JAK2V617F promotes replication fork stalling with disease-restricted impairment of the intra-S checkpoint response

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Cancers result from the accumulation of genetic lesions, but the cellular consequences of driver mutations remain unclear, especially during the earliest stages of malignancy. The V617F mutation in the JAK2 non-receptor tyrosine kinase (JAK2V617F) is present as an early somatic event in most patients with myeloproliferative neoplasms (MPNs), and the study of these chronic myeloid malignancies provides an experimentally tractable approach to understanding early tumorigenesis. Introduction of exogenous JAK2V617F impairs replication fork progression and is associated with activation of the intra-S checkpoint, with both effects mediated by phosphatidylinositide 3-kinase (PI3K) signaling. Analysis of clonally derived JAK2V617F-positive erythroblasts from MPN patients also demonstrated impaired replication fork progression accompanied by increased levels of replication protein A (RPA)-containing foci. However, the associated intra-S checkpoint response was impaired in erythroblasts from polycythemia vera (PV) patients, but not in those from essential thrombocythemia (ET) patients. Moreover, inhibition of p53 in PV erythroblasts resulted in more gamma-H2Ax (γ-H2Ax)–marked double-stranded breaks compared with in like-treated ET erythroblasts, suggesting the defective intra-S checkpoint function seen in PV increases DNA damage in the context of attenuated p53 signaling. These results demonstrate oncogene-induced impairment of replication fork progression in primary cells from MPN patients, reveal unexpected disease-restricted differences in activation of the intra-S checkpoint, and have potential implications for the clonal evolution of malignancies.

myeloproliferative neoplasm | JAK2V617F | replication stress

Next-generation sequencing studies are starting to reveal the genetic complexity of tumors but are limited in their ability to infer the details of antecedent clonal evolution (1, 2). It remains unclear how mutations accrue in the early stages of tumor formation, how preneoplastic cells respond to DNA damage, and how these events potentiate secondary transformation.

The myeloproliferative neoplasms (MPNs) provide a powerful model for investigating mechanisms of mutation accumulation and disease acceleration. They encompass a spectrum of chronic myeloid malignancies characterized by overproduction of morphologically normal cells of the myeloerythroid lineages and include polycythemia vera (PV) and essential thrombocythemia (ET) (3, 4). Several aspects of MPNs make them useful in the investigation of clonal evolution: they reflect subtle modulation of hematopoiesis and are often diagnosed after an incidental blood count, thus providing investigators a glimpse of the earliest stages of tumorigenesis; these disorders are experimentally tractable, as hematopoietic progenitors are readily cultured from peripheral blood of MPN patients and are amenable to clonal analysis; and PV and ET patients have near-normal life expectancy, thus permitting serial biological sampling over many years.

A majority of PV and ET patients harbor a V617F mutation in the JAK2 non-receptor tyrosine kinase (JAK2V617F) that activates downstream signaling and is associated with cytokine-independent growth (5–8). Moreover, several studies have demonstrated that JAK2V617F is associated with increased DNA damage. Mutant JAK2 inhibits Bcl-xL deamidation and the apoptotic response to DNA damage in primary cells from patients with PV, providing a potential mechanism for accumulation of DNA damage (9). JAK2V617F expression leads to increased frequency of γH2Ax-marked double-stranded breaks (DSBs) (10, 11), mutations of the *HPRT* gene locus (12), and spontaneous homologous recombination events (13). Increased DSB repair was also observed in CD34+ hematopoietic cells obtained from JAK2V617F-positive PV and myelofibrosis patients (12). However, the molecular basis for JAK2V617F-mediated DNA damage remains poorly understood.

Replication-associated errors are one of the largest endogenous sources of DSBs and are particularly problematic for cells harboring oncogenes that stimulate S-phase entry under inappropriate circumstances, such as limiting levels of nutrients (14). This can promote stalling of the replisome during DNA replication, a phenomenon called "replication stress." Stalled forks that collapse can generate single-strand DNA nicks (15, 16), which can in turn be converted to DSBs if a replication fork subsequently attempts to

Significance

Cancers arise through a succession of enabling genetic lesions, but the consequences of many driver mutations remain unclear, especially in the earliest stages of tumor formation. The myeloproliferative neoplasms (MPNs) encompass a group of chronic hematologic disorders that can collectively provide a window into these early stages of leukemia evolution. This study reveals a role for the JAK2V617F mutation, the most frequent genetic abnormality in MPN patients, in impairing replication fork progression during cell division of MPN patient-derived tumor cells. Moreover, analysis of different MPN disease subtypes reveals unexpected differences in DNA repair activity in response to JAK2V617F-induced perturbations in replication dynamics. These findings have potential implications for tumor clonal evolution and individualized cancer therapy.

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replicate past the nick (17). The intra-S checkpoint guards against such replication-induced genomic damage by stabilizing stalled forks and, in the absence of appropriate repair, promoting senescence or apoptosis. Activation of the intra-S checkpoint is therefore an important defense against transformation (18).

