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Splicing factor Ybx1 mediates persistence of Jak2-mutated neoplasms

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Janus kinases (Jak) mediate cytokine, hormone and growth factor responses in hematopoietic cells[1,2]. Jak2 is one of the most frequently mutated genes in the aging hematopoietic system [3,4] and in hematopoietic cancers [5]. Mutations in Jak constitutively activate downstream signaling and are drivers of myeloproliferative neoplasms (MPN). In clinical use, Jak-inhibitors have incomplete effects on overall disease burden of Jak2 mutated clones [6,7], prompting us to investigate the mechanism underlying disease persistence. By in-depth phospho-proteome profiling we here identify proteins involved in mRNA processing as targets of mutant Jak2. Inactivation of the post-translationally modified Jak2-target Ybx1 sensitizes Jak-inhibitor persistent cells to apoptosis and results in RNA mis-splicing, retained intron enrichment and disruption of the transcriptional control of extracellular signal-regulated kinase (ERK) signaling. In combination with pharmacological Jak-inhibition it induces apoptosis in Jak2-dependent murine and primary human cells, leading to *in vivo* regression of the malignant clones and inducing remission. This identifies and validates a novel cell-intrinsic mechanism how differential protein phosphorylation results in splicing-dependent alterations of Jak2-ERK-signaling and the maintenance of Jak2V617F malignant clones. Therapeutic targeting of Ybx1 dependent ERK-signaling in combination with Jak2-inhibition may eradicate Jak2-mutated cells.

Functional phosphoproteome profiling

Jak2 is essential for the development of hematopoietic cells and its mutation is a frequent event during aging [3,4] that pre-disposes to the development of hematologic cancers such as MPN [5]. While Jak-inhibitors (Jak-i) reduce inflammatory activity and hyperproliferation, Jak2-mutated clones persist [6,7]. To identify downstream effectors of the mutant Jak2 kinase that may be responsible for this, we performed in-depth mass spectrometry(MS)-based phosphoproteomics [8] in murine hematopoietic cells expressing erythropoietin-receptor and either Jak2-wildtype (Jak2WT) or mutated Jak2-V617F (Jak2VF) kinase (Fig. 1a). We exposed cells to erythropoietin (EPO) alone or combined with Jak-inhibitor (Jak-i; Ruxolitinib, RUX) to investigate EPO-independent and RUX-responsive signaling in Jak2VF compared to Jak2WT. In total, we quantified 21,764 distinct and localized phosphorylated sites on 4135 different proteins (Extended Data Fig. 1a-e). Of 6517 phosphosites regulated in Jak2VF and Jak2WT at a False Discovery Rate (FDR) of 5%, 5191 were distinctly regulated in Jak2VF cells on 1758 proteins, including *bona fide* Jak2 targets such as STAT5, STAT3, and PIM (Fig. 1b,c). Kinase motifs of glycogen synthase kinase-3 (GSK3), ERK, and cyclin-dependent kinases (CDKs) were also enriched (Fig. 1d). Notably, the most highly enriched cellular processes in Jak2VF cells were mRNA splicing and processing (Fig. 1e and Extended Data Fig. 1f). To assess the potential functional role of mRNA splicing and processing pathway members (Fig. 1f), we chose the top 15 ranked of a total of 47 members by p-values for an RNAi based screen (Fig. 1g). We used 4-5 shRNAs per gene, confirmed knock-down efficiencies and assessed their growth with and without Jak-i (70 shRNAs against 15 candidates and 4 non-targeting controls (Extended Data Fig. 1g-i and Extended Data Fig. 2a). Targeting Hnrnpk, Srsf2 and Srsf9 resulted in dropouts, with at least 3 of 4 shRNAs displaying significant growth impairment. Some of the targets appeared dispensable for proliferation and survival of Jak2VF cells but their inactivation sensitized persistent cells to Jak-i treatment (Fig. 1h). Given the persistence of Jak2VF-clones upon Jak-i therapy in MPN patients, we reasoned that these proteins may offer novel strategies to target disease persistence *in vivo*. The most prominent and only candidate with positive results in 4 of 4 shRNAs and exclusively in the presence of Jak-i (Extended Data Fig. 2a) was the pleiotropic Y-box binding protein 1 (Ybx1). Ybx1 sensitized Jak2VF cells to Jak-i treatment and is a core spliceosomal protein [9] that regulates mRNA splicing in various cellular contexts [10-13] (Fig. 1h).

Dependency of Jak2V617F cells on Ybx1

We found Ybx1 to be highly expressed in 76 primary bone marrow (BM) biopsies of patients diagnosed with Jak2-mutated MPN (Fig. 2a,b) compared to normal BM (n=18). Moreover, Ybx1 colocalized with and bound to Jak2 in Jak2VF-positive cells (Extended Data Fig. 2b,c). Genetic inactivation of Ybx1 resulted in reduced proliferation *in vitro* when exposed to Jak-i with the IC50 reduced from 1000nM to 275nM (Fig. 2c). Ybx1-depletion alone did not affect viability, proliferation, cell cycle activity, ROS production and DNA damage in Jak2WT and Jak2VF cells (Extended Data Fig.2d-i). Instead, reduction in growth of Jak2VF cells exposed to Jak-i in absence of Ybx1 could be attributed to induction of apoptosis (Fig. 2d-f). We confirmed these findings in Jak2VF mutated murine (Fig. 2d) and human (Fig. 2e) cell lines, as well as primary lineage depleted Jak2VF (Jak2+/VF) murine BM cells (Fig. 2f and Extended Data Fig. 2j).

To assess Ybx1 as a potential therapeutic target in Jak2-mutated neoplasms, we asked whether genetic inactivation would reduce Jak-i persistent clones *in vivo*. We generated a conditional knockout mouse model with exon 3 of Ybx1 flanked by loxP sites (Extended Data Fig. 3a-b) and crossed this with conditional Jak2VF knock-in mice harboring an inducible Mx1-Cre recombinase. BM from Ybx1F/F Jak2VF Mx+ and Ybx1+/+ or Ybx1+/- Jak2VF Mx+ littermate controls (CD45.2) was transplanted in a competitive manner along with 45.1 competitor cells. Following engraftment of transplanted cells, recipient animals received pIpC injections to activate Mx1-Cre and genetically delete Ybx1. Concomitantly, Jak-i was administered by gavage. Recipients of Jak2VF Ybx1+/+ BM showed hyperleukocytosis, thrombocytosis, and onset of symptomatic splenomegaly. In contrast, Ybx1-deficient Jak2VF clones failed to induce symptomatic disease within 16 weeks after transplantation (Fig. 2g-i). Peripheral blood (PB) chimerism revealed an increasing percentage of PB CD45.2/Jak2VF positive cells, while genetic inactivation of Ybx1 resulted in suppression or loss of the Jak2-mutated clone when co-treated with Jak-i (Fig. 2j and Extended Data Fig. 3c). After 20 weeks, 5 of 9 recipients had lost Jak2VF cells (<1% CD45.2 cells in PB and BM) while conversely, all controls notably increased PB chimerism (Fig. 2k). Four of 9 recipients of Ybx1F/F Jak2VF Mx+ BM showed counter-selection of clones with incomplete excision of Ybx1, indicating selective pressure. Consistently, development of myeloid hyperplasia in the BM and organ infiltration (Extended Data Fig. 3d,e) was blunted in recipients of Ybx1-depleted cells. In a xenograft model of Jak2VF mutated human cells, RNAi mediated inactivation of Ybx1 followed by saline injection did not result in significant delay of disease progression (Fig. 2l). However, concurrent Jak-i treatment with inactivation of Ybx1 significantly prolonged survival and loss of disease penetrance in 4 of 12 recipients (Fig. 2m). Notably, Jak-i alone delayed disease progression only to a minor extent (NT+diluent: OS 27 vs. 30 days; NT+Jak-i: OS 27 vs. 72 days). We conclude that the persistence of Jak2-mutated clones under pharmacologic Jak inhibition can be disrupted in human and murine cells by eliminating Ybx1.

To be clinically relevant, such treatment must have a suitable therapeutic index between hematopoietic stem- and progenitor cells (HSPCs) and their malignant counterpart. This is particularly important considering the role of Ybx1 in mRNA processing & splicing. Of note, genetic inactivation of Ybx1 did not perturb steady-state hematopoiesis (Extended Data Fig. 3f-j) and Ybx1-deficient HSPCs (Lineage-Sca-1+Kit+ cells; LSK) did not reveal any significant disadvantage in colony forming potential or lineage commitment compared to Ybx1+/+ controls (Extended Data Fig. 3k-n). Furthermore, functional impairment of adult Ybx1-deficient LT-HSCs was excluded by serial transplantation into primary (Fig. 2n, Extended Data Fig. 3o) and secondary recipient hosts (Fig. 2o, Extended Data Fig. 3p). Thus, inactivation of Ybx1 does not impair HSPC function *in vitro* and *in vivo*.

Ybx1 is required for Mknk1 splicing

To gain further insight into the regulation of Ybx1 by mutant Jak2, we investigated the phosphoprofile of Ybx1 in Jak2VF cells. Following treatment with EPO and Jak-i, Ybx1 was specifically phosphorylated at several phosphosites in Jak2VF compared to Jak2WT cells (Extended Data Fig. 4a-c). To further characterize post-translational modulators of Ybx1, we performed phosphoproteome analysis following pharmacologic short-term inhibition of several bona fide Jak2 downstream effectors (Extended Data Fig. 4d,e). This identified significant changes in Ybx1 phosphorylation, and specifically the only MEK/ERKi (PD0325901) responsive pS30 and pS34 residues of this protein that are conserved across species (Extended Data Fig. 4f,g). To corroborate the Ybx1-MAPK interaction, we performed Ybx1 affinity purification combined with quantitative interaction proteomics, which revealed 260 Jak2VF dependent interactors (Extended Data Fig. 5a). Among these, ribo-nucleoproteins, mRNA splicing factors and ribosomal proteins were significantly enriched (Extended Data Fig 5b-e), which also explains the large number of interactors ($p<0.05$). Most notably, several *bona fide* members of mRNA splicing complexes were identified as Ybx1-interactors in Jak2-mutated cells, that are known to interact during spliceosome formation and activation (44/134 core splicing factors that participate in several steps of dynamic spliceosome assembly; Extended Data Fig. 5b-d). We also discovered that Jak2 and Mapk1 interact (Extended Data Fig. 5e,f) indicating that Ybx1 may be recruited and regulated by the Jak2-Mapk1 axis. Furthermore, the interactome of Jak-i treated VF cells confirmed that the Ybx1-Mapk1 interaction is Jak-i independent (Extended Data Fig. 5g,h). To delineate the relevance of MEKi induced regulation of Ybx1 phosphorylation sites, we expressed Ybx1 phospho-mutants mimicking hyper- or hypo-phosphorylated states at conserved and relevant serine residues in Jak2-mutated cells (Extended Data Fig. 6a). Expression of pS30A, pS34A and pS30A/34A double phosphomutants (but not pS30D, pS34D, pS30D/34D or pS170A/S172A mutants) resulted in reduction of nuclear Ybx1 translocation (Extended Data Fig. 6b,c). Moreover, these modifications recapitulated the phenotype of genetic inactivation and sensitized Jak2-mutated cells to Jak-i mediated cell death (Extended Data Fig. 6d-f). Consistently, MEKi treatment alone or in combination with RUX significantly prevented nuclear import of Ybx1, an effect that was not detectable on RUX treatment alone, in murine and human Jak2-mutated cells (Extended Data Fig. 7a-e). These data suggest that MAPK signaling stabilizes nuclear Ybx1, a notion that was further supported by impaired binding of Ybx1 pS30A and pS34A-containing phosphomutants to Mapk1 (Extended Data Fig. 7f). Collectively our data demonstrate that Ybx1 undergoes Jak2VF-Mapk1 dependent phosphorylation and that Ybx1-Mapk1 interaction is crucial for Ybx1 nuclear translocation and persists despite Jak-i treatment.

