**Second generation tyrosine kinase inhibitors prevent disease progression in high-risk (high CIP2A) chronic myeloid leukaemia patients.**

**Authors:** Claire M Lucas,[1] Robert J Harris,[1] Alison K Holcroft,[1] Laura J Scott,[1] Natasha Carmell,[1] Elizabeth McDonald,[1] Fotios Polydoros,[2] and Richard E Clark.[1]

**Affiliation:** 1. Section of Haematology, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, United Kingdom. L69 3GA. 2. CR-UK Liverpool Cancer Trials Unit, University of Liverpool

**Correspondence:** Prof. Richard E Clark,
Section of Haematology,
Department of Molecular and Clinical Cancer Medicine,
University of Liverpool,
Liverpool,
United Kingdom
L69 3GA.
Email: cml@liv.ac.uk.
Telephone: +44 (0)151-706-4373
FAX: +44 (0)151-706-5810

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**ABSTRACT**

High CIP2A protein levels at diagnosis of chronic myeloid leukaemia (CML) are predictive of disease progression in imatinib treated patients. It is not known whether this is true in patients treated with second generation tyrosine kinase inhibitors (2G TKI) from diagnosis, and whether 2G TKIs modulate the CIP2A pathway. Here, we show that patients with high diagnostic CIP2A levels who receive a 2G TKI do not progress, unlike those treated with imatinib (p=<0.0001). 2G TKIs induce more potent suppression of CIP2A and c-Myc than imatinib. The transcription factor E2F1 is elevated in high CIP2A patients and following 1 month of *in vivo* treatment 2G TKIs suppress E2F1 and reduce CIP2A; these effects are not seen with imatinib. Silencing of CIP2A, c-Myc or E2F1 in K562 cells or CML CD34+ cells reactivates PP2A leading to BCR-ABL suppression. CIP2A increases proliferation and this is only reduced by 2G TKIs. Patients with high CIP2A levels should be offered 2G TKI treatment in preference to imatinib. 2G TKIs disrupt the CIP2A/c-Myc/E2F1 positive-feedback loop, leading to lower disease progression risk. The data supports the view that CIP2A inhibits PP2Ac, stabilising E2F1, creating a CIP2A/c-Myc/E2F1 positive-feedback loop which imatinib cannot overcome.

**INTRODUCTION**

Chronic myeloid leukaemia (CML) is a malignant disease of the primitive haematological cell in which a reciprocal translocation between chromosomes 9 and 22 creates the fusion gene *BCR-ABL1* which is the molecular hallmark of the disease.[1] CML treatment has been significantly improved by the tyrosine kinase inhibitor (TKI) imatinib, but at least one-third of patients will eventually fail imatinib treatment[2,3] and a significant proportion of these will progress towards blast crisis (BC), which is usually rapidly fatal.

Impairment of PP2A activity by its negative regulators SET and cancerous inhibitor of PP2A (CIP2A) plays an important role in the pathogenesis and progression of CML.[4,5] CIP2A protein is associated with increased proliferation in several human malignancies[6-10] and its over-expression can cause cellular transformation.[7,11] High levels are an adverse prognostic indicator in many malignancies including AML,[12] breast,[8, 13-15] bladder,[16] cervical,[17,18] and lung cancers.[10,20,21] In CML, we have shown that high levels of CIP2A protein at chronic phase diagnosis is a strong prospective predictor of subsequent development of BC.[5] CIP2A functions by preventing PP2A driven dephosphorylation of c-Myc at residue serine 62, which results in stabilisation of c-Myc.[2,7,9,15] SET also inhibits c-Myc degradation.[22] c-Myc is essential for BCR-ABL mediated cellular transformation[23] and is over-expressed at transformation to BC.[24] In CML, elevated levels of c-Myc may promote aneuploidy, contributing to disease progression.[25,26] Furthermore, protein levels of c-Myc at diagnosis correlate with imatinib treatment response.[5]