In this article we explore the effect of JAK2V617F on DNA replication and S-phase checkpoint function.

Results

JAK2V617F Expression Causes Replication Stress. To investigate whether JAK2V617F influences DNA replication, BJ human diploid fibroblasts were engineered to express wild-type JAK2 or JAK2V617F (hereafter referred to as BJ^{WT} and BJ^{V617F}, respectively). Western blotting confirmed ectopic expression of JAK2 in both BJ^{WT} and BJ^{V617F} lines compared with parental BJ cells (*SI Appendix*, Fig. S1*A*). Expression of JAK2V617F protein was somewhat lower than that of wild-type JAK2 protein but was consistently associated with increased activation of downstream signaling, as indicated by increased phosphorylation of STAT1, STAT5, and AKT (*SI Appendix*, Fig. S1*A*).

Chromosome combing permits visualization and analysis of replication tracts on individual, BrdU-labeled DNA fibers (Fig. 1*A*). Using this approach, we quantified several aspects of DNA replication in the JAK2-expressing BJ fibroblasts. First, 355 individual replication fork structures were scored to differentiate among "origins of replication," "normally progressing forks," and "stalled forks." JAK2V617F expression had no effect on frequency of origin firing. However, we observed a significant increase in the proportion of stalled forks (as defined by a >30% reduction in the length of the tract labeled by the second labeling pulse compared with the first pulse) in BJ^{V617F} cells compared with BJ^{WT} cells (23% versus 5%, respectively; $P < 0.05$) (Fig. 1*B* and *SI Appendix*, Fig. S1*B*). It is worth noting that this analysis is likely to underestimate the actual frequency of fork stalling, as only forks that stall during the second labeling step would be scored. Second, replication fork processivity was assessed, as it is another indicator of fork stalling. We observed a significant decrease in the mean replication rate, going from 1.5 ± 0.04 Kb/min ($n = 110$) in BJ^{WT} cells to 0.80 ± 0.04 Kb/min ($n = 65$) in BJ^{V617F} cells ($P < 0.0001$) (Fig. 1*C*). Third, as two replication forks emerging bidirectionally from the same origin tend to exhibit the same replication rate, replication stress can be intimated by the presence of asymmetric progression of the two outgoing forks. We therefore analyzed all origin structures to compare the length of their left and right replication tracts and used the ratio of >0.7 between left and right forks to represent symmetric progression (19). Replication origins displayed increased asymmetry in BJ^{V617F} cells compared with BJ^{WT} cells (Fig. 1*D*). In BJ^{WT} cells, the mean ratio between left and right forks was 0.99 ± 0.2 ($n = 18$), with only 6% of the replication bubbles showing asymmetric dynamics, whereas in BJ^{V617F} cells, the mean ratio was 1.24 ± 0.4 ($n = 19$), with 58% of the origins showing asymmetry. Taken together, these data demonstrate that JAK2V617F causes increased replication fork stalling.

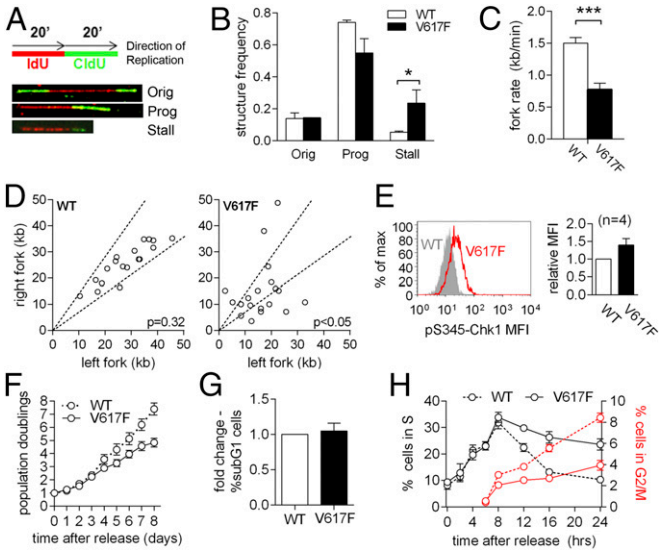

Fig. 1. JAK2V617F induces replication fork stalling and activates the intra-S checkpoint in BJ human diploid fibroblasts. (*A*) Replication structures of single combed DNA molecules from BJ^{WT} and BJ^{V617F} cells labeled with IdU (red) and CldU (green), showing origins of replication (Orig), progressing forks (Prog), and stalled forks (Stall). (*B*) Scoring of fibers for different fork structures from BJ^{WT} and BJ^{V617F} cells. Fibers from each slide were scored by two independent observers. The data represent the mean ± SD of three independent experiments. (*C*) Quantitation of fork rate from BJ^{WT} and BJ^{V617F} cells. Results represent the mean ± SD for 110 fibers from BJ^{WT} cells and 65 fibers from BJ^{V617F} cells. (*D*) Origin symmetry in BJ^{WT} and BJ^{V617F} cells. Each point represents a single origin, with the length of one arm (arbitrarily designated as "left") plotted relative to its opposite moving counterpart (designated as "right"). The dotted line represents a ratio of 0.7 between the two arms. (*E*) Analysis of pS345-CHK1 levels in BJ^{WT} and BJ^{V617F} cells by intracellular flow cytometry. The results are representative of four experiments. (*F–H*) Analysis of doubling time (*F*), cell death (*G*), and S-phase duration (*H*) of BJ^{WT} and BJ^{V617F} cells. (*B–D*) Testing for significance was performed using an unpaired *t* test (*C* and *D*) or a one-sample *t* test (*E*). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. WT, wild-type.