To identify the transcriptional pathways controlled by Ybx1, we performed RNA sequencing analysis (RNAseq) of murine and human Jak2-mutated cells following inactivation of Ybx1 by RNAi. Gene-ontology (GO) analysis of the differentially expressed coding genes revealed strong signatures of inflammation, chemotaxis and cytokine production but also of MAPK and ERK signaling and programmed cell death (Fig. 3a,b). Given the established role of Ybx1 in mRNA splicing, we analyzed altered splicing and detected a 70% increase in intron retention (IR; 1064 events, $p< 0.05$ & $\Delta PSI>0.1$) compared to the controls (Fig. 3c and Extended Data Fig. 8a,b). GO term analysis of IR highlighted genes enriched for RNA splicing, nonsense-mediated decay, apoptosis and MAPK signaling (472 events, $p< 0.01$ & $\Delta PSI>0.1$, Extended Data Fig. 8c). Depending on localization, IR results in initiation of translation, nuclear degradation, nonsense mediated mRNA decay (NMD) or mRNA stabilization. We then asked whether ERK-signaling molecules that had increased intron retention such as Araf, Braf and Mknk1 were regulated in Jak-mutated cells (Fig. 3d). Comparing global mRNA and protein abundance of ERK signaling proteins showed a significant decrease of Braf (mRNA) and Mknk1 (mRNA and protein) (Fig. 3e). Western blot further confirms loss of Mknk1 expression in Ybx1 depleted Jak2VF murine and human cells (Fig. 3f,g). Treatment with mRNA

splicing inhibitors Herboxidiene and Pladeinolide B targeting spliceosome machinery confirmed that Mknk1 protein expression is dependent on efficient Mknk1 splicing (Fig. 3h,i). *In vitro* splicing assays confirmed that Ybx1 is required for Mknk1 pre-mRNA splicing in murine Jak2VF cells (Extended Data Fig. 8d). Additionally, targeting NMD pathway by dorsomorphin (compound c, termed CC) or VG-1 inhibitor treatment resulted in dose dependent rescue of Mknk1 translation (Extended Data Fig. 8e,f). Of note, Ybx1 ChIP-seq revealed no significant binding to Mknk1 genomic regions in murine and human Jak2VF cells (Extended Data Fig. 8g,h). Consistently, expression of Ybx1 phosphomutants (S30A, S34A, and S30A/S34A) resulted in reduction or abrogation of Mknk1 (Extended Data Fig. 8i), confirming that pS30/pS34 phosphorylation of Ybx1 in Jak2VF cells is a critical requirement for efficient mRNA splicing and transcriptional regulation of Mknk1 transcripts and a mechanistic link to the Ybx1 dependent disruption of ERK-signaling.

Targeting of Mknk1-ERK-signaling

Phosphoproteome profiling of Ybx1 targeted murine and human Jak2VF cells revealed significant downregulation of phosphosites with key phosphorylation changes in substrates enriched for ERK1/2, GSK3 and CDK motifs (2012 sites in murine and 2390 sites in human, FDR < 0.01; Fig. 4a,b and Extended Data Fig. 9a,b). Overall, approximately 40% of ERK substrate motif containing phosphoproteins were shared between mouse and human Jak-mutated cells (Extended Data Fig. 9c). Of these, Mknk1 and Mcl-1 were identified as relevant ERK targets (Fig. 4c). To confirm these effects on cell signaling we investigated Jak2-dependent pathways in the presence or absence of pharmacologic Jak-i treatment. Inactivation of Ybx1 again led to a considerable reduction of ERK-phosphorylation in murine and human cells (Fig. 4d and Extended Data Fig. 9d-f), while STAT signaling was largely unaffected. Concomitant pharmacologic Jak-inhibition abrogated ERK-signaling, while decreasing STAT signaling, irrespective of Ybx1 inactivation. Thus, Ybx1 is required for maintenance of ERK-signaling downstream of Jak2VF. Genetic inactivation of Mknk1 by RNAi likewise abrogated ERK signaling and sensitized the cells to Jak-i induced cell death (Extended Data Fig. 9g-j), which links Ybx1 dependent Mknk1 expression to maintenance of ERK signaling in Jak2VF cells. Besides RNA splicing and processing, disturbed cellular functions indicated by proteome changes in Ybx1 depleted Jak2VF cells included positive regulation of programmed cell death and apoptosis (Extended Data Fig. 10a). Supporting this, inactivation of Ybx1 in Jak2VF cells by RNAi reduced Mcl-1 phosphorylation (Extended Data Fig. 10b,c). Likewise, Ybx1 loss concomitantly decreased Mcl-1 protein abundance and induction of Bim in a gene-dose dependent manner (Extended Data Fig. 10d) and induction of apoptosis after Ybx1-KD and RUX treatment could be rescued by forced expression of Mcl-1 (Extended Data Fig. 10e).

Finally, pharmacological modulation of Mknk1- or ERK-signaling in combination with two different Jak-i resulted in induction of apoptosis in murine (Fig. 4e-g) and primary human VF-mutated CD34+ BM cells but not in CD34+ healthy donor controls (Fig. 4h). This therapeutic combination of Jak2 and ERK inhibition dramatically downregulated Mknk1 protein levels in both murine and human cells, indicating that Mknk1 is the primary target of this combination (Fig. 4i and Extended Data Fig. 10f). Likewise, treatment of human Jak2VF-mutated cell-lines with a combination of RUX and the ERK-inhibitor Trametinib (Tram) in a xenograft model reduced persistent cells (Fig. 4j) and disease penetrance *in vivo* (Fig. 4k). These findings were confirmed using primary human Jak2VF-mutated BM cells in a patient-derived xenograft (PDX) model of MPN. Here, *in vivo* treatment with a combination of RUX and Tram following engraftment of human cells resulted in significant decrease of hCD45 PB (Extended Data Fig. 10g) and BM chimerism (Fig. 4l) and abrogated the Jak2VF clone in 2 of 5 recipients as confirmed by pyrosequencing of FACS-isolated hCD45 cells (Fig. 4m,n). To further consolidate ERK as a relevant target in Jak2 mutant

cells, we performed in-depth phosphoproteome analysis following *in vitro* and *in vivo* exposure to Jak-i (RUX) in primary human Jak2-mutated cells. In primary cells, Jak-i treatment significantly altered 618 phosphosites including relevant mRNA splicing factors (Extended Data Fig. 10h,i). Consistent with our previous findings, Jak-i treatment did not affect MAPK phosphorylation to a major extent (Extended Data Fig. 10j), unlike NfKB and STAT signaling (Extended Data Fig. 10k).

Many cancers that harbor mutated kinases can be targeted with kinase inhibitors due to their inherent dependency on this oncogenic hit. However, Jak-inhibitors fail to eliminate Jak-mutated clones, which has restricted their use to rather symptomatic approaches [2]. As a result of an oncogenic mutation, cancer cells may also develop secondary dependency on pathways or molecules that are not originally oncogenic [14]. Using in-depth phosphoproteomic analysis, our data provides a first unbiased and global view on relevant downstream effectors of mutated Jak2. We identified phosphorylation of Ybx1 by mutated Jak2 as a critical event required for Mknk1 mRNA splicing. Mknk1 is an essential component of ERK-signaling and required for maintenance of Jak2-mutated cells during Jak-i treatment, which exposes a major regulatory mechanism of disease persistence. While recent studies have highlighted the role of ERK-signaling in Jak2-mutated cells [15-17], we here discover how cell-intrinsic mechanisms of differential protein phosphorylation can result in splicing-dependent alterations of cell signaling (Extended Data Fig. 10l). Direct and specific inhibitors of Ybx1 are not available for clinical use so far, but identification of relevant protein domains using genetic screens may facilitate such developments in the future. Confirmation of dual Jak2-ERK targeting using primary cells *in vivo* establishes a therapeutic principle for Jak-mutated neoplasms and a strong experimental rationale for incorporating the Jak2-Ybx1 mediated regulation of Mknk1-dependent ERK signaling into therapies directed at the elimination of the persistent Jak-mutated clone.

FIGURES

Figure 1 | Functional phosphoproteomics screen identifies mRNA splicing and processing factor Ybx1 downstream of Jak2V617F. a, Experimental setup of the phosphoproteome screen. **b,** Unsupervised hierarchical clustering of significantly (t-test with permutation-based FDR < 0.01) regulated phosphosites in Jak2WT and Jak2VF cells. The panel highlight shows the top-upregulated phosphoproteins in WT (cluster-1) and VF (cluster-2) cells and the individual phosphosites with the amino acid position of significantly regulated Jak2-Stat signaling pathway members. Heatmap on the right shows enrichment of known Jak2 targets in WT and VF cells. **c**, Venn diagram showing differentially phosphorylated proteins in Jak2 WT (722 proteins), Jak2VF (1758 proteins), and shared (1655 proteins) between Jak2WT and Jak2VF. **d,** Heat map representation of significantly enriched motifs in Jak2VF assessed by Fisher's exact test (Benjamin-Hochberg FDR value ($-1\log_{10}$) shown) **e**, Sub-network map of significantly enriched GO terms ($p < 0.01$) of differentially phosphorylated proteins in Jak2VF. The node of the subnetwork color-range from bright to transparency based on their p-value. Highlighted nodes indicate the core components of the sub-network. Two-sided hypergeometric test, pV correction-Bonferroni step down (also Ext. Data Fig. 2a). **f,** Venn diagram depicting number of proteins common to highlighted nodes in **e. g,** Ranking of significantly phosphorylated proteins in Jak2VF. Phosphorylated proteins participating in mRNA splicing and processing are highlighted in red with top 15 proteins displayed in alphabetical order. **h,** shRNA validation of selected top 15 targets essential for Jak2VF cell survival and growth in the presence and absence of Jak-i (RUX, 0.5μM) measured by MTS assay (4 biologically independent experiments with 8 technical replicates each).

Figure 2 | Inactivation of Ybx1 selectively sensitizes Jak2V617F positive cells to Jak-inhibitor induced apoptosis. a, Immunohistochemistry for Ybx1 in bone marrow (BM) biopsies of MPN patients compared to healthy donor (HD) controls. Scale bar: 200μm. HD (n=18), MPN: essential thrombocythemia (ET; n=32), polycythemia vera (PV; n=23), myelofibrosis (MF; n=21) **b,** Quantification of Ybx1 abundance in Jak2VF+ BM biopsies (multiplied M-score). Box plot indicates mean ± SD, 10-90 percentile. **c**, Cell growth of Jak2VF cells following lentiviral infection with shRNAs targeting Ybx1 or non-targeting control (shNT) treated with Jak-inhibitor (Ruxolitinib; 1nM-10μM) measured by MTS assay (n=4 with 8 technical replicates). **d-f,** Percentage of apoptotic cells upon RUX treatment of Jak2VF+ cells following knockdown of Ybx1 (shYbx1-1/2) versus shNT. Mean ± SD **d,** Murine Ba/F3-Jak2VF cells (n=3) **e,** human Jak2VF+ cell lines HEL and SET-2 (n=5, each). **f,** primary murine lineage-negative BM cells (Jak2+/+, n=3 and Jak2+/VF, n=6). **g-k,** Persistence of Jak2VF+ clones upon Jak-i treatment following deletion of Ybx1. RUX treatment (90mg/KG, BID by oral gavage) and LMW-pIpC treatment (5mg/KG i.p. QD) days 10-15 following engraftment of donor cells. **g-i,** Peripheral blood (PB) counts of recipient mice, week 8 & 16. **g,** White blood count (WBC), **h,** hemoglobin (HGB) and **i,** platelet count (PLT) of Jak2VF-Ybx1-/- animals compared to Jak2VF-Ybx1+/+ controls (n≥5; mean ± SD). **j,** PB chimerism of irradiated (12Gy) recipient mice. Percent of Jak2VF-Ybx1+/+ or Jak2V617F-Ybx1-/- (CD45.2) cells / CD45.1-competitors, weeks 4-16 (n=9; mean ± SD) **d-j,** p-values determined by two-tailed t-test. **k,** Percentage of animals with loss (green) or persistence (gray) of Jak2VF clones in Ybx1-WT or -KO recipients, respectively. **l-m**, Kaplan-Meier survival curves of irradiated (2Gy) recipient NSGS mice transplanted with HEL cells expressing shRNAs as indicated. **l,** shYbx1-1/2 (n=12) or shNT (n=12). **m,** shYbx1-1/2 (n=12) or shNT (n=9) followed by RUX treatment (90mg/KG BID, by gavage, days 5-10). Logrank-test. **n-o,** PB chimerism of CD45.2 $Ybx1^{+/+}$ (n=9) or $Ybx1^{-/-}$ (n=9) cells / CD45.1/2 (competitors) of **n,** primary and **o,** secondary recipient mice weeks 4-16. Two independent cohorts; mean ± SD **a-o**, n indicates independent biological replicates.