E2F1 is a transcription factor which is over-expressed in CML.[27] E2F proteins are involved in cell cycle regulation by controlling genes required for cell-cycle progression at the G1/S checkpoint.[28] E2F1 transcriptionally regulates CIP2A[8] and c-Myc.[29] E2F1 activates cell cycle and over-expression of E2F1 results in uncontrolled cellular proliferation.[30] There is cross-talk between E2F transcription factors, as depletion of E2F1 has no effect and E2F1 deficient mouse embryos develop normally.[31] E2F1 activity can be regulated by phosphorylation on residue serine 364, which is controlled by PP2A.[32] When phosphorylated, E2F1 is stabilised and cannot be degraded. In addition to phosphorylation, E2F1 transcriptional activity can be negatively regulated via the p53/p21/Rb pathway.[8,32,33]

We have previously shown that patients with a high CIP2A protein level at diagnosis and treated with imatinib have a 100% actuarial risk of progressing to BC.[5] However, the second generation TKIs (2G TKIs) dasatinib and nilotinib are now increasingly used for first-line CML treatment and both drugs

may have superior clinical outcomes compared to imatinib.[34,35] It is not known whether high CIP2A levels confer a poor prognosis in patients treated with a 2G TKI at initial diagnosis or whether the 2G TKIs modulate the CIP2A pathway. In this study we assessed the clinical outcome of 74 patients treated with imatinib or a 2G TKI (dasatinib or nilotinib) in relation to their diagnostic CIP2A level. Here we confirm our earlier report that imatinib treated patients with high diagnostic CIP2A have a high risk of developing BC, in a larger cohort with longer follow up. We also report that in contrast, patients with high diagnostic CIP2A do not progress to BC if treated with a 2G TKI. We show that this benefit is because unlike imatinib, 2G TKIs suppress CIP2A, leading to decreased c-Myc and E2F1, reactivation of PP2A, a decrease in BCR-ABL tyrosine kinase activity and reduced cellular proliferation.

## METHODS

### Patient cohort

The study was approved by the Liverpool Central Research Ethics Committee; all 74 patients gave informed consent and were aged 18 or over. All have been seen since original diagnosis of chronic phase CML at our centre and have been followed for at least 9 months (median follow-up: 50 months). Patient characteristics are shown in Table 1. Patients were diagnosed between 2005-2013 and we include all patients who progressed during this time.

### Sample Collection and Preparation

At diagnosis and after 1 month of TKI treatment, mononuclear cells (MNC) from chronic phase CML patients were separated by density-dependent centrifugation (Lymphoprep Axis-Shield, Norway), washed in RPMI 1640 (BioSera, UK), and resuspended in 10% DMSO/10% FCS (BioSera), /RPMI at 4°C. Wherever possible, samples were enriched for CD34+ cells using the CliniMACS kit (Miltenyi Biotec, USA).

### Measurement of CIP2A, PP2Ac, phosphorylated PP2A, c-Myc and E2F1.

Flow cytometry methodology has been previously described[5, 36] and was used for the detection of PP2Ac, PP2A$^{Y307}$, CIP2A, c-Myc, c-Myc$^{S62}$, E2F1 and E2F1$^{S364}$. The following antibodies were used: Anti-PP2A catalytic subunit (Merck Millipore, UK), PP2A$^{Y307}$ (Epitomics, USA), CIP2A, E2F1 and E2F1$^{S364}$ (Santa Cruz Biotechnology, USA), c-Myc (New England Biolabs, UK), c-Myc$^{S62}$ (Abcam, UK), anti-mouse and anti-rabbit Alex fluor 488 (Invitrogen, UK). Levels of pCrKL and CrKL were used as an assay of BCR-ABL1 activity, measured by flow cytometry as previously described.[36] Supplementary information is available at Leukemia's website.

### CIP2A status

CIP2A protein level was assessed by flow cytometry as described above and previously.[5] In this study the diagnostic CIP2A protein level (mean fluorescence intensity (MFI)) for all 74 patients was calculated (supplementary Table 1). This range was 0-52, the interquartile range was 0.48 – 8.65, the median was 2.85 and the mean was 6.5. High CIP2A patients are defined as those patients with a CIP2A level greater than or equal to 7.3. The cut-off value was derived using receiver operating characteristics (ROC) curve analysis for the prediction of BC based on the diagnostic CIP2A protein level; minimisation of the Euclidian distance between the ROC curve and the corner (0, 1) was the criterion used. The optimal cut-off value produced an $AUC_{ROC}$ = 0.902 (95% CI: 0.832, 0.973).