JAK2V617F Expression Activates the Intra-S Checkpoint. The checkpoint protein Chk1 is phosphorylated at Ser345 (pS345-Chk1) in response to stalled replication forks, and it is also the principle mediator of the intra-S checkpoint response. Levels of pS345-Chk1 were increased in BJ^{V617F} cells relative to BJ^{WT}cells (Fig. 1*E* and *SI Appendix*, Fig. S1*C*). This was associated with longer doubling times for BJ^{V617F} cells compared with BJ^{WT} cells (Fig. 1*F*), a difference that did not reflect increased apoptosis (Fig. 1*G*). Instead, BJ^{V617F} cells exhibited a delayed exit from S and entry into M, as shown by synchronizing cells at the G1/S border with mimosine, followed by release into standard growth medium (Fig. 1*H*). Together, these data demonstrate that JAK2V617F-induced replication stress is associated with the activation of an intra-S checkpoint and prolongation of S-phase duration.

We next investigated which signaling pathways were necessary for the effect of JAK2V617F on replication stress. Levels of phosphorylated Chk1 were assessed after transient transfection of JAK2-expressing BJ fibroblasts with pools of siRNAs (three to five siRNAs per pool) targeting STAT1, STAT5, or the p85 catalytic subunit of phosphatidylinositide 3-kinase (PI3K). Efficient knockdown of corresponding protein species was confirmed by immunoblot (*SI Appendix*, Fig. S2 *A* and *B*). In BJ^{V617F} cells, knockdown of PI3K, but not STAT1 or STAT5, reduced pS345-Chk1 to a level comparable to that of BJ^{WT} cells (Fig. 2*A*). To ensure the siRNA pools were not generating off-target effects, each pool was deconvoluted and three individual siRNAs were transfected into BJ cells. All three individual siRNAs targeting PI3K abrogated pS345-Chk1 levels in BJ^{V617F} cells, whereas none of the individual STAT1 or STAT5 siRNAs did (Fig. 2*B*). Furthermore, treatment of BJ^{V617F} cells with the PI3K inhibitor PI103 also led to decreased Chk1 phosphorylation (*SI Appendix*, Fig. S3*A*) accompanied by reduced AKT phosphorylation (*SI Appendix*, Fig. S3*B*).

It remained formally possible that the reduced Chk1 phosphorylation after PI3K knockdown was a phenomenon independent of fork stalling. To investigate this possibility, we also performed chromosome combing on JAK2-expressing BJ fibroblasts after siRNA knockdown of STAT1, STAT5, and PI3K. Consistent with the Chk1 phosphorylation results, we observed that increased fork stalling (Fig. 2*C*), decreased fork rate (Fig. 2*D*), and increased origin asymmetry (*SI Appendix*, Fig. S4) were

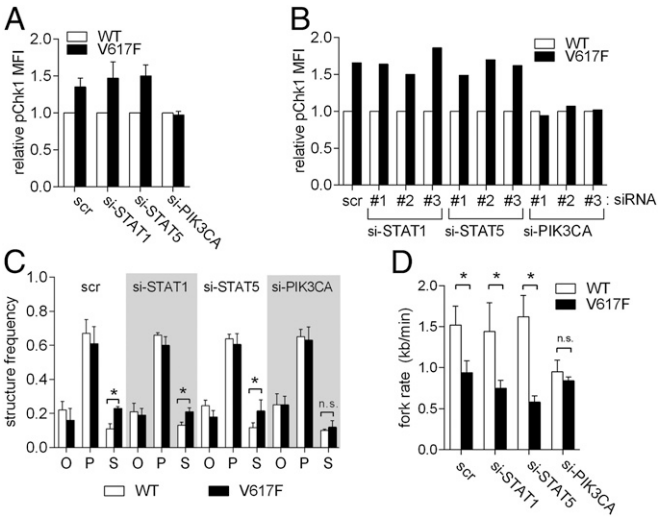

Fig. 2. JAK2V617F-induced fork stalling is PI3K-dependent. (*A* and *B*) pS345-CHK1 levels in BJ^{WT} and BJ^{V617F} cells after transfection with scrambled siRNA control (scr) or either pools of three to five siRNAs targeting STAT1, STAT5, or the p85 subunit of the PI3K (PIK3CA) (*A*) or individual siRNAs targeting STAT1, STAT5, or PIK3CA (*B*). (*C* and *D*) BJ^{WT} and BJ^{V617F} cells transfected with control siRNA or siRNAs targeting STAT1, STAT5, or PIK3CA were scored for frequency of origin (O), normal progressing forks (P), or stalled forks (S) (*C*) or fork rate (*D*). (*A–D*) Data represent the mean ± SD of three independent experiments. Testing for significance was performed using an unpaired *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. WT, wild-type.

only mitigated in cells transfected with siRNAs targeting PI3K, not STAT1 or STAT5. Collectively, these results indicate that the PI3K pathway is an important mediator of JAK2V617F-induced replication stress and intra-S checkpoint activation.