Figure 3 | Targeting Ybx1 in Jak2V617F cells promotes intron retention. a, Gene ontology enrichment analysis of differentially expressed genes after inactivation of Ybx1 in murine Jak2VF cells. Bar plot shows significantly deregulated pathways with Fisher exact test p-values on the x-axis. **b,** Gene-set-enrichment analysis (GSEA) depicting negative enrichment of ERK-signaling related genes following inactivation of Ybx1. **c,** Percentage and type of splicing events (1943 differential splicing events with two-tailed test p-value <0.05) differentially regulated following inactivation of Ybx1; here 'Retained intron' (RI, yellow) is the significantly (70%) upregulated splicing event. **d,** RI read density profile between indicated exon-intron locations of Araf, Braf and Mknk1 genes in control (n=4) and Ybx1 targeted (n=4). **e,** Transcript (TPM by RNAseq) and protein (Log2-fold change by proteome analysis) expression levels of Araf, Braf and Mknk1 in control and Ybx1-depleted murine Jak2VF cells (n=4, two-tailed t-test). **f-g,** Representative Western blot (of n=3) validation of Mknk1 protein abundance following inactivation of Ybx1 in murine Ba/F3 Jak2VF and human HEL cells. **h,** Representative flow cytometry histograms showing Mknk1 levels of murine Jak2VF cells after 8h treatment with Herboxidiene (25nM) and Pladeinolide B (10nM). **i,** Violin-plot showing Mknk1 abundance (indicated by MFI) after Herboxidiene and Pladeinolide B treatment. (n=4; two-tailed t-test). **f-i**, n indicates independent biological replicates.

Figure 4 | Jak2-mutated clones are selectively vulnerable to inhibition of ERK signaling. a, Unsupervised hierarchical clustering of significantly down regulated 2012 phosphosites in Ybx1 targeted murine Jak2-mutated cells (2 shRNAs, NT-control). Averaged replicates (n=4); heatmap shows Z-scored $\log_2$ transformed phosphosite intensity. **b,** Kinase-substrate motifs significantly down-regulated in Ybx1 targeted Jak2VF cells (Benjamin-Hochberg FDR value ($-\log_{10}$)). **c,** Network map: proteins assigned as ERK substrates (significantly downregulated phospho-Serine (blue) and phospho-Threonine (purple) residues, indicated by position). **a,c,** ANOVA, permutation-based FDR <0.01. **d,** Representative flow cytometry histograms showing pERK levels in Jak2-mutated HEL- (left) and primary patient cells (right) following RNAi (shNT; shYbx1) and/or drug treatment (RUX 500nM, MEK/ERK-inhibitor Trametinib (Tram) 200nM). **e-g,** Pharmacologic inhibition of murine Jak2VF cells using **e,** RUX alone or in combination with the Mknk1 inhibitor (2µM; n=6, mean ± SD), **f,** RUX alone or RUX/Tram (100, 200nM; n=6, mean ± SD), **g,** Jak2-i Pacritinib (PAC) alone or PAC/Tram (100, 200nM; n=4, mean ± SD). **h,** Proportion of dead cells in FACS sorted CD34+ Jak2VF+ MPN BM cells (n=6) and CD34+ non-malignant controls (n=6) using RUX (500nM), PAC (200nM) or combinations with Tram (200nM). **i**, Representative Western blot (of n=3) showing reduction of Mknk1 protein abundance in HEL cells following RUX/Tram treatment. **j-k,** Xenograft model using NSGS mice transplanted with Jak2VF+ HEL cells and treated with RUX (n=12) or RUX/Tram (n=12). **j,** Percentage of hCD45+ BM cells (n=12/cohort; mean ± SD). **k,** Disease penetrance (%) in NSGS animals. hCD45 positivity (>1%, grey) or negativity (<1%, green) following RUX or RUX/Tram treatment, respectively. **l-n,** Patient derived xenograft (PDX) model to investigate primary Jak2VF+ patient BM cells of 3 different donors in humanized mice (NSGW41). Treatment of recipient animals with RUX (90mg/kg BID) or RUX/Tram (1mg/kg QD per gavage; 5d q 4wk). **l,** BM analysis of human cell chimerism in week 20 (n=5 per cohort). Box plot indicates mean ± SD, Min-Max. **m,** Quantification of JAK2V617F allelic burden by pyrosequencing in sorted hCD45+ BM cells of recipient animals. **d-m** p-values determined by two-tailed t-test. **n,** Pie charts of JAK2V617F allelic burden per individual mouse that were either RUX (grey) or RUX/Tram (green) treated. **d-n**, n indicates independent biological replicates.

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Disclosure of Potential Conflicts of Interest

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METHODS

Cell culture

Murine Ba/F3 cells stably expressing Jak2WT and Jak2VF, human SET-2 and HEL cells (purchased from and authenticated by DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Life Technologies). HEK293T cells (purchased from DSMZ) were cultured in DMEM+GlutaMAX™ medium (Life Technologies) supplemented with 10% FBS (Life Technologies). All cell lines were maintained in a humid atmosphere of 5% CO_2 at 37 °C. Cell lines were tested and maintained mycoplasma free throughout the study. Primary murine and human cells were cultured in StemSpan SFEM medium (Stemcell Technologies, Vancouver, Canada) supplemented with cytokines (murine: 100 ng/ml mSCF, 10 ng/ml mTPO, 6 ng/ml mIL-3 and 10 ng/ml mIL-6; human: hFLT3 100 ng/ml, hSCF 50 ng/ml, hIL-6 10 ng/ml, hIL-3 6 ng/ml; all Pepro Tech, Rocky Hill, NJ, USA).

Primary patient samples

All types of patient samples and healthy donor controls derived during routine biopsies (peripheral blood, bone marrow aspirates and biopsies) and investigated in this study were obtained after informed consent and according to the Helsinki declaration from the Tumor Banks in Jena and Magdeburg. Scientific protocol, patient information and patient approval forms have been approved by the respective local ethics committees ('Ethics Committee, University Hospital Jena' #4753/04-16 and 'Ethics Committee, Medical Faculty, OvGU Magdeburg' #115/08).

Focused lentiviral shRNA library screen

In brief, the Mission TRC lentiviral pLKO.1 shRNA vectors from Sigma targeting the top15 hits of mRNA processing and splicing factors enriched in Jak2VF were selected (in total 74 shRNAs, 4-5 different shRNAs per hit and 4 non targeting controls, the sequence of the shRNA is provided in the supplement) and lentiviruses were individually produced per shRNA by co-transfecting with 3rd generation packaging plasmids pMDL, pRSV and pVSVG in HEK293T cells seeded in a 10 cm culture dishes. The viruses were collected at 48hrs and 72hrs after transfection, syringe filtered through a 0.45µ syringe filters, concentrated using 30K Amicon Ultra-15 centrifugal filters (Merck) and the target murine Ba/F3 Jak2V617 cells were infected with polybrene (8mg/ml, Sigma). For screening purpose 200,000 murine Ba/F3 Jak2V617F cells in 2ml RPMI media with 10%hiFBS were seeded in each well of a 6 well plates. Concentrated virus was added per well (with polybrene 8mg/ml), centrifuged at 500 x g for 1 hour at 35ºC, subsequently 48hrs after transduction cells were selected with 1µg/ml puromycin for 2days. On day3 cells were washed, viable cells were counted and plated in 96 well plates (8 technical replicates per sample condition per experiment. In addition, each experiment was performed as technical duplicates) for growth assay with and without Jak2 inhibitor RUX (0.5µM). The plates were incubated at 37ºC and 5%CO_2 for 72 hours and subjected to Cell Titer 96 aqueous one solution (Promega) according to the manufacturers' protocol. Further viable cells were counted after 72 hours with the countess automated cell counter (Thermo Fischer Scientific) using trypan blue. Western blot was carried out to assess knock down efficiency on protein level for Pcbp1 (Abcam, ab168378, dilution 1:1000) and Ybx1(Abcam, ab76149, dilution 1:1000). For data analysis, the 8 technical duplicates were averaged and the values were normalized against non-targeting

controls included in each plates. The analysis of the normalized data between biological replicates showed correlation coefficient between r = 0.963 to 0.988 indicating high reproducibility of the procedure. Targets were considered potential candidates if 2 or more shRNA responded only in the Jak2 inhibitor treated group but not in the untreated controls of all the biological replicate experiments performed. Using these criteria, we choose our candidates for further characterization.

Genetic inactivation of murine and human Ybx1 by RNAi

To target Ybx1, 4 murine and 5 human shRNAs targeting Ybx1 (all received from Sigma-Aldrich, St. Louis, MO, USA) were validated and two selected shRNAs were used thereafter (mentioned in the supplementary shRNA table) with respective non-targeting controls. In brief, Ba/F3, SET-2, and HEL cells were lentiviral transduced with lentiviral particles by spinfection (872 x g for 1.5 hours at 33 °C). The cells were cultured for 2 days, puromycin selected for 48 hours and seeded ($5x10^6$ cells) followed by inhibitor treatment or addition of diluent control as indicated below. Cells were harvested and Ybx-1 knock-down was analyzed by qPCR and Western blotting.

Jak-i dose dependent cell growth and viability

shNT control or shYbx1 (two different shRNA targeting Ybx1 and Mknk1) targeted murine Ba/F3 Jak2VF cells were counted with the countess automated cell counter (Thermo Fischer Scientific) using trypan blue cell viability in 96 well plates for viable cells after 2 days of 1µg/ml puromycin selection. $3x10^4$ viable cells per well (8 technical replicates per row per sample condition) in 96 well plate was seeded in RPMI medium with 10%hi-FBS exposed to different concentrations of Jak-i RUX ranging from 1nM-10µM. The plates were incubated at 37ºC and 5%CO_2 for 72 hours and subjected to Cell Titer 96 Aqueous One Solution (Promega) according to the manufacturer's protocol. Viable cells were counted after 72 hours by trypan blue. Determination of IC50 inhibitory concentration of the Jak-i was calculated using GraphPad Prism™.