**c-Myc protein and phosphorylation status**

Briefly, whole-cell lysates were prepared from MNC using NP40 lysis buffer as previously described.[5]

***In-vitro* TKI or PP2A activation assay**

The effect of TKIs or PP2A activation was examined using MNC at a density of 2 x $10^6$/ml were cultured either untreated or with 5μM imatinib or 150nM dasatinib or 5μM nilotinib, or 2.5μM FTY720 for 24 hours.

**PP2A activity assay**

PP2A phosphatase assays were carried out using the PP2A IP phosphatase assay kit (Millipore). An optimised manufacturer's protocol for this assay was kindly provided by Professor Danilo Perrotti, University of Maryland, Maryland, USA.

**small interfering RNA (siRNA) treatment.**

K562 or CD34+ cells were transfected using Nucleofector Kit V (Lonza, Switzerland) using the Amaxa instrument. After nucleofection cells were cultured for 72 hours prior to analysis. The following siRNAs were used: Control siRNA, CIP2A, and E2F1 (Santa Cruz Biotechnology, USA), c-Myc siRNA (Thermo Scientific, USA).

**CIP2A overexpression**

K562 cells were transfected using pCMV6AC-CIP2A-GFP or pCMV6AC-GFP (OriGene, USA) as a control, using the Nucleofector Kit V (Lonza, Switzerland) using the Amaxa instrument. After nucleofection cells were cultured for 72 hours prior to analysis.

**Measurement of bromodeoxyuridine (BrdU) incorporation**

Cellular proliferation was assessed using a BrdU cell proliferation ELISA (Roche Diagnostics, UK). Briefly, 1x$10^5$ cells were incubated with BrdU for 24h. Cells were then fixed and DNA denatured, anti-BrdU-POD antibody was added, cells washed and incubated with TMB. The reaction was stopped by addition of sulphuric acid. The plate was read at 450nm.

**Statistical Analysis**

Statistical analysis and comparisons were performed using the statistical programmes SPSS 16.0 (SPSS Inc. USA) and R (R Core Team 2013, Austria). Continuous variables were summarised by descriptive statistics (median, IQR, range) while frequencies and percentages were calculated for categorical data.

For continuous variables, the Mann-Whitney U test was used for comparisons between independent samples and the Wilcoxon signed-rank test was used for paired data; for categorical variables Fisher's exact test was used. Overall and progression-free survival functions were estimated by the Kaplan-Meier estimator and the log-rank test was used for comparisons between groups. Cumulative incidence functions adjusted for the competing risk of death/ progression were estimated for Time to CCR, MMR and MR4 and differences across groups were assessed using Gray's test. All tests were two-sided at a 5% significance level. No adjustment for multiple testing was performed while no missing data imputation was required since the analysed dataset was complete.

## RESULTS

### High CIP2A expressing patients do not progress to blast crisis if treated with 2G TKI from initial diagnosis.

At diagnosis of chronic phase CML, CIP2A protein levels were assessed by flow cytometry [5] in 74 patients. Patients were stratified into high (>7.3 MFI) and low CIP2A (<7.3 MFI) groups according to their diagnostic level, as defined in the methodology.

For patients with high diagnostic CIP2A level treated with imatinib, the overall and progression-free survival probability at 24 months was estimated to be 41% (95% CI: 6% - 75%) and 17% (95% CI: 1% - 51%) respectively, compared to 100% for patients in any other group ($p<0.001$, Figure 1). Disease progression to BC only occurred in those patients with a high diagnostic level of CIP2A and treated with imatinib. All progressions occurred within 32 months from diagnosis with the median time to progression being 12.5 months (IQR: 10.3 - 20.15). Progression was not associated with the development of BCR-ABL kinase domain mutations.