Replication Stress Is a Feature of JAK2 V617F-Positive Erythroblasts from MPN Patients. The results described here demonstrate that exogenous JAK2V617F causes replication stress in fibroblasts. We next investigated whether a similar phenomenon operates in primary JAK2-mutant cells obtained from MPN patients. To this end, we studied clonally pure populations of JAK2V617F-heterozygous cells together with autologous JAK2-wild-type cells obtained from the same patient (Fig. 3*A*). This strategy controls for differences in sex, age, therapy, constitutional genetic background, and other confounding variables (20). Cells from a given patient were pooled by genotype and subjected to chromosome combing.

We scored 1,163 individual DNA fibers obtained from six MPN patients (three ET, three PV). Compared with autologous JAK2-wild-type cells, JAK2-mutant cells from both ET and PV patients exhibited increased frequencies of stalled forks (Fig. 3*B*), slower fork processivity (Fig. 3*C*), and greater replication origin asymmetry (Fig. 3*D*). Culturing colonies in the presence of the STAT5 inhibitor pimozide (*SI Appendix*, Fig. S5*A*) or the STAT1 inhibitor fludarabine (*SI Appendix*, Fig. S5*B*) did not abrogate fork stalling and processivity in JAK2-mutant colonies (*SI Appendix*, Fig. S5 *D* and *E*). In contrast, culturing JAK2-mutant colonies in the presence of PI103 reduced fork stalling and processivity to their normal levels (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S5*C*). These findings accord with our fibroblast data and represent, to our knowledge, the first demonstration of replication stress induced by an oncogenic tyrosine kinase in primary patient samples.

Impaired Intra-S Checkpoint in PV. To investigate whether JAK2V617F-induced replication stress is associated with a detectable expression signature, we reanalyzed expression array data from clonally derived JAK2-mutant and JAK2-wild-type cells obtained from 36 MPN patients (20 ET, 16 PV) (20). Gene set enrichment analysis revealed that genes regulated by exposure to UV light were enriched among genes differentially expressed in mutant cells from ET patients (*SI Appendix*, Fig. S6*A* and Table S1). In addition, connectivity map analysis was applied to JAK2-mutant gene signatures to look for similarities to gene expression modulations induced by various pharmacologic agents. Expression changes in JAK2-mutant cells from ET patients were similar to those observed in cell lines treated with the topoisomerase inhibitors camptothecin or irinotecan (*SI Appendix*, Fig. S6 *B* and *C* and Table S2). UV light, camptothecin, and irinotecan share the common attribute of interfering with DNA replication, so these data are consistent with our results demonstrating replication stress in JAK2-mutant cells from ET patients. In marked contrast, JAK2-mutant cells from PV patients displayed no enrichment for UV-regulated gene sets or for genes modulated in response to camptothecin or irinotecan (*SI Appendix*, Fig. S6*D* and Tables S3 and S4). Because replication stress was observed in JAK2-mutant cells from patients with ET, as well as those with PV, these data suggest the possibility of a defective response to replication stress in PV.

To explore this hypothesis, intracellular flow cytometry was performed on autologous wild-type and JAK2V617F-heterozygous erythroblasts from 16 MPN patients (seven ET and nine PV) to