Drug combination treatments

Viable Ba/F3 Jak2VF cells were seeded at a density of 30,000 cells per well in RPMI medium with 10% heat inactivated FBS (Invitrogen) and exposed to Jak-i (0.5µM RUX) alone or in combination with different concentration of Mknk1 inhibitor CGP57380 (Sigma), MEK/ERK inhibitor Trametinib (Novartis), PI3K inhibitor LY294002, and p38 SB203580 (Merck) and incubated for 72 hours. Cell Titer 96 AQueous One Solution was added to the plates according to the manufacturers' protocol and measurements were performed after 4 hours. The plates were read at 490 nm in Tecan Infinite M200 and the response were analyzed using GraphPad Prism™.

Apoptosis assays

Cells stably infected with either non-targeting or target specific shRNA (Ybx1, Mknk1) were seeded in six-well plates and selected for 24 hours with puromycin. Primary murine lineage-depleted cells or FACS-sorted human CD34+ cells were incubated in 48 well plates. Inhibitor treatment was performed at concentrations as indicated for 48 hours unless otherwise stated. Apoptosis was measured by flow cytometry on a BD FACSCanto™ cytometer using Annexin V (1:50 dilution) in combination with SYTOX Blue or SYTOX Green or 7AAD as dead cell stains.

Proliferation assay with PCNA

After puromycin selection of shNT and shYbx1 murine Ba/F3 Jak2VF, washed in ice cold 1xPBS twice, fixed in 70% ethanol and permeabilized with 0.1% Tween-20. Cells were stained for PCNA-AlexaFluor488 conjugate (Biozol) on ice for 20 min.

Cell cycle analysis

For cell cycle measurements, 2x 10^6 murine Ba/F3 Jak2VF cells expressing shNT or shYbx1 were washed in ice cold 1x PBS twice, fixed in ice cold 70% ethanol for 30mins on ice and stored at 4ºC. After collection of biological replicates, samples were Ribonuclease A treated and stained with Propidium Iodide. The PI stained cells were measured using a BD FACSCantoII™ cytometer and the data was analyzed using FlowJo™ software (Treestar, Ashland, OR, USA).

ROS measurements using Carboxy-H_2DFFDA

In brief, 1x 10^6 murine Ba/F3 Jak2VF cells stably expressing shNT or shYbx1 were washed in 1xPBS twice and resuspended in 20µM carboxy-H_2DFFDA for 30 mins in dark at room temperature. Further the cells were washed in 1xPBS thrice and measured using BD FACSCanto™ cytometer. Data were analyzed in FlowJo™.

DNA damage analysis using yH2AX pS139

1x 10^6 murine Ba/F3 Jak2VF cells stably expressing shNT or shYbx1 cells were seeded on Poly-L-Lysin coated dishes for 2-4 hours, washed in PBS, fixed with 4% Paraformaldehyde, blocked in blocking buffer (0.2% Triton-X,1%BSA and 5%Normal rabbit serum) and incubated over night with rabbit yH2AX pS139 antibody (Cell Signaling, 2577, 1:800) overnight. After overnight incubation, samples were washed and incubated with secondary antibody (anti-rabbit-Alexa 568). DAPI was used for nuclear staining (NucBlue™, ThermoScientific). Positive control samples were prepared by exposing the Ba/F3 Jak2VF cells expressing shNT to 20 mins UV under the cell culture hood. Imaging was performed using Zeiss LSM 780 microscope and processed in Zen Black software tool.

Label free phospho-proteome sample preparation

Samples were processed according to the protocol previously described [8]. Briefly, samples were collected as quadruplicate biological replicates for each condition, lysed in Gmdcl buffer (6M Gdmcl, 100mM Tris pH8.5, 10mM TCEP and 40mM CAA), heated for 5 mins at 95°C and cooled on ice for 15min. Lysed samples were then sonicated (Branson probe sonifier output 3-4, 50% duty cycle, 10x 30 sec) and heated again. Proteins were precipitated with acetone, BCA quantified. 2mg were digested with LysC and Trypsin overnight at room temperature and phosphopeptides enriched by TiO2 beads. The enriched peptides were desalted, washed and eluted on StageTips with 2 layers of SDB-RPS material with elution buffer (80% Acetonitrile and 5% NH4OH). The eluted peptides were vacuum centrifuged until dryness and reconstituted in 2% ACN /0.1% TFA. All the samples were stored in -20ºC until measurement.

Phosphoproteome of primary patient samples

In brief, peripheral blood samples from patients with Jak2 mutated myeloproliferative neoplasms were collected, granulocytes isolated, and treated with DMSO or Ruxolitinib 0.5µM for 2 hours *in vitro.* Also, for *in vivo* exposure, cells were isolated from MPN patients before and 2 hours after dosing of Ruxolitinib (10mg to 20mg oral dose during routine treatment). Cells were lysed and processed in 4% SDC buffer (4%SDC,100mM Tris pH8.5, 10mM TCEP and 40mM CAA), heated for 5 mins at 95°C and cooled on ice for 15min. Lysed samples were sonicated, heated again for 5 mins and BCA quantified. Approximately 350µg of proteins were digested with LysC and Trypsin overnight at room temperature and phosphopeptides were enriched by TiO2 beads as described elsewhere [18].

Deep-proteome quantification

Cell were lysed and peptides were prepared as previously described [19]. Proteome samples of phosphoproteome analysis were

collected after TiO_2 enrichment. In brief, cells were lysed in 1% SDC buffer (1%SDC, 100mM TrispH8.0, 40mM CAA and 10mM TCEP), heated for 5mins at 95°C, cooled on ice for 15mins and sonicated (Branson probe sonifier output 3-4, 50% duty cycle, 10x 30 sec). 25µg were digested with LysC and Trypsin overnight and peptides were eluted on Stage Tips with 3 layers of SDB-RPS material with elution buffer. The eluted peptides were vacuum centrifuged until dryness and reconstituted in 2% ACN /0.1% TFA. All the samples were stored in -20ºC until measurement.

Drug-perturbed phosphoproteome profiling

In order to profile the kinase inhibitor action on Jak2VF, murine Ba/F3 expressing Jak2VF cells were treated with 0.5µM Jak2 inhibitor Ruxolitinib (Selleckchem, S1378) for 2 hours and 10µM MEK inhibitor PD0325901 (Sigma), 10µM p38 inhibitor SB203580 (Merck), 20µM JNK inhibitor SP600125 (Sigma), 50µM PI3K inhibitor LY294002 (Merck), 10µM AKT inhibitor MK2206 (Enzo Life) and 100nM mTOR inhibitor Torin-1 (Millipore) for 1 hour. The cells were lysed in Gmdcl buffer and processed as mentioned in the phospho-proteome sample preparation protocol.

Ybx1 interactome preparation

For Ybx1 affinity purification, cells were lysed in 150mM NaCl, 50mM Tris (pH7.5), 5% glycerol, 1% IGPAL-CA-630 (Sigma), protease inhibitors (EDTA free, Roche), 1% Benzonase, and 1mM MgCl2 for 30 min on ice. 1mg of total lysate was precleared with protein A sepharose beads, incubated with validated Ybx1 antibody (Abcam ab76149) overnight and 30µl of rec-protein A sepharose 4B conjugates (Invitrogen) for 2 hours. Non-specific binders were removed by three washes with wash buffer I (150mM NaCl, 50mM Tris (pH7.5), 5% glycerol, 0.05% IGPAL-CA-630) and three washes with wash buffer II (150mM NaCl, 50mM Tris (pH7.5)). The bound proteins were on-beads digested with Trypsin and LysC overnight. The peptides were desalted on C_{18} Stage tips and analyzed by mass spectrometry.

Liquid chromatography (LC) -MS/MS measurement

For the LC-MS/MS analysis, Q-Exactive mass spectrometer with a nanospray ion source connected online to an Easy-nLC 1000 HPLC system was used. Peptides were separated on an in-house prepared 50cm C_{18} columns (75µM inner diameter with 1.9µM C_{18} ReproSil particle, Dr. Maisch GmbH) in a 140 minute gradient from 5%-65% in buffer B (0.5% formic acid, 80% acetonitrile). The column temperature was maintained at 50°C using column oven (in-house made). Peptides were analyzed with a full scan (300-1600 m/z, R=60,000 at 200 m/z) at a target of 3e6 ions, followed by high energy collisional disassociation-based fragmentation (HCD) of top10 most abundant isotope patterns with a charge ≥2 MS/MS scan, detected in the orbitrap detector (R=15,000 at 200 m/z). Dynamic exclusion of sequenced peptides was set to 40s and apex trigger (4 to 7s) were on. All data were acquired using X-caliber software (Thermo Scientific).

Data processing with Maxquant

Mass spectrometric raw files were processed using the Andromeda search engine integrated into Maxquant [20] environment (1.5.5.2 version). The MS/MS spectra were matched against the mouse (UniProt FASTA 2015_08) database with an FDR < 0.01 at the level of proteins, peptides and modifications. The search included fixed modification for carbamidomethyl and in the variable modifications table phosphoSTY was added additionally for the phosphorylated peptide search to the default settings. Peptides with at least seven amino acids were considered for identification. Maximum two missed cleavages were allowed for protease digestion. Match between run was enabled with the matching window of 1 min to transfer peptide identification to across runs based on normalized retention time and high mass accuracy.

Phosphoproteome data analysis

Perseus[21] software (1.5.2.11 version) environment was used for all Maxquant output table analysis. For phosphoproteome analysis, sample for class –I phosphosites (localization probability >0.75) and required a minimum of 3 or 4 valid values in each of the biological quadruplicates Statistical analysis of was performed on the logarithmized intensities values. Significance was assessed by Student's t-test using permutation-based FDR, to identify the significantly regulated phosphosites. In group comparisons two sample t-test or for multiple samples comparison ANOVA test was performed with permutation-based FDR cut-off 0.01 or 0.05. The significantly regulated phosphosites were filtered, Z-scored and represented as either unsupervised hierarchical clustered heat maps or profile plot. Annotations were extracted from UniprotKB, Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome. Kinase-substrates relationships and Kinase motifs were extracted from phosphosite plus database (phosphosite.org). Fischer exact test was performed to discover motifs and annotations that are significantly regulated in the sample groups. For phosphosite occupancy calculation, the proteome and phosphoproteome of corresponding samples were matched in maxquant to estimate occupancy, occupancy ratio and occupancy error scale using the extracted signal difference of modified peptide, unmodified peptide and the corresponding protein ratios (described earlier, [8]). Phosphoprotein network architecture were obtained using String database and further networks and sub-networks were analyzed and visualized in *Cytoscape (version 3.5.1)*.