The estimated cumulative incidence functions of complete cytogenetic response (cumulative CCR rate) is presented in Figure 2A; for low CIP2A level imatinib treated patients at 18 months was 85% (95% CI: 70%-95%). Only one patient with a high diagnostic CIP2A level and treated with imatinib achieved a CCR ($p<0.001$); this patient subsequently progressed. Again, this deleterious effect of high CIP2A was not seen if patients were treated with a 2G TKI from diagnosis, where the estimated cumulative CCR rate at 18 months was 86% (95% CI: 68%-96%) and 56% (28%-86%) for the low and high CIP2A 2G TKI patients respectively. A similar trend was observed in time to major molecular response (MMR), as shown in Figure 2B.

The cumulative rate of MR4 (*BCR-ABL1* transcript level of ≤0.01%) for low CIP2A imatinib treated patients was 16% (95% CI: 7-34%) at 18 months. No patient with a high diagnostic CIP2A level and treated with imatinib achieved MR4 ($p=0.008$, Figure 2C). High CIP2A patients treated with a 2G TKI have a lower rate of MR4 compared to the low CIP2A 2G TKI treated patients, at 11% and 48% at 18 months respectively. High diagnostic CIP2A levels in patients treated with a 2G TKI predict for poor molecular response ($p=0.019$).

Early molecular response (EMR, BCR-ABL1/ABL1 ratio of <10% at 3 months) is an excellent predictor of clinical outcome in imatinib treated patients.[37-39] 55% of imatinib treated low CIP2A patients achieved an EMR, compared to only 9% of high CIP2A patients ($p=0.01$ Figure 2D). 90% of low CIP2A

patients treated with a 2G TKI achieved an EMR compared to 44% of those with a high CIP2A level. These data demonstrate that patients with high CIP2A and treated with a 2G TKI have a higher rate of achieving an EMR. In summary, patients with high diagnostic CIP2A levels and treated with a 2G TKI at initial diagnosis do not progress to BC and have a similar clinical outcome to low CIP2A patients.

**2G TKIs but not imatinib suppress the CIP2A pathway.**

We investigated if the superior clinical outcome in the high CIP2A 2G TKI cohort could be explained by the 2G TKIs modulating the CIP2A pathway. MNC from 20 newly diagnosed chronic phase CML patients were treated with the three TKIs for 24 hours in an *in vitro* assay. CIP2A levels significantly decreased in high CIP2A patients following dasatinib or nilotinib treatment (p=0.007 and p=0.001 respectively), but imatinib had no significant effect (Figure 3A). This 2G TKI associated decrease in CIP2A was accompanied by a significant decrease in c-Myc following dasatinib and nilotinib treatment (p=0.002, Figure 3B). All three TKIs decreased c-Myc and c-Myc$^{S62}$ levels compared to untreated control; however dasatinib and nilotinib also caused a significant decrease beyond that achieved with imatinib (p=0.03 and p=0.008 for c-Myc and p=0.01 and p=0.008 c-Myc$^{S62}$).

PP2A activity can readily be assessed using two methods. PP2A is inactive when it is phosphorylated at Y$^{307}$, and levels of PP2A$^{Y307}$ phosphorylation can be measured by flow cytometry. PP2A activity assay can also be assessed directly, though this requires many cells; where cell availability was restricted the flow cytometry approach was used. Supplementary Figure 1 shows the correlation between PP2A activity assay and flow cytometry assessment of PP2A$^{Y307}$.

*In vitro* TKI treatment of cells from high CIP2A patients resulted in a decrease in PP2A$^{Y307}$ phosphorylation and hence increased PP2A and this activity was only observed in dasatinib (p=0.03) and nilotinib treated cells (p=0.04) and not with imatinib. This is consistent with our earlier in vivo observation that PP2A$^{Y307}$ is not reduced following 12 months of imatinib treatment in high CIP2A patients.[5] In line with this, Figure 3K shows that in high CIP2A expressing K562 cells, there is a trend for 2G TKIs to increase PP2A activity to a greater degree than imatinib. Figure 3E and J demonstrate that these differences in TKI effects are not mediated by differential suppression of BCR-ABL activity (as assessed using the pCrKL assay,[36,40,41]), since all three TKIs suppress the pCrKL/CrKL ratio to a similar degree. The differences observed were not attributable to changes in viability (Supplementary Figure 2).