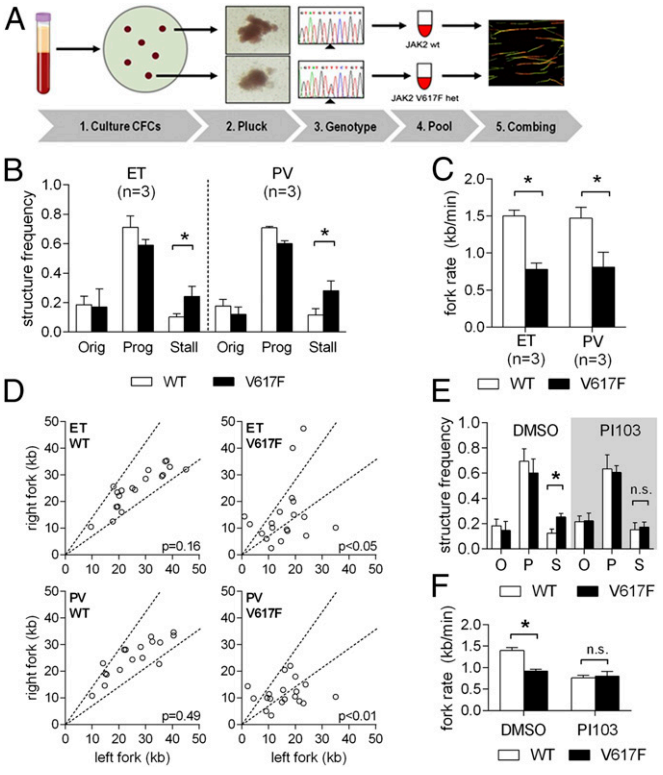

Fig. 3. JAK2V617F causes replication fork stalling in primary MPN patient samples. (*A*) Strategy for analysis of paired normal and JAK2V617F samples from MPN patients. (*B*) Scoring of fibers for structures resembling origins (Orig), progressing (Prog), or stalled (Stall) forks from erythroblasts from PV and ET patients. (*C* and *D*) Measurement of fork rate (*C*) and origin symmetry (*D*) in wild-type and JAK2-heterozygous erythroblasts from ET and PV patients. (*E*) Scoring of fork structures in control and PI103-treated wild-type and mutant erythroblasts. The results represent the mean ± SD for three MPN patients (one PV, two ET). (*F*) Fork rates of control and PI103-treated wild-type and mutant erythroblasts. The results represent the mean ± SD for three MPN patients (one PV, two ET). (*B–F*) The data are represented as mean ± SD. Each patient's fibers were scored by two independent observers. Testing for statistical significance was performed using an unpaired *t* test or a one-sample *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. WT, wild-type.

quantify pS345-pChk1 levels. Relative to autologous wild-type erythroblasts, mean pS345-Chk1 levels were higher in JAK2-mutant erythroblasts from ET patients compared with those from PV patients (Fig. 4 *A* and *B*). We also assessed Chk1 activity in the hematopoietic stem and progenitor cell compartment of normal individuals and JAK2-mutant MPN patients. Higher levels of pS345-Chk1 were also observed in the cycling fraction of peripheral blood $CD34^+$ cells from ET patients compared with PV patients (*SI Appendix*, Fig. S7 *A–C*). These data accord with our findings in erythroblasts and demonstrate that differential Chk1 phosphorylation is also seen in the hematopoietic stem and progenitor cell compartment.

To investigate the mechanism for impaired Chk1 phosphorylation in JAK2-mutant PV erythroblasts, we performed immunocytochemistry on 1,034 individual nuclei from seven MPN patients (three ET and four PV) to assess the formation of foci containing replication protein A (RPA) and Ataxia telangiectasia and Rad3 related (ATR), two checkpoint proteins critical for the recognition of stalled replication forks and signaling to Chk1. Relative to wild-type erythroblasts, median RPA focus formation was significantly increased in JAK2-mutant cells, a phenomenon common to both ET and PV patients (Fig. 4*C*). This finding is consistent with a role for RPA in marking sites of exposed single-stranded DNA at stalled fork intermediates. No significant difference was observed in the number of RPA foci in JAK2-mutant cells of ET and PV patients (Fig. 4*C* and *SI Appendix*, Fig. S8*A*), suggesting stalled replication forks had similar stability in the two diseases. Consistent with this, direct staining for single-stranded DNA by flow cytometry also showed increased levels of single-stranded DNA in mutant erythroblasts in all patients, but no difference between ET and PV patients (*SI Appendix*, Fig. S9 *A* and *B*). In contrast, patterns of ATR foci differed significantly between PV and ET. In all three ET patients tested, JAK2-mutant cells harbored increased numbers of ATR foci, the majority of which colocalized with RPA foci, whereas there was no increase in the number of ATR foci in JAK2-mutant cells from four PV patients (Fig. 4*C* and *SI Appendix*, Fig. S8*A*). Moreover, a significant reduction in the numbers of nuclei exhibiting more than 20 RPA-ATR colocalizing foci was observed in JAK2-mutant erythroblasts from patients with PV relative to those with ET (*SI Appendix*, Fig. S10*A*). Real-time quantitative PCR revealed no differences in levels of ATR transcript between mutant erythroblasts of ET and PV patients (*SI Appendix*, Fig. S11). In addition, RPA-ATR colocalizing loci were readily detected in BJ fibroblasts expressing JAK2V617F, but not in wild-type JAK2 (*SI Appendix*, Fig. S12), suggesting that the lack of ATR foci formation represents an acquired deficit in PV patients. Collectively, these findings demonstrate that there is impaired physical recruitment of ATR to sites of stalled replication forks in JAK2-mutant erythroblasts from PV, which accounts for impaired Chk1 phosphorylation in response to JAK2V617F-mediated replication stress.