RNA sequencing and ChIP sequencing

Transcriptome profiling of Ba/F3 Jak2VF cells transduced with shNT or shYbx1 cells was performed using a strand-specific RNA sequencing protocol. In brief, total RNA was isolated from 2x 10^6 cells using NucleoSpin RNA Kit according to the manufacturers' protocol (Macherey Nagel). RNA library for sequencing was prepared using NEBNext Poly(A) mRNA Magnetic Isolation Module. The quality was analyzed on a Bioanalyzer (Agilent 2100 Bioanalyzer) high sensitivity DNA assay. Samples were sequenced on Illumina Nexseq500 and multiplexed reads were demultiplexed on the basis of their barcodes. Sequencing reads were filtered, trimmed and then mapped to the Ensembl gene annotation and the mouse genome assembly GRCm38 using STAR aligner [22] with ENCODE settings in two-pass mode considering splice junctions across all samples in the second mapping step. Gene counts were quantified using *featureCounts* [23] and differential expression calculated with the *limma-voom* pipeline [24]. Gene and transcript expression levels were quantified using RSEM. Event level differential splicing was calculated with the *EventPointer* package [25] in R. Chromatin-immunoprecipitation (ChIP) was performed using a ChIP-validated Ybx1 antibody (Abcam #76149). Cells were crosslinked (1% formaldehyde, 10 min) and nuclei were isolated, lysed, and sonicated to yield chromatin fragments of about 200–500 bp. Chromatin was diluted with ChIP buffer, pre-cleared with Protein A/G Sepharose that has been blocked with 1 mg/ml BSA and 1% gelatin from cold water fish skin. Chromatin from 5x10^6 cells was incubated with 5µg anti-YBX1 antibody overnight followed by immobilization on sepharose. After extensive washing, immuno-precipitated material was eluted with 1% SDS/0.1 M NaHCO3 and chromatin was de-crosslinked (65C, 6h) [26]. ChIP-sequencing libraries were prepared using the ThruPLEX DNA-library kit (Takara) and sequenced on an Illumina NextSeq platform. To remove the adaptors and low-quality parts of DNA reads trimming was done using FASTX-Toolkit (Quality threshold was specified as 20 and the minimum length of trimmed reads is 1, version 0.0.14) and TrimGalore (version 0.4.4). After trimming, the DNA read mapping to reference sequences is performed by Segemehl with default parameters (version 0.2.0-418). Samtools (version 0.1.19) was used for conversion from SAM format to BAM format. Reads were aligned using Bowtie 2 (version 2.4.0). For peak calling, MACS2 (version 2.1.1) was applied, and Q value is assigned as 0.01. HOMER (version 4.11) was used for call motifs. Analysis was performed using Basepair^{TM} software tool default settings (version3).

***In vitro* transcription**

Intron retained Mknk1 region (Exon12-Exon13) was cloned with T7 promoter. Biotin labelled Mknk1 mini pre-mRNAs (Biotin-11-dUTP, Cat# NU-803-BIOX, Jena Bioscience) was invitro transcribed at 37°C for 4 hours using MegaScript T7 RNA polymerase (AM1333, Thermo Fisher) with 10:1 ratio of Ribo m7G Cap Analog to GTP (P171A, Promega), DNase I treated for 15mins at 37°C followed by RNA purification using NucleoSpin RNA columns (Cat#740955,Macherey-Nagel).

***In vitro* splicing assay**

Nuclear extract from BaF3 cells with and without Ybx1 by shRNA targeting was prepared and splicing assay was performed as described elsewhere[27]. Briefly splicing reaction of 25µl contained: 50µg of nuclear extract, 10nM RNA substrate, 1nM DTT, 3.7% polyvinyl alcohol (low molecular weight, Sigma), 1mM magnesium acetate, 1.7mM ATP, 17 mM phospho creatine, 20 mM glycine, 2.5µl 10U RNasin Plus (Promega). Splicing reaction was incubated at 30°C for 2hours; reaction was stopped by adding Proteinase K for 30mins at 45°C followed by streptavidin pull down of biotinylated RNA. The purified RNA was reverse transcribed using RevertAid first strand cDNA synthesis protocol using random hexamer as primers (Cat# K1621, Thermo Scientific), PCR was performed with the following Mknk1 primer (Forward 5'-AACAAGCTGTTTGAGAGCATCCAG-3' and Reverse 5'- CTCTGAAGGACTTGCGGCGTG-3'), and the products were resolved in Agarose gel and visualized with safe red gel staining.

Genetic inactivation by genome editing using CRISPR/Cas9

Guide RNAs targeting *Ybx1* were designed using the Broad GPP tool and sequences of sgRNAs are provided in Supplementary Table-1. For cloning of sgRNA sequences, the improved-scaffold-pU6-sgRNA-EF1Alpha-PURO-T2A-RFP (ipUSEPR) vector system[28], with puromycin resistance and RFP selection marker was used (kindly provided by Prof. Scott Armstrong, Department of Pediatric Oncology, Dana Farber Cancer Institute, Harvard University, Boston, MA, USA). HEK293T cells were transfected using FUGENE®HD Transfection Reagent (Promega) to generate lentiviral particles as described above. Ba/F3 Jak2V617F-GFP_Cas9-Blast cells were infected twice (8 hours gap in between) by spinfection (872xg, 1.5 hours, 33°C). The cells expressing sgRNAs were selected with 1 µg/ml puromycin starting on day 2 post-infection. For knockout confirmation, cells were collected for RT-qPCR or Western Blotting on day 8 post-infection.

NMD and spliceosome machinery inhibition

For treatment with inhibitors of nonsense-mediated decay (NMD), Ba/F3 Jak2V617F_Cas9 cells transduced with either sgLuc or 2 different sgRNAs targeting Ybx1 were treated with Dorsomorphin (Compound C; 10 µM, 24 hours; Abcam, Cambridge, UK;) or VG-1 (5 µM, 10 µM, 20 hours), respectively. DMSO treated cells were used as a control. For inhibition of splicing machinery, Ba/F3 Jak2V617F cells were treated with either Pladienolide B (10nM, 8 hours; Santa Cruz Biotechnologies, Dallas, TX, USA) or Herboxidiene (25nM, 8 hours; Cayman Chemical, Ann Arbor, MI, USA), respectively. DMSO treated cells were used as a control.

Mouse genotyping

Genotyping of tails from conditional Ybx1 knockout mice was performed using the following primers: Ybx1_cond_for (GCCTAAGGATAGTGAAGTTTCTGG), Ybx1_con_rev (CCTAGCACACCTTAATCTACAGCC),

Cre_for (CGTATAGCCGAAATTGCCAG), Cre_rev (CAAAACAGGTAGTTATTCGG). Genotyping PCR was performed using the Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' protocol.

Histology staining and immunohistochemistry

Formalin-fixed and paraffin-embedded bone marrow biopsies with proven myeloproliferative neoplasia or primary samples without histopathologic abnormalities were retrieved from the archival files of the Institute of Pathology, Otto-von-Guericke University Medical Center, Magdeburg, Germany. All MPNs were diagnosed and classified according to the World Health Organization (WHO) 2008 classification in synopsis with clinical data and presentation. The study comprises of biopsies derived from 76 MPN patients (PV (n=23), ET (n=32) and MF (n=21)) compared to healthy donor controls (n=18) or BCR-ABL positive CML (n=17). Immunohistochemistry was performed using a monoclonal Rabbit-anti-human Ybx1 antibody (Abcam; ab76149) in a dilution of 1:100. Spleen, liver and lung from Ybx1+/+ Jak2VF and Ybx1-/- Jak2VF mice treated with RUX were fixed in 4% paraformaldehyde for 24 hours followed by incubation in 30% ethanol for 30 min and 50% ethanol for 24 hours. Organs were embedded in paraffin and paraffin sections were cut on a rotary microtome (Microm HM 355S, Thermo Fisher Scientific), mounted on microscope slides (Thermo Fisher Scientific) and air-dried in an oven at 37°C overnight. Tissue section slides were then processed automatically for H&E staining (Leica AutoStainer XL, Leica Biosystems, Wetzlar, Germany). Images were acquired at 10x magnification on an AxioImager A.2 (Carl Zeiss Microscopy, Jena, Germany). Images were processed using the ImageJ software (NIH, Bethesda, MD, USA).

Experimental animals

All mice were housed under pathogen-free conditions in the accredited Animal Research facility of the Otto-von-Guericke University – Medical Faculty, Magdeburg or the University Hospital Jena. Animals were maintained in groups in single-ventilated type II long IVC-cages. Room temperature was 22°C +/- 2°C and humidity was maintained at 55% +/- 10%. Mice were kept in rooms with light/dark cycle (6am-20pm: light; 20pm-6am: dark; including a twilight phase in between). All animal experiments have been approved by the respective ethics committees for animal welfare either at the Landesverwaltungsamt Saxony-Anhalt, Halle (State Administration Office Saxony-Anhalt) or at the Landesamt für Verbraucherschutz, Abteilung Gesundheitlicher und technischer Verbraucherschutz, Referat Verbraucherschutz und Veterinärangelegenheiten (Thuringian State Administration Office), Bad Langensalza, Thuringia, Germany. Conventional Ybx1 knockout mice have been generated as previously described [29]. Mice harboring a 'floxed' (flanked with loxP sites) allele of Ybx1 have been generated at Taconic-Artemis in a C57BL/6 background. Conditional Jak2V617F knock-in mice have been described previously[30]. In pre-clinical patient-derived xenograft mouse models paired-samples of patient cells were investigated for response to drug-treatment versus diluent control. Therefore, no randomization was necessary. Likewise, murine cells that were either genetically modified or pharmacologically treated were injected at equal distribution into recipient mice. Due to the analysis in paired samples, with pharmacologic therapy or diluent control, no blinding was necessary and possible at the stage of cell transplantation or drug treatment. Animals were maintained in groups in single-ventilated type II long IVC-cages. Room temperature was 22°C +/- 2°C and humidity was maintained at 55% +/- 10%. Mice were kept in rooms with light/dark cycle (6am-20pm: light; 20pm-6am: dark; including a twilight phase in between). Specification of sex and gender for the different mouse strains: Ybx1 +/+ or Ybx1 -/- donor mice, females and males were used, age 6-8 week old; Ybx+/+ VF-Mx or Ybx-/- VF-Mx donor mice, females and males were used, age 6-8 week old; C57BL/6J (CD45.2) recipients, only females were used, age 6-8 week old; Ly5.1

(CD45.1) recipients, only females were used, age 6-8 week old; CD45.1/2 competitors, males and females were used (female competitors were paired with female donors and male competitors were paired with male donors), age 6-8 week old; NSGS recipients, only females were used, age 6-8 week old. NSGW41 mice were generated and maintained as previously described [31].

Hematopoietic progenitor cell assays

Colony formation assay: For investigation of colony formation in methylcellulose, LSK (Lin−Sca1+KIT+) cells were sorted from bone marrow of the respective donor mice as previously described. $1x10^3$ cells were seeded in MethoCult M3434 (Stem Cell Technologies), respectively. Colony numbers were counted on day 8 after plating as previously described [32].

Spleen colony formation assays (CFU-S12): bone marrow cells were collected from donor mice and $1x\ 10^2$ LSK cells were FACS sorted and injected via tail vein into lethally irradiated (12Gy TBI) C57BL/6 recipient mice. At day 12 post-injection, spleens from recipient mice were harvested and stained with Bouin's fixative solution (Sigma-Aldrich), and colonies were counted as previously described [33].

Transplantation assays and *in vivo* treatment

For competitive repopulation assays $2x\ 10^6$ BM cells (for Jak2WT) or $5x\ 10^4$ sorted LSK cells (for Jak2VF) of 6-8 week old $Ybx1^{-/-}$ or $Ybx1^{+/+}$ (CD45.2) littermates and $2x\ 10^6$ (CD45.1/2) competitor cells (derived from intercrossing CD45.1 animals with CD45.2 animals purchased from Charles River) were transplanted via lateral tail vein injection into lethally irradiated (12Gy, single dose) 6-8 week old Ly45.1 mice (Jackson Laboratories, Bar Harbor, ME). For serial transplantation experiments whole BM of primary recipient mice was harvested and $2x\ 10^6$ whole BMC were injected into lethally irradiated secondary recipients. Ruxolitinib was purchased at Selleckchem (Selleckchem, S1378) and formulated for administration by oral gavage as previously described [34]. Mice received the Jak1/2 inhibitor Ruxolitinib at a dose of 90 mg/kg or vehicle control by oral gavage BID. For xenografting of Jak2-mutated human cells, HEL cells were either infected with lentiviral particles for transduction of the respective shRNAs (shNT or shYbx1) or incubated for 24 hours with inhibitors as indicated. $1x\ 10^6$ viable cells were injected in each irradiated (2Gy) recipient NSGS mouse via lateral tail vain injection. Engraftment and expansion of human cells was monitored weekly by the presence of hCD45-positive cells in the peripheral blood. For patient derived xenograft experiments (PDX) we used an improved model for human HSC transplantation and analysis, that has been developed by her group from immune-deficient mouse strains containing Kit mutations (NSGW41) [31]. These mice can be engrafted without prior conditioning and therefore maintain an intact niche and microenvironment. Primary bone marrow samples were acquired during routine biopsies and cells were isolated by Ficoll gradient centrifugation followed by depletion of CD3 positive cells. $1.8\text{-}2x\ 10^6$ stem- and progenitor cells (HSPCs) were engrafted (pairwise) per animal. Mice were followed for 4 weeks and peripheral blood chimerism of human CD45 positive cells was measured by flow cytometry. Between weeks 4 and 20 all animals were treated for 5 days every 4 weeks with either Ruxolitinib (90mg/kg BID per gavage) or the combination of Ruxolitinib with the MEK/ERK-inhibitor Trametinib (1mg/kg QD per gavage).