The TKIs had no effect on CIP2A or c-Myc in patients with a low diagnostic CIP2A level. PP2A activity increased (demonstrated by decreased $PP2A^{Y307}$ Figure 3I); suggesting PP2A inhibition occurs by another mechanism, possibly involving SET.[4, 5]

**E2F1 acts like a control switch in the CIP2A pathway.**

Laine *et al*[8] recently demonstrated that in breast cancer, a p53-mediated increase in E2F1 leads to the up-regulation of CIP2A. In CML, p53 mutations only occur in about 15-20% of patients in BC and are rarely observed at diagnosis.[42-44] No patient with a high diagnostic CIP2A level had a 17p deletion or rearrangement, although the TP53 gene was not sequenced to unequivocally determine a mutation. E2F1 has previously been shown to be over-expressed in CML.[27] E2F1 was significantly higher in CML MNC compared to normal MNC (p=0.02). E2F1 is high in patients with high diagnostic CIP2A protein levels compared to those with low CIP2A protein levels (p=0.04, Figure 4A). In high CIP2A patients, following 1 month of clinical 2G TKI treatment, E2F1 protein levels significantly decreased (p=0.01, Figure 4B) and this was accompanied by a decrease in mRNA levels of its transcriptional target *CIP2A* (Figure 4C). In sharp contrast, imatinib did not suppress E2F1, and thus *CIP2A* mRNA levels remain unchanged. Furthermore, no difference in BCR-ABL1 mRNA expression was observed after one month of imatinib or 2G TKI treated patients.

E2F1 is stabilised by phosphorylation at $S^{364}$ and PP2A can regulate E2F1 by controlling its phosphorylation status.[8] To investigate whether E2F1 is stabilised in patients with high CIP2A protein as a consequence of impaired PP2A function, K562 cells were treated with the PP2A activator FTY720. FTY720 activates PP2A by binding and sequestering SET from PP2Ac.[22, 45-47] FTY720 increased PP2A activity (p=0.009, Figure 4D) and decreased CIP2A (p=0.01, Figure 4E). FTY720 reactivation of PP2A resulted in a decrease in $E2F1^{S364}$ (p=0.04, Figure 4F) leading to a decrease in total E2F1 protein (p=0.01, Figure 4G). These data imply that CIP2A acts to inhibit PP2A leading to stabilisation of E2F1, creating a positive feedback loop generating constant transcription and stabilisation of both CIP2A and c-Myc. This model is summarised in Figure 4H.

This putative positive feedback loop was interrogated by sequentially depleting CIP2A, c-Myc and E2F1 in K562 cells (Supplementary Figure 3). Inhibition of CIP2A reduced both c-Myc (p=0.002) and E2F1 (p=0.004). A decrease in BCR-ABL tyrosine kinase activity was also noted (data not shown). c-Myc inhibition resulted in a decrease in CIP2A (p=0.03) and E2F1 (p=0.003); again a decrease in BCR-ABL tyrosine kinase activity was observed. Finally E2F1 was inhibited and this caused a decrease in both CIP2A (p=0.004) and c-Myc (p=0.003). These data support our model in Figure 4H that a positive

feedback loop exists between CIP2A/c-Myc and E2F1. We next repeated the siRNA experiments using CD34+ selected CML cells from high CIP2A patients (Figure 5). Depleting CIP2A in CD34+ cells results in the reactivation of PP2A resulting in the dephosphorylation of E2F1; thus a decrease in E2F1 and c-Myc. This may occur by two mechanisms; firstly the decrease in CIP2A level means that CIP2A can no longer stabilise c-Myc, and secondly the reduction in E2F1 removes its drive to transcribe c-Myc (Figure 5A-C). In CD34+ cells, c-Myc knockdown resulted in a decrease in CIP2A ($p=0.04$) and E2F1 ($p=0.04$, Figure 5D-F). This is consistent with our model (Figure 4H), whereby decreased CIP2A will reactivate PP2A, leading to the dephosphorylation of E2F1 and therefore a decrease in E2F1. Finally E2F1 was depleted in CD34+ cells. This resulted in a decrease in CIP2A ($p=0.002$) and c-Myc (Figure 5G-I).