We next assessed whether JAK2V617F-positive cells from PV patients were able to respond to camptothecin, a potent inducer of replication stress. Although camptothecin treatment yielded robust ATR foci and RPA-ATR colocalizing foci in wild-type erythroblasts, this response was significantly blunted in JAK2-mutant erythroblasts (Fig. 4*D* and *SI Appendix*, Fig. S8*B*). Moreover, compared with autologous JAK2-wild-type erythroblasts, fewer JAK2-mutant erythroblasts developed more than 20 colocalizing foci after camptothecin treatment (*SI Appendix*, Fig. S10*B*). When grown in camptothecin, compared with autologous wild-type erythroid burst forming units (BFU-Es) or ET-derived JAK2-mutant BFU-Es, JAK2-mutant PV BFU-Es exhibited less apoptosis (Fig. 4*E*), more cells in S/G2/M (Fig. 4*F*), and increased colony size (Fig. 4*G*). Collectively, these findings demonstrate that in patients with PV, JAK2V617F-positive cells display impaired activation of the intra-S checkpoint and that this is associated with sustained proliferation and survival, despite replication stress.

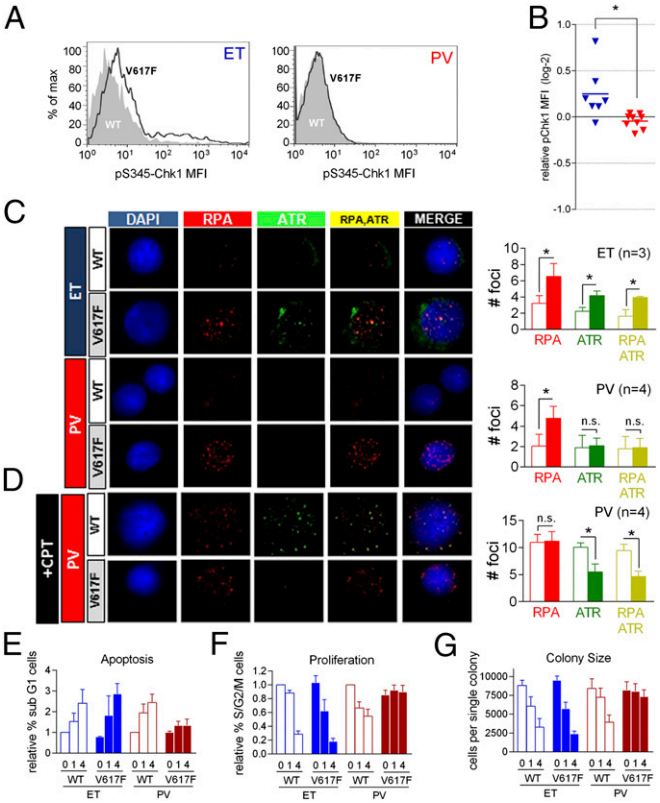

Fig. 4. Disease-specific impairment of intra-S checkpoint activity. (*A*) Exemplary intracellular phospho-flow cytometry of pS345-CHK1 expression in autologous wild-type and V617F-heterozygous BFU-E colonies from ET and PV patients. (*B*) Dot plot depicting ratio of pCHK1 MFI in JAK2-mutant erythroblasts relative to autologous wild-type erythroblasts from seven ET and nine PV patients. (*C*) Immunofluorescent staining of cytospins from wild-type and V617F-heterozygous BFU-E pools from PV and ET patients for RPA (red), ATR (green), and DAPI (blue). The bar graphs depict median foci number per cell from three ET and four PV patients. (*D*) RPA, ATR, and DAPI staining of cytospins from wild-type and V617F-heterozygous BFU-E pools from PV patients grown in 4 nM camptothecin. Bar graph depicts data from four PV patients. (*E–G*) Effect of camptothecin treatment on cell death (*E*), cell proliferation (*F*), and colony size (*G*). For proliferation and cell death, the data are depicted as a normalized fold change relative to the untreated wild-type sample for each disease group. The results represent the mean ± SD for five ET and three PV patients. (*B–G*) Testing for statistical significance was performed using a Student *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. WT, wild-type.

p53 Inhibition Increases DNA Damage in PV. To assess the effect of impaired intra-S checkpoint function on genomic integrity in ET and PV patients, we performed immunocytochemistry on erythroblasts from six MPN patients (three ET and three PV) to assess the formation of γ-H2Ax-labeled foci, which mark the sites of DSBs. We found a significant increase in the number of γ-H2Ax-positive foci in mutant erythroblasts cultured under normal growth conditions (Fig. 5 *A* and *B*), a finding common to both ET and PV patients. However, no significant difference in the numbers of γ-H2Ax-positive foci was observed between the two diseases (Fig. 5 *A* and *B*). These data indicate that mutant JAK2 expression is associated with increased DNA damage, but differential intra-S checkpoint activity is not associated with further increased frequency of DNA damage in chronic phase disease.