Quantification of JAK2VF mutant cells by pyrosequencing

In order to assess for the relative abundance of JAK2-mutated cells within the patient derived xenograft (PDX) model, we sorted human CD45 positive cells from the bone marrow at week 20 and performed pyrosequencing for the JAK2V671F mutation. DNA isolation and whole-genome amplification were carried out on FACS-sorted hCD45 positive cells using the REPLI-g Single Cell Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplicons were generated

using AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA; biotinylated forward primer: GAAGCAGCAAGTATGATGAGCA; reverse primer: TGCTCTGAGAAAGGCATTAGAA) according to standard protocols. Samples were then analyzed by pyrosequencing (PyroMark Q96 ID, Qiagen, Hilden, Germany; sequencing primer: TCTCGTCTCCACAGA) to assess for the mutational status of the *JAK2*V617F variant of the individual subpopulations.

Rescue of shRNA-inactivated endogenous Ybx1 with exogenous enforced expression of Mcl-1

Ba/F3 cells expressing EpoR (MSCV-EpoR-Neo) and Jak2VF-GFP (MSCV-Jak2VF-GFP) were infected with retrovirus expressing empty vector (MSCV-Puro) or Mcl-1 (MSCV-Mcl-1-Puro). Knockdown of Ybx1 was performed as indicated.

Immunoprecipitation and Immunoblotting

For western blot analysis samples were collected in freshly prepared RIPA buffer containing 1X Protease Inhibitor cocktail (Roche) and 1X PhosphoSTOP (Roche). For immunoprecipitation samples were collected in freshly prepared IP buffer (150mM NaCl, 50mM Tris (pH 7.5), 5% glycerol, 1% IGPAL-CA-630, protease inhibitors (EDTA-free), 1mM MgCl2, Benzonase, 1X Protease Inhibitor cocktail and 1X PhosphoSTOP). In case of Jak2-Ybx1 interaction analysis Ba/F3 EpoR and wild-type Jak2 or Jak2V617F cells were washed twice with PBS and starved for 4 h in serum-reduced (0.5%) medium at a density of $1x10^6$/ml. TrueBlot Anti-Rabbit Ig IP Beads Kit (Rockland Immunochemicals, Gilbertsville, PA, USA) was used following the manufacturer`s instruction. The following antibodies were purchased from Cell Signaling (Danvers, MA, USA) and used at a 1: 1000 dilution: GFP (2555S), Mknk1 (2195), p-Akt (9271), Akt (9272), p-p44/42 MAPK (9106), p44/42 MAPK (9102 and 4695), cRaf (9422), p-JAK2 (3771), JAK2 (3230), pSTAT3 (9134), STAT3 (9139), Bim (2933), and Bcl-XL (2764). GAPDH antibody (H86504M, 1: 5000) was purchased from Meridian Life Sciences (Memphis, TN, USA), p-Stat5 antibody (05–495, 1: 1000) was purchased from Millipore (Darmstadt, Germany) and Stat5 (sc-1081, 1: 100) antibody from Santa Cruz Biotechnologies (Dallas, TX, USA). Mcl-1 antibody (600-401-394, 1:1000) was received from Rockland Immunochemicals (Limerick, PA, USA), Vinculin from Sigma (V9131, 1:5000) and Ybx1 antibody (ab76149, 1:1000) was delivered by Abcam (Cambridge, UK). All the uncropped western blot images were provided as source data in the supplement data figure.

Flow Cytometry

For immunophenotype analysis, peripheral blood cells, bone marrow or spleen cells were resuspended in PBS/1% FBS after erythrocyte lysis (PharmLyse™, BD Pharmingen). Unless otherwise stated, the following antibodies were used: Sorting and analysis of LSK-cells or Sca-1^+cells were performed as previously described [35,36]. Biotinylated antibodies against Gr-1 (RB6-8C5), B220 (RA3-6B2), CD19 (6D5), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), TER119 and IL7Ra (A7R34) (all Biolegend, San Diego, CA, USA) were used for lineage staining in a 1:500 dilution. An APC-Cy7- or BV421-labeled streptavidin-antibody (Biolegend) was used for secondary staining together with an APC-anti-KIT (clone 2B8) and a FITC- or PE-anti-Sca-1 antibody (clone E13-161.7). All surface marker antibodies were used in a 1:100 dilution. Cells were sorted on a BD FACSAria™ III, analysis of the cells was performed using a FACSCantoII™ (Becton-Dickinson) cytometer and FlowJo™ software (Treestar, Ashland, OR, USA). Fix & Perm Kit (Life Technologies) was used for intracellular staining according to the manufacturer's protocol. The following antibodies were used: MNK1 antibody (Thermo Fisher Scientific, 711542; 1:100), p-p44/42 (ERK1/2)-Alexa Fluor 647 (Cell Signaling, 13148S, 1:50).

Immunofluorescence analysis (Jak2-Ybx1 co-localization)

2x 10^4 cells were washed and seeded onto adhesion slides (Marienfeld). Attached cells were fixed in PBS/2 % PFA/0.01 %

glutaraldehyde for 15 min on ice followed by permeabilization for 10 min with PBS/0.02% Triton X100 at room temperature. After blocking for 30 min with PBS/1%BSA/0.1% Tween 20, Ybx-1 (Abcam ab76149, 1:250) or was incubated in blocking solution 1h. The samples were washed 5 times 5 min with PBS followed by incubation of Alexa Fluor 488 or 568 donkey anti-rabbit antibody (Life Technologies A21206, A11036 at 1:200 dilution) for 1h. After additional washing, Jak2 labeled with Cy3 (BIOSSUSA bs-0908R-Cy3, 1:50) was incubated in blocking solution for 1h. In the following, the samples were washed, incubated with DAPI (Thermo Scientific #R37606) for 10 min and mounted using ProTaqs® Mount Fluor (Quartett, 401603095). Samples were analyzed using confocal microscope Leipa SP8 or Zeiss LSM-780 and ImageJ for quantification.

Generation of Ybx1 phosphorylation mutants

Phophorylation mutants mimicking hyperphosphorylation or de-phosphorylation of Ybx1 were generated by site-directed mutagenesis (as previously described [37]) at amino acid residues that were (i) highly conserved and (ii) differentially phosphorylated in the absence or presence of mutated Jak2 kinase. These aspects applied to the murine serine residues S30, S34, S172 and S174. In detail the following mutants were generated by site directed mutagenesis using a retroviral MSCV-IRES-GFP backbone: (1) MIG-mYbx1-S30A/S34A; (2) MIG-mYbx1-S30A; (3) MIG-mYbx1-S34A; (4) MIG-mYbx1-S30D/S34D; (5) MIG-mYbx1-S30D; (6) MIG-mYbx1-S34D and (7) MIGmYbx1-S172A/S174A. Constructs were expressed in murine Ba/F3-JAK2VF cells. In brief, cells were infected by co-localization of virus supernatant (containing the respective constructs as indicated above) with Ba/F3-Jak2-V617F(VF) cells on retronectin-coated plates. Infection has been repeated after 24 hours and GFP-positive cells were sorted to ensure expression of the mutants in a homogeneous population.

Data availability

The MS raw data files and Maxquant output files reported in this manuscript are available at ProteomeXchange Consortium with the data identifier PXD006921. RNA-seq data have been deposited in the Gene expression Omnibus database with accession number GSE123417. ChIP-Seq data has been deposited to the Gene expression Omnibus database with the accession code GSE154025 for mouse and GSE146717 for human. All other data supporting the findings of this study are available from corresponding authors upon reasonable request.

Statistical Analysis

For survival analysis, Kaplan-Meier curves were plotted using GraphPad Prism™ version 6.0h (GraphPad Software, SanDiego, CA). Differences between survival distributions were analyzed using the logrank test. Statistical analyses were performed using Student`s t test (normal distribution) or Mann-Whitney U test (when normal distribution was not given). P less than 0.05 was considered statistically significant.

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Extended Data Figure 1 | Phospho-proteomic analysis uncovers differential regulation of splicing factors in Jak2-mutated cells. a, Schematic of the phosphoproteome workflow. Following sample collection, phosphopeptides were enriched using EasyPhos work flow[8] and analyzed in single-run LC-MS/MS. Data were analyzed in Maxquant and Perseus. **b,** Quantified phosphosite depth per sample. Samples were measured as biological quadruplicates**. c,** Heatmap of sample correlation matrix of all measured samples (in b) based on Pearson correlation values. The reproducibility between the phosphoproteome sample is highlighted**. d,** Summary of identified and quantified class-I phosphosites (localization probability of >0.75) corresponding to number of proteins of this experiment. **e,** Principal component analysis of the samples. **f,** Network map of significantly enriched GO terms (p-value<0.05) of differentially phosphorylated proteins in Jak2V617F. Phosphorylated proteins significantly regulated in Jak2V617F were subsequently used as an input for *Cytoscape* to obtain the network. The highlighted sub-network was obtained with p-value <0.01 and Kappa score >0.6. Two-sided hypergeometric test, pV correction- Bonferroni step down **g-h,** Western blot validation of shRNA library targets. **g,** Pcbp1 protein and **h,** Ybx1 protein in murine Jak2-V617F cells. n=3 with comparable results. **i,** Pearson correlation profile of the independent shRNA experiments.

Extended Data Figure 2 | Functional consequences of Ybx1 depletion in Jak2-mutated cells. a, *In vitro* shRNA validation of selected top 15 targets essential for Jak2VF cell survival and growth in the presence (green) and absence (grey) of Jak-i (Rux, 0.5μM) measured by proliferation assays (16 technical replicates measured in 2 plates independently, mean). **b,** Immunofluorescence analysis of Ybx1 and Jak2 localization in Jak2V617F positive Ba/F3 cells (left) and murine Jak2V617F positive stem- and progenitor cells (lineage-negative cells) (right panel) (representative image of n=3). **c,** Immunoprecipitation of Jak2 receptor from murine Jak2VF cells showing binding of Ybx1 to mutated Jak2-receptor (n=3 with similar results). **d-e,** Percentage of cell growth **d,** in Jak2WT and **e,** in Jak2VF cells following lentiviral infection with shRNAs targeting Ybx1 or non-targeting control (shNT) measured by MTS assay **f,** Representative histogram and bar plot showing ROS levels measured in Jak2VF cells with shRNAs targeting Ybx1 or control. **g,** Representative histogram and bar plot showing proliferative marker PCNA levels in Jak2VF cells with shRNAs targeting Ybx1 or control **h,** Cell cycle analysis after Ybx1 inactivation in Jak2VF cells. **d-h,** n=4 independent experiments, mean ± SD, two-tailed t-test with equal variance**. i,** Representative confocal images of DNA damage marker yH2AX pS139 in Jak2VF cells following shRNAs targeting Ybx1 or non-targeting control. Cells exposed to UV light for 20 minutes were used as positive controls (n=4 independent experiments). **j,** FACS plots showing percentage of apoptotic cells in Jak2+/+ and Jak2VF/+ murine BM cells following Jak-i treatment (RUX 0.5μM) after lentiviral knockdown of Ybx1 (sh1 and sh2) compared to non-targeting control (shNT).