Taken together, these data suggest that the E2F1 level is a major factor regulating the CIP2A/c-Myc pathway, whereby the CIP2A/c-Myc complex inhibits PP2A activity and thus E2F1 remains phosphorylated at $S^{364}$ thereby creating a positive feedback loop. The 2G TKIs but not imatinib reduce E2F1, which is controlling the CIP2A/c-Myc pathway. This explains the clinical observation of Figures 1 and 2 that patients with high diagnostic CIP2A level do not progress to BC if treated with a 2G TKI as the CIP2A/c-Myc/E2F1 pathway is suppressed.

**CIP2A alters cellular proliferation rates**

c-Myc and E2F1 have well documented effects on proliferation, and CIP2A overexpression in neural progenitor cells increases proliferation.[48] We next examined whether high levels of CIP2A affected the rate of proliferation in CML. Inhibition of CIP2A in K562 cells by siRNA (transfection efficiency 50%) resulted in a 20% decrease in cellular proliferation ($p=0.02$, Figure 6A). Conversely, when CIP2A was over-expressed using pCMV6AC-CIP2A-GFP plasmid (transfection efficiency 70%), a 35% increase in the rate of proliferation was seen ($p=0.02$, Figure 6B). To determine if the reduction in proliferation observed when CIP2A was inhibited was a result of PP2A reactivation, K562 cells were treated with the PP2A activator FTY720. FTY720 treatment reduced the rate of proliferation ($p=0.02$, Figure 6C). Figure 6D shows that all three TKIs significantly inhibit proliferation, to a similar degree, in unmanipulated K562 cells. However, in K562 cells overexpressing CIP2A (by transfection of pCMV6-CIP2A-GFP plasmid), imatinib treatment did not alter proliferation, whereas dasatinib and nilotinib both reduced proliferation ($p=0.03$ and $p=0.03$ respectively, Figure 6E). This again demonstrates a differential effect on CIP2A between imatinib versus 2G TKI.

**DISCUSSION**

The early identification of CML patients likely to progress and die from BC is important. CIP2A has previously been identified as a biomarker which identifies those patients likely to progress into BC, if treated with imatinib.[5] Here, we extend these observations, showing that patients with a high diagnostic CIP2A level and treated with a 2G TKI do not progress to BC, unlike those treated with imatinib. The survival and molecular outcomes of those patients with a high CIP2A level at diagnosis and treated with a 2G TKI are similar to those with a low CIP2A diagnostic level, although high CIP2A patients treated with a 2G TKI have an inferior MR4 rate than low CIP2A patients, suggesting that high CIP2A in 2G TKI treated patients is a marker for poor molecular response.

Both dasatinib and nilotinib significantly decrease CIP2A levels in primary CML cells, and this is associated with a decrease in both c-Myc and BCR-ABL tyrosine kinase activity, and reactivation of PP2A. Imatinib did reduce the BCR-ABL tyrosine kinase activity, it did not affect CIP2A levels nor did it significantly reactivate PP2A.

The present data are consistent with a positive feedback loop within the CIP2A/c-Myc/E2F1 pathway (Figure 4H). E2F1 is significantly higher in patients with a high diagnostic CIP2A level; this is a likely consequence of PP2A inactivation. Reactivation of PP2A by FTY720 decreases E2F1 levels. In high CIP2A patients, CIP2A stabilises c-Myc and inactivates PP2A. When PP2A is inactivated it is no longer able to dephosphorylate E2F1 thus E2F1 remains stabilised, whereby it transcriptionally activates both CIP2A and c-Myc which sustains PP2A inactivation via a positive feedback loop. Active PP2A can cause the proteasomal degradation of non-phosphorylated BCR-ABL by activating SHP1.[4] SHP1 down-regulation is associated with disease progression.[49] If PP2A is inactivated by CIP2A then it will no longer be able to activate SHP1 and thus BCR-ABL remains phosphorylated and active. We have shown that FTY720 reduces CIP2A, but since FTY720 is also known to inhibit the PP2A-SET interaction[45-47] it is likely that both CIP2A and SET play a role in PP2A inhibition in CML.