The lack of disparity in DNA damage between ET and PV (despite differential activation of the intra-S checkpoint) may be a result of the presence of intact p53 pathways downstream of the intra-S checkpoint to guard against genomic instability. Consistent with this idea, we detected activation of the p53 pathway

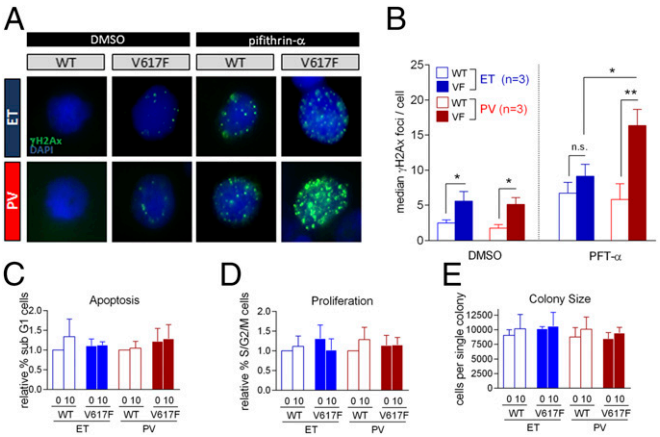

Fig. 5. Loss of p53 activity increases DNA damage in PV JAK2-mutant cells. (*A*) Representative immunocytochemical micrographs of γ-H2Ax (green) and DAPI (blue) staining of cytospins from wild-type and V617F-heterozygous BFU-E pools from PV and ET patients cultured in the presence of DMSO vehicle or 10 nM PFT-α. (*B*) Histogram depicts median γ-H2Ax foci per cell for three ET and three PV patients cultured with and without PFT-α. Testing for statistical significance was performed using a Student *t* test. $*P < 0.05$; $**P < 0.01$. (*C*–*E*) Effect of PFT-α treatment on cell death (*C*), cell proliferation (*D*), and colony size (*E*). For proliferation and cell death, the data are depicted as a normalized fold change relative to the untreated wild-type sample for each disease group. The results represent the mean ± SD for three ET and three PV patients. WT, wild-type.

in mutant erythroblasts from both ET and PV patients; mutant erythroblasts exhibited heightened levels of phosphorylated p53 protein (*SI Appendix*, Fig. S13*A*) and increased expression of two p53 target genes: *CDKN1A* (which encodes the p21 protein) and *TP53I3* (*SI Appendix*, Fig. S13*B*). We therefore next assessed whether inhibiting the p53 pathway would have differential effects on the accumulation of DNA damage in ET- versus PV-derived erythroblasts. To inhibit p53 signaling, pifithrin-alpha (PFT-α) was used, which has been described to specifically prevent transactivation of p53-responsive genes (21). Strikingly, PV JAK2-mutant cells cultured in the presence of PFT-α exhibited a significant increase in the number of γ-H2Ax foci compared with autologous wild-type cells or ET-derived JAK2-mutant cells under identical conditions (Fig. 5 *A* and *B*). This was not associated with alterations in the frequency of apoptosis (Fig. 5*C*), proliferation (Fig. 5*D*), or colony size (Fig. 5*E*), suggesting these DNA damage-laden cells can persist in vitro. Cumulatively, these data suggest that the p53 pathway plays a key role in maintaining genomic stability during chronic-phase MPN and that its inhibition in the context of an impaired intra-S checkpoint (as in PV patients) leads to increased DNA damage without affecting cell survival or proliferation.

Discussion

Cancers evolve as a consequence of the stepwise accumulation of genetic lesions with subclonal evolution. However, the cellular consequences that flow from the acquisition of a driver mutation are poorly understood, particularly in the early stages of tumor development. Here we demonstrate that JAK2V617F increases the frequency of replication fork stalling. As JAK2 mutations arise early during MPN evolution and may represent initiating events in a significant proportion of MPN patients (22, 23), these findings support the hypothesis that replication stress is among the earliest events in tumorigenesis. This concept is consistent with observations in other early premalignant conditions, such as dysplastic epithelia (24) and precursor lung lesions (25, 26), in which activation of checkpoint kinases was used as a surrogate for fork stalling and was assessed in bulk populations of cells. Furthermore, we have performed chromosome combing on clonally derived and genotyped populations of primary cells from MPN patients. To our knowledge, our data represent the first direct demonstration of replication fork stalling in primary cells from any malignancy.

Several models have been proposed to explain how different oncogenes induce replication stress. Overexpression of the human papillomavirus E6/E7 oncoproteins and cyclin E drives transition from G1 to S phase under inappropriate circumstances, such as limiting levels of nutrients (14), and H-RasV12 causes increased activation of dormant origins and rereplication (26). Our data, however, preclude both of these mechanisms for JAK2V617F. We observe that wild-type and mutant-JAK2 expressing fibroblasts entered S phase at similar rates. Moreover, the frequency of origin structures was similar in both wild-type and mutant-JAK2-expressing fibroblasts and patient-derived colonies, arguing against activation of dormant origins. Instead, our results show that JAK2V617F causes replication fork stalling by activating the PI3K signaling pathway. Indeed, analogous findings have been reported in *Saccharomyces cerevisiae*, in which increased growth factor signaling and AKT activity are associated with replication stress (27), but a similar correlation has not previously been reported in metazoans. It is unclear how increased AKT activity perturbs replication dynamics. However, it may be relevant that AKT phosphorylation of epigenetic regulators such as EZH2 and p300 histone deacetylase can result in unscheduled euchromatinization during S-phase, with the ensuing interference between the transcription and replication machineries causing replication fork stalling (28, 29). In addition, AKT may directly destabilize the replication fork through phosphorylation of the DNA methyltransferase DNMT1, thus impairing its retention at the replication fork (30).