Extended Data Figure 3 | Functional consequences of Ybx1 deletion *in vivo*. a, Schematic representation of the wild-type Ybx1 allele (Genomic Locus), the targeting vector (Targeting Vector), the desired targeted allele (Targeted Allele), the desired conditional allele flanked by LoxP sequences (Conditional Allele), and the null recombined allele (Recombined Allele) after Cre-mediated recombination of the conditional allele. Triangles indicate LoxP sequences. **b,** Excision control by genomic PCR on whole bone marrow cells at week 16 following genetic inactivation of Ybx1 in conditional knockout mice. Representative micrograph of n=5 animals from a total of n=9 controls (+/+) and n=9 knockout (-/-) replicates. **c,** Schematic as in Fig. 2j. FACS plots showing percentage of Jak2V617F (CD45.2) cells of $Ybx1^{+/+}$ or $Ybx1^{-/-}$ recipients. **d,** Histology of liver, spleen and lung of Jak2V617F-$Ybx1^{+/+}$ and Jak2V617F-$Ybx1^{-/-}$ recipient mice at week 20 after BMT. Representative micrographs of n=9 individual mouse replicates. Hematoxylin and eosin stain (H&E) at 10x magnification. Focal leukocyte infiltration (arrows) and hemorrhage (stars) of liver, spleen and lung, respectively. Scale bar: 200μm. **e,** Peripheral blood chimerism of lethally

irradiated (12Gy) recipient mice. FACS plots showing abundance of CD45.2 myeloid cells in Jak2V617F-Ybx1$^{+/+}$ and Jak2V617F-Ybx1$^{-/-}$ recipient mice at week 20 after BMT. **f,** Design for assessment of steady state hematopoiesis. **g,** White blood count (WBC)**,** Gr-1 positive cells (Gr1+), hemoglobin (HGB) and platelets (PLT) following genetic inactivation of Ybx1 (Ybx1$^{-/-}$ mice, n=10) compared to Ybx1$^{+/+}$ controls (n=10). Data represented as mean ± SEM. **h,** FACS plots showing comparable percentage of LSK-cells and HSCs (SLAM^{+}CD34^{-}L^{-}S^{+}K^{+} cells) following genetic inactivation of Ybx1 in conditional knockout mice (compared to wildtype littermate controls). **i,** stem- and progenitor cell numbers per 1x 10^6 whole bone marrow cells at week 16 after genetic inactivation of Ybx1 (n=6; mean ± SD). **j,** FACS plot showing comparable abundance of mature myeloid and erythroid cells following genetic deletion of Ybx1. **k,** Total numbers of mature blood cells of the myeloid (Gr1+), erythroid (TER119+), B-lymphoid (CD19) and T-lymphoid (CD3) lineage at week 16 after genetic inactivation of Ybx1 (n=6; mean ± SD). **l,** Experimental protocol for investigation of hematopoietic progenitor cell function. **m,** Colony numbers of Ybx1$^{+/+}$ versus Ybx1$^{-/-}$ murine stem/progenitor cells. Colonies were counted at day 8 after plating (each sample plated in duplicate, n=3 independent experiments, mean ± SD). **n,** Spleen colony numbers counted on day 12 after injection of Ybx1$^{+/+}$ or Ybx1$^{-/-}$ LSK-cells into lethally irradiated (12Gy) recipient mice (CFU-S12) (n=12 Ybx1+/+; n=12 Ybx1-/- independent biological mouse replicates in n=3 independent cohorts). **o,** BM chimerism of primary recipient mice (n=10 individual biological replicates) at week 20 after BMT. Whole bone marrow chimerism (WBMC) and chimerism of myeloid cells (Gr1+ BMC) (left panel). HSPC chimerism (LSK) and HSC chimerism (CD34- LSK) (right panel). Data shown as mean ± SD. **p,** BM chimerism of secondary recipient mice (n=5 individual biological replicates) at week 20 after BMT. Whole BM chimerism (WBMC) and chimerism of myeloid cells (Gr1+ BMC) (left panel). HSPC chimerism (LSK) and HSC chimerism (CD34- LSK) (right panel). Data shown as mean ± SD.

Extended Data Figure 4 | Regulation of Ybx1 phosphorylation dynamics in Jak2VF cells. **a,** Profile plot showing significantly regulated individual phosphorylated residues of Ybx1 in Jak2WT and Jak2VF. Each data point is the averaged median of biological quadruplicate and significance was tested using two sample test. **b,** Profile plot showing significantly regulated individual phosphorylated residues of Ybx1 in Jak2WT under unstimulated (control), stimulated with EPO and combination with Jak-i+EPO. **c,** Profile plot showing significantly regulated individual phosphorylated residues of Ybx1 in Jak2V617F under unstimulated, stimulated with EPO and combination with Jak-i+EPO. **b-c** Each data point is the averaged median of biological quadruplicate, Z-scored (log2 phosphosite intensity) and significance was tested using multiple sample test. **d,** Experimental design for phosphoproteome analysis of short-term Jak2 downstream effector kinase inhibitor treatment in Jak2-VF cells. n=4 per group, phosphopeptides were enriched using EasyPhos work flow and analyzed in single-run LC-MS/MS. **e,** Dot plot showing the successful inhibition of respective targets of the corresponding kinase inhibitor used in this study (ANOVA test with permutation-based FDR< 0.01). **f,** Dot plot showing changes in quantified Ybx1 phosphosites after various kinase inhibitor treatment (ANOVA test, permutation-based FDR< 0.01). The highlighted Ybx1 pS30 phosphosite is the only site highly significantly downregulated upon MEK/ERK inhibitor treatment compared to controls. **e-f,** size and color of the dots are proportional to the phosphosite intensity, Z-scored (log2 intensity). **g,** Ybx1 amino acid sequence alignment across different species shows the mouse Ybx1 S30 and S34 is conserved.

Extended Data Figure 5 | Ybx1 interaction with spliceosome components and validation of Ybx1-Mapk1 partnership.

a, Study design of murine Ybx1 interactome. n=4 biological replicates and Ybx1 interactome analyzed in LC-MS/MS. In *Perseus*, samples were filtered for minimum 3 valid values in at least one group. In total 614 high confident interactors of Ybx1

were identified (t-test with Permutation based FDR< 0.05) with 260 Jak2VF-specific interactors. **b,** Network representation of Ybx1 interacting spliceosomal proteins in Jak2VF cells. The size and color of the node indicates the abundance of the corresponding proteins (Z-scored protein intensity) and the edges are connected by STRING database interactions. **c,** List of significant Ybx1 interacting spliceosomal proteins presented according to their spliceosome complex. **d,** Spliceosome proteins interacting with Ybx1 participate in spliceosome assembly reaction in a stepwise manner to excise intronic sequences from immature mRNA to form a mature mRNA. **e,** Network representation of Ybx1 interactome (regulated in both Jak2WT and Jak2VF) based on annotation keywords. The keywords are highlighted in colors according to the protein function. n=4 biological replicates, t-test with permutation-based FDR < 0.05. **f,** Scatter plot of Ybx1 interactome in Jak2VF vs control. Ybx1 interactome is enriched for GO term mRNA splicing factor (green) and Ribonucleoproteins (blue) assessed by Fisher's exact test. Fold enrichment of Ybx1 and Mapk1 in Jak2VF cells compared to IgG control plotted against -$\log_{10}$ student t-test p-value. **g,** Scatter plot of Ybx1 interactome in DMSO vs Jak-i (RUX 0.5μM, 4 hours) treated Jak2VF cells. Fold enrichment of Mapk1 in DMSO vs. Jak-i plotted against -$\log_{10}$ Student`s t-test p-value. **h,** Immunoprecipitation of Ybx1 from murine Jak2VF cells ± RUX, 0.5μM for 4 hours and analyzed for interaction with Mapk1 by Western blot analysis using ERK1/2 antibody. Representative images from n=3 biological experiments.

Extended Data Figure 6 | Ybx1 phospho-null mutants display impaired nuclear localization and increased sensitivity to Jak-i. a, Western blot showing the expression of Ybx1 phospho mutants in Ba/F3 Jak2VF cells as indicated. **b,** Confocal images of Ybx1 localization (Red) in Ybx1 phospho mutants expressing Ba/F3 Jak2VF cells. Cells were counterstained with DAPI and GFP confirms the phospho-mutant expression. **a-b** Representative images from n=3 biological experiments. **c,** Bar plot showing quantification of nuclear Ybx1 expression in Ybx1 phospho-mutants expressing Ba/F3 Jak2VF cells, p-values were determined by two tailed t-test. Control (n=67): min=15.39; max=33.01; whisker=[15.39-33.01]; median=[24.74], S1(n=70): min=4.14; max=9.75; whisker=[4.14-9.75]; median=[7.0053], S2(n=64): min=5.32; max=14.11; whisker=[5.32-14.11]; median=[7.9], S3(n=57):min=4.44; max=17.6; whisker=[4.44-17.6]; median=[11.4], S4(n=72): min=13.32 ;max=23.82; whisker=[13.32-23.82]; median=[19.3], S5(n=61): min=13.08; max=27.97; whisker=[13.08-27.97]; median=[22.05], S6(n=57):min=18.89; max=30.04; whisker=[18.89-30.04]; median=[22.92], S7(n=60): min=16.69; max=32.53; whisker=[16.69-32.53]; median=[24.65]. **d,** Cell growth curve of Ybx1 phospho mutants expressing Ba/F3 Jak2VF cells following treatment with increase doses of Jak-inhibitor (1nM-10μM RUX) measured by MTS assay. n=4 independent experiments each with 8 technical replicates. **e**, FACS plots showing induction of apoptosis in Ba/F3 Jak2VF cells expressing Ybx1 phospho-mutants following Jak-i treatment (RUX 0.5μM) compared to untreated Ybx1 wildtype Ba/F3 Jak2VF cells. **f,** Bar plot shows quantification of percentage of Annexin V/ 7-AAD positive cells. n=6 independent experiments since Ybx1 phospho mutants endogenously express GFP, n=3 for Annexin V-APC staining and n=3 for 7-AAD-APC staining (error bars represent mean ± SD).

