progenitors). Even at these low doses of 15mg/kg, combined CHZ868 and binimetinib reduced Jak2V617F mutant allele burden reflected by the CD45.2/CD45 ratio of bone marrow (BM) cells (F). This reduction in Jak2V617F mutant allele burden was also confirmed in CD71-Ter119 erythroid BM progenitors as shown by a representative FACS panel (G) and by quantitation (H). Results are from recipients of Jak2V617F (CD45.2) and WT (CD45.1) BM treated for 2 weeks (n=3-5/group). Quantitative results were analyzed by student’s t-test with p<0.05 considered significant and shown as median with boxes representing 25th to 75th percentiles and whiskers indicating minimum to maximum (A-C) or as mean ± SD (E-F, H).
Supp.Figure 14. Therapeutic benefit of combined JAK2/MEK inhibition is partially maintained after treatment cessation in the MPLW515L model. Mice treated for 4 weeks were followed for 4 additional weeks after treatment discontinuation (n=5-6/group, histology assessed for n=2-3/group). A. Survival of MPLW515L mice was significantly improved by combined JAK2/MEK inhibition as compared to single agent therapies also after stopping treatment. B. Correction of splenomegaly was significantly improved with combined JAK2/MEK inhibition followed by recurrence of splenomegaly 4 weeks after cessation of treatment as shown by sequential spleen sonography (mice deceased during treatment period included in post-treatment analysis, mice deceased during observation period included in post-cessation analysis). C. Correction of leukocytosis (top) and thrombocytosis (bottom) was improved by combined JAK2/MEK inhibition with a partially maintained corrective impact on white blood cell (WBC) counts 4 weeks after stopping therapy. D. Gross fibrosis characteristic of the MPLW515L model showed a residual benefit of combined JAK2/MEK inhibition after cessation of treatment (top) with a lower mean fibrosis grade according to WHO-grading (myelofibrosis grade 0-3, bottom). E. Extramedullary hematopoiesis of liver and lung was most potently reduced by combined JAK2/MEK inhibition after stopping therapy. F. Body weights were similar between the single and combined therapy groups at end of treatment as well as off-treatment observation periods (mice alive at end of period included). G. Histopathological analysis of kidney tissue did not indicate nephrotoxic effects. Quantitative results were analyzed by one-way ANOVA with p<0.05 considered significant and shown as mean ±SD (B, D, F) and ±SEM (C).
Supp. Figure 15. Therapeutic benefit of combined JAK2/MEK inhibition is partially maintained after treatment cessation in the \textit{Jak2V617F} model. A. Splenomegaly showed improved correction upon combined JAK2/MEK inhibition and recurred upon cessation of treatment with a residual benefit most pronounced for the combination at 4 weeks off-therapy. B-D. Red cell parameters including hematocrit, hemoglobin and reticulocytes rebounded 4 weeks after cessation of treatment with residual benefit on reticulocyte counts most pronounced with combined JAK2/MEK inhibitor therapy. E. Ter119\textsuperscript{+}CD71\textsuperscript{-} erythroid bone marrow (BM) progenitors also showed a partial, maintained reduction after stopping treatment as compared to vehicle-treated \textit{Jak2V617F} mice. F. Fibrosis correction according to WHO-grading (myelofibrosis grade 0-3) in BM and spleen by combined treatment was still detectable 4 weeks after cessation of treatment as reflected by Gomori reticulin stain. G-H. Body weights 4 weeks after stopping treatment were similar between treatment groups and kidney histopathological analysis was normal, suggesting no late toxic effect of combined JAK/MEK inhibitor treatment. N=4-6/group. Quantitative results were analyzed by one-way ANOVA with p≤0.05 considered significant and shown as mean ±SD.