Our data also demonstrate that some MPN patients, especially those with PV, exhibit an attenuated intra-S checkpoint response to the presence of JAK2V617F. This observation is unlikely to reflect constitutional genetic differences between PV and ET patients, as autologous JAK2-wild-type colonies were used as controls. Moreover, wild-type colonies from both patient groups exhibited normal patterns of RPA and ATR foci after camptothecin exposure. Instead, our evidence suggests the existence of additional genetic or epigenetic events that modulate intra-S checkpoint activity in most PV patients and in rare ET patients. A recent survey of the mutational landscape of MPN patients did not reveal any recurrent mutations in intra-S checkpoint regulators in PV patients (31), although many of the lesions identified in PV patients have not been functionally characterized with respect to their ability to modulate the intra-S checkpoint.

How might impairment of the intra-S checkpoint response relate to MPN biology? PV and ET are distinguished primarily by the enhanced erythrocytosis seen in PV, which is thought to reflect increased STAT5 and/or reduced STAT1 activity (20, 32, 33). However, our data demonstrate that reduced activity of STAT5 or STAT1 did not influence Chk1 activation, replication fork stalling, or rate of fork progression. Instead, it seems more plausible that the reduced intra-S checkpoint response seen in PV patients may contribute to increased genomic instability. This mechanism might contribute to the increased prevalence of JAK2V617F homozygosity observed in PV patients, although when assessed at a clonal level, the prevalence of JAK2V617F homozygosity is only modestly increased in PV patients (34). Moreover, the concept that a reduced intra-S checkpoint gives rise to enhanced DNA damage runs counter to evidence that the genomes of PV patients are generally stable; exome sequencing revealed a median of only 6.5 somatic mutations per patient for both ET and PV (31), and cytogenetic lesions are infrequent (35). We also found that JAK2-mutant cells from ET and PV patients have similar frequencies of DSBs, which is in agreement with other studies that suggest ET and PV are not significantly different with respect to genomic stability (35).

So what is the biological significance of impaired intra-S checkpoint in PV? We observed activated p53 signaling in JAK2-mutant

erythroblasts, indicating that p53-dependent processes initiated downstream of JAK2V617F-induced replication stress may be crucial in maintaining the genome stability of ET and PV. Indeed, p53 mutations are common in MPN patients who transform to AML (36), and p53-deficient secondary AML exhibits high rates of cytogenetic abnormalities (37). We therefore hypothesized that impaired intra-S checkpoint function could synergize with p53 loss to exacerbate genomic instability during leukemic transformation. Strikingly, when cultured in the presence of p53 inhibitors, PV JAK2-mutant cells had significantly more DSBs compared with ET-derived counterparts. Our data are consistent with murine studies that have shown that *Atr* haploinsufficient mice only develop genomic aberrations and malignant phenotypes after further compromise of DNA repair checkpoints, such as through loss of p53 function (38). Moreover, a recent study showed a higher frequency of copy number-neutral loss-of-heterozygosity in leukemic blasts from individuals who had an antecedent PV compared with those who had an antecedent ET (39). Co-occurrence of the JAK2V617F mutation with copy number-neutral loss-of-heterozygosity was also associated with poorer prognosis (39). Although this study was limited to unmatched samples and needs to be reproduced in a larger cohort, it is consistent with the idea that defective intra-S checkpoint activity seen in PV patients is associated with heightened genomic instability during leukemic transformation, and possibly even a decrease in overall survival.

The prognosis of leukemias with an antecedent MPN phase is exceedingly poor. The selection for clones with impaired DNA damage repair checkpoints may contribute to clonal evolution and the well-recognized recalcitrance of these advanced malignancies to respond to therapy with genotoxic agents. Further understanding of the aberrant DNA repair processes in these disorders may facilitate the development of synthetic lethal or other therapeutic approaches to these treatment-refractory malignancies.

Materials and Methods

Chromosome Combing. For chromosome combing, at least 1×10^6 BJ cells or five colonies of each genotype were pooled and resuspended in StemSpan Serum-Free Expansion Medium (SFEM) supplemented with 30% (vol/vol) FBS, 50 μg/mL stem cell factor, 3 U/mL erythropoietin, and 40 ng/mL insulin-like growth factor-1. Replicating DNA was first labeled with 25 μM 5-iodo-2′-deoxyuridine (IdU; Sigma), followed by 250 μM 5-chloro-2′-deoxyuridine (ClDU; Sigma) for 20 min each. Cells were then harvested, genomic DNA was extracted, and individual DNA molecules were stretched on glass slides, stained with anti-IdU (1:300) and anti-CldU (1:150), and analyzed under oil immersion on a Zeiss Axioscop 2 fluorescent microscope. Methodology for calculating fork stalling, origin symmetry, and fork rate are described in the *SI Appendix, Materials and Methods*. Additional information on all other experimental protocols also is provided in the *SI Appendix, Materials and Methods*.

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