Extended Data Figure 7 | MEK-inhibition prevents Ybx1 nuclear localization in Jak2-mutated cells. a, Confocal images of Ybx1 localization (Red) in Ba/F3 Jak2VF cells following treatment with Ruxolitinib (0.5μM), MEKi (2μM), Trametinib (100nM and 200nM), Ruxolitinib in combination with Trametinib and DMSO treated control for 2 hours. Cells were counterstained with DAPI. **b,** Bar plot shows quantification of nuclear Ybx1 expression in Ba/F3 Jak2VF cells (n≥3 independent imaging experiments, p-value was determined by Student`s t-test). DMSO (n=67): min=7.05; max=27.27; whisker=[7.05-27.27]; median=[15.01], RUX (n=77): min=6.6; max=35.8; whisker=[6.6-35.8]; median=[16.39], MEKi

(n=89): min=3.2; max=16.35; whisker=[32.-16.35]; median=[8.35], Tram (n=67): min=2.44; max=14.18; whisker=[2.44-14.18]; median=[12.01], Tram (n=62): min=2.5; max=17.96; whisker=[2.5-17.96]; median=[12.01], Tram+RUX (n=64): min=3.44; max=19.23; whisker=[3.44-19.23]; median=[13.04], MEKi+RUX (n=68): min=4.06 max=14.68; whisker=[4.06-14.68]; median=[13.01]. **c,** Bubble plot showing the regulation of human Ybx1 pS32 and pS36 phosphorylation in HEL cells ± Ruxolitinib (0.5µM), MEKi (10µM), Trametinib (500nM and 2µM) for 4 hours *in vitro*. Phosphorylation status of Mapk and Jak2 is shown as successful inhibition of respective targets of the corresponding kinase inhibitors. n=4 biological samples per group. Size and color of the bubbles are proportional to the Z-scored log2 phosphosite intensity, significance using multiple sample test. **d,** Confocal images of Ybx1 localization (Red) in HEL cells ± inhibitors for 2 hours. Cells were counterstained with DAPI. n=3 biological experiments. **e,** Bar plot shows quantification of nuclear Ybx1 expression in HEL cells, p-values were determined by Student`s t-test. DMSO (n=44): min=17.51; max=34.56; whisker=[17.51-34.56];median=[24.30], RUX (n=44): min=13.04; max=36.92; whisker=[13.04-36.92]; median=[23.9], MEKi (n=41): min=7.62; max=15.36; whisker=[7.62-15.36]; median=[11.89], Tram (n=48): min=6.38; max=19.03; whisker=[6.38-19.03]; median=[12.01], Tram+RUX (n=55): min=3.31; max=19.19; whisker=[3.31-19.19]; median=[13.04], MEKi+RUX (n=49): min=4.32; max=19.46; whisker=[4.32-19.46]; median=[13.01]. **f,** Immunoprecipitation of Ybx1 from Ba/F3 Jak2VF cells expressing Ybx1 phospho mutants. n=3 independent experiments.

Extended Data Figure 8 | Nuclear Ybx1 regulates Mknk1 mRNA splicing in Jak2VF cells. a, Experimental design of RNA sequencing and data analysis. **b,** Bars represent number of retained intron events significantly upregulated in Ybx1 depleted cells with two-tailed test p-value 0.05 (1064 RI events) then filtered for p-value 0.01 and delta-PSI 0.1 are down to 472 highly significant RI events. **c,** Network map displaying enrichment of gene sets in the 472 highly significant RI events. Each node represents significantly enriched gene sets. Clusters of functionally related gene sets are circled, and labels are highlighted. Two-sided hypergeometric test, pV correction- Bonferroni step down. **d,** Visualization of spliced Mknk1 mRNA product after *in vitro* splicing assay. Nuclear extracts with and without Ybx1 knockdown and ± ATP were incubated for 2 hours with biotin labelled Mknk1 pre-mRNA (Exon12-13). mRNA was isolated, reverse transcribed, PCR was performed using the primers at the indicated arrow, products were agarose gel resolved and visualized using gel-red stain. Representative images from n=3 biological experiments. **e-f,** Flow cytometric analysis of Mknk1 protein expression rescue experiment in CRISPR/Cas9 induced Ybx1 knock out murine Jak2VF cells upon nonsense-mediated decay (NMD) inhibition. Representative flow cytometry histogram and Violin-plot showing quantification of Mknk1-DyLight649 mean fluorescence intensity (MFI) upon **e,** Compound C treatment (NMD inhibitor- dorsomorphin, termed CC, 10µM, for 24h) and **f,** VG-1 treatment (5µM and 10µM, for 20h) (n=3, two-sided t-test; mean ± SD). **g,** Genomic track profile of human Mknk1, Araf and Braf loci in HEL cells from Ybx1 ChIP sequence dataset (n=2). **h,** Genomic track profile of mouse Mknk1, Araf, and Braf loci in Ba/F3 Jak2VF cells from Ybx1 ChIP sequence dataset. n=3 biological experiments. Genomic track profile of human and mouse Prkcb shown as positive control from the Ybx1 ChIPseq dataset compared to IgG controls. **i,** Western blot showing the regulation of Mknk1 protein abundance in Ybx1 phosphomutants expressing Ba/F3 Jak2VF cells as indicated. n=3 independent experiments.

Extended Data Figure 9 | Targeting Mknk1 deregulates ERK signaling in Jak2-mutated cells. a, Unsupervised hierarchical clustering of significantly down regulated phosphosites (n=2390 sites) in human HEL cells following inactivation of Ybx1 by 2 independent shRNAs compared to non-targeting control. n=4 biological replicates. Heatmap represents Z-scored and averaged $\log_2$ phosphosite intensity, significance by ANOVA test with permutation-based FDR< 0.01. **b,** Kinase-substrate

motifs significantly down-regulated in Ybx1 targeted HEL cells are shown including Benjamin-Hochberg FDR value ($-\log_{10}$). **c,** ERK substrate motifs significantly downregulated and shared between Ybx1 targeted mouse and human Jak2VF cells. **d,** Western Blot analysis of total protein abundance and phosphorylation of Jak2-downstream targets upon Jak-i treatment and/or genetic inactivation of Ybx1 by RNAi. GAPDH used as loading control. Representative images from n= 3 independent experiments. **e-f,** Bar plots show the mean fluorescence intensity of pERK levels measured **e,** in human HEL (n=3) and **f,** patient Jak2 mutated cells (n=4 independent biological replicates from 4 individual patients) following genetic inactivation of Ybx1 by RNAi with or without drug treatment as indicated. Representative FACS plots shown in **Fig. 4d**. Data shown as mean ± SD and p-value determined by two-tailed Student`s t-test. **g,** Western blot validation of Mknk1 targeting shRNAs in murine Ba/F3 Jak2VF cells. **h,** Representative western blot analysis of pERK upon genetic inactivation of Mknk1 in Ba/F3 Jak2VF cells. n=4 with comparable results. **i,** Growth curve of Jak2VF cells following lentiviral infection with shRNAs targeting Mknk1 or non-targeting control and treatment with increase doses of Jak-inhibitor (1nM-10μM RUX) measured by MTS assay. n=4, each with 8 technical replicates. **j,** Percentage of apoptotic Jak2VF cells following lentiviral knockdown of Mknk1 (sh2, sh3 ± RUX 0.5μM) compared to non-targeting control (shSCR) (n= 4, two-tailed student t-test, mean ± SD).

Extended Data Figure 10 | Mcl-1 rescue of Ybx1 targeted cells and phosphoproteome analysis of primary Jak2-mutated cells upon Jak-i treatment. a, Proteome analysis of murine Jak2-mutated cells following inactivation of Ybx1 by 2 shRNAs compared to non-targeting control. Heat map representation of significantly enriched GO term biological processes in Ybx1 depleted Jak2VF cells assessed by Fisher's exact test (P-value ($-1\log_{10}$) shown). **b,** Quantification of Mcl-1 phosphosite pT144 in sh1Ybx1, sh2Ybx1, shNT control in murine Jak2VF cells. The y-axis is the $\log_2$ intensity of the phosphopeptide. (n=4 biological replicates, two tailed student t-test) shNT: min=23.8; max=24.7; whisker=[23.8-24.7]; box=[24-24.2], sh1Ybx1: min=19.8; max=22; whisker=[19.8-22]; box=[21.3-21.6], sh2Ybx1: min=19.2; max=22.4; whisker= [19.2-22.4]; box=[21.8-22.4]. **c,** Scatter dot plot of Mcl-1 phosphosite pT144 after respective kinase inhibitor treatment (n=3, biological replicates, p-values using two tailed student t-test, error bars represent ± SD). The y-axis is the z-scored, $\log_2$ phosphopeptide intensity. DMSO: min=-0.09; max=1.02; whisker=[-0.09-1.02], RUX: min=-0.554; max=0.135; whisker=[-0.55-0.135], AKTi: min=1.071; max=1.263; whisker= [1.07-1.2634], PI3Ki: min=-1.008; max=-0.89; whisker=[-1.008--0.89], MEKi: min=-1.266; max=-0.699; whisker=[-1.266--0.699]. **d,** Western blot analysis of Mcl-1, Ybx1, Bim and Bcl-XL following genetic inactivation of Ybx1 with 4 different shRNA constructs, compared to non-targeting control. n=3 independent experiments. **e,** Measurement of apoptosis (AnnexinV/Sytox-positive cells) after genetic inactivation of Ybx1 and concomitant Jak-i treatment (RUX 100nM, 500nM). Rescue by ectopic overexpression of Mcl-1 (n=4 independent experiments, two-tailed t-test). **f,** Western blot analysis of Ba/F3 Jak2VF cells showing down-regulation of Mknk1 protein abundance following overnight combination treatment with Ruxolitinb and Trametinib. n=3 biological independent experiments. **g,** PB analysis of human cell chimerism in NSGW41 humanized mice at week 4 and 20 (n=5 per cohort). **h,** Heatmap shows unsupervised hierarchical clustering of significantly regulated (t-test with permutation-based FDR< 0.01) phosphosites with (n=24) and without (n=24) Jak-i treatment in Jak2 mutated primary patient samples. Phosphoproteome analysis of Jak2 mutated primary patient samples (total n= 48) samples following *in vitro* (n=18, Jak-i treatment for 2 hours) or *in vivo* (n=6, 2 hours post dosing of RUX samples) exposure to Ruxolitinib. **i,** Network map of significantly enriched GO terms (p-value < 0.01) of dephosphorylated proteins upon Jak-i treatment. Two-sided hypergeometric test, pV correction- Bonferroni step down. **j,** Box plot shows no significant changes in the Mapk1 and Mapk3 phosphorylation in control (n=24) vs Jak-i treated (n=24) patient samples. MAPK1pT185 (DMSO): min=19.61; max=24.82; whisker=[19.61-24.82]; box=[21.34-23.11], MAPK1pT185 (RUX): min=19.5; max=24.81;

whisker=[19.5-24.81]; box=[21.64-23.72], MAPK1pY187 (DMSO): min=21.04; max=26.45; whisker=[21.04-26.45];box=[23.22-25.46], MAPK1pY187(RUX): min=21.09; max=25.89; whisker=[21.09-25.89]; box=[22.63-24.77], MAPK3pT202(DMSO): min=19.72; max=26.06; whisker=[19.72-26.06]; box=[21.46-24.78], MAPK3pT202 (RUX): min=20.41; max=26.93; whisker=[20.41-26.93]; box=[22.05-23.88], MAPK3pY204 (DMSO): min=20.70; max=26.51; whisker=[20.70-26.51]; box=[22.02-24.99], MAPK3pY204 (RUX): min=20.31; max=25.76; whisker=[20.31-25.76]; box=[22.72-24.42]. **k,** Box plot shows significant changes in Ikbkb, Stat3 and Stat5 phosphorylation in control (n=24) vs Jak-i treated (n=24) patient samples. p-values as determined by Mann-Whitney test. IKBKBpS697 (DMSO): min=20.66; max=25.79; whisker=[20.66-25.79]; box=[22.89-24.40], IKBKBpS697 (RUX): min=20.66; max=25.04; whisker=[20.66-25.04]; box=[21.88-23.88], IKBKBpS672 (DMSO): min=20.71; max=26.30; whisker=[20.71-26.30]; box=[22.5-23.86], IKBKBpS672 (RUX): min=18.85; max=24.73; whisker=[18.85-24.73]; box=[21.44-23.24], STAT5pY699 (DMSO): min=22.02; max=25.33; whisker=[22.02-25.33]; box=[22.73-23.63], STAT5pY699 (RUX): min=19.90; max=24.83; whisker=[19.90-24.83]; box=[21.8-23.2], STAT3pY705 (DMSO): min=21.87; max=26.66; whisker=[21.87-26.66]; box=[22.41-25.62], STAT3pY705 (RUX): min=20.07; max=26.17; whisker=[20.07-26.17]; box=[21.22-23.26]. **l,** Schematic depicting the mechanism of Ybx1-mediated Jak-i persistence.