Our study highlights a role for E2F1 in the CIP2A pathway, and others have shown that E2F1 is a target of BCR-ABL kinase activity and might have a role in BCR-ABL leukaemogenesis in vitro.[29, 50] Moreover, it cannot be assumed that E2F1 is the only transcription factor regulating CIP2A and others may influence this dynamic process. Furthermore, the role of E2F1 is complex, as there is a degree of redundancy between the family members and other members of the E2F family such as E2F3 have been shown to play a role in CML BC.[51] Although loss of E2F1 is generally associated with a decrease in proliferation, one study has shown that impaired DNA replication of haematopoietic progenitors in

E2F1/E2F2 mutant mice allows BCR-ABL expressing progenitors to outcompete non-mutated cells, promoting leukaemogenesis.[52] Thus, CIP2A remains an attractive therapeutic target since high levels are only found in malignant cells.

Using both K562 and CML CD34+ cells we demonstrate that inhibition of CIP2A decreases c-Myc and reactivates PP2A, resulting in a reduction in E2F1 levels. c-Myc inhibition decreases CIP2A and reactivates PP2A, again leading to a decrease in E2F1. Furthermore we have recently demonstrated that c-Myc inhibition the small molecule inhibitor 10058-F4 leads to a reduction in both CIP2A and BCR-ABL tyrosine kinase activity.[53] Moreover, E2F1 inhibition results in a decrease in both CIP2A and c-Myc. These data suggest that disruption of the CIP2A/c-Myc/E2F1 interaction is important for the reactivation of PP2A and suppression of BCR-ABL.

CIP2A is known to stabilise c-Myc. c-Myc plays a critical role in proliferation and cell cycle, and levels are elevated in those patients with a high diagnostic CIP2A level.[5] E2F1 results in uncontrolled cellular proliferation.[30] CIP2A has been shown to be involved in cell cycle progression through chromosome separation and mitotic spindle dynamics,[54] supporting the notion that CIP2A plays a role in proliferation. Here we have shown that CIP2A, c-Myc and E2F1 interact and regulate each other. Inhibition of CIP2A reduces and overexpression increases proliferation, suggesting that patients with a high diagnostic CIP2A level potentially have cells which will proliferate faster. Importantly, we show that 2G TKIs but not imatinib reduce proliferation when CIP2A is overexpressed.

In those patients with a low diagnostic level of CIP2A, imatinib treatment can suppress both BCR-ABL and SET, and this leads to a degree of PP2A reactivation allowing SHP1 to dephosphorylate BCR-ABL. In patients with high prevailing CIP2A levels, their PP2A activity is suppressed to a much greater degree by both CIP2A and SET and imatinib is unable to relieve this suppression. 2G TKIs can suppress CIP2A and E2F1, leading to reactivation of PP2A and suppression of BCR-ABL. Moreover, we show that E2F1 is elevated in high CIP2A patients and this is reduced by 2G TKIs but not imatinib. We speculate that PP2A inactivation may be the key event. When BCR-ABL is inhibited with a TKI, PP2A is reactivated, and E2F1 and CIP2A are reduced. If CIP2A is inhibited by siRNA, then again PP2A is reactivated leading to a reduction in E2F1 and BCR-ABL.

In conclusion, our study highlights the importance of CIP2A/c-Myc/E2F1 pathway in CML. A patient with a high diagnostic CIP2A level is far less likely to progress to BC if treated with a 2G TKI from original diagnosis, because the 2G TKIs modulate the CIP2A/c-Myc/E2F1 pathway. Overall, these data suggest

that if a patient has a high CIP2A level at diagnosis then they should not be treated with imatinib due to the high risk of disease progression; these patients should be offered either dasatinib or nilotinib upfront. There is therefore now a case for routine CIP2A testing at diagnosis. Further study to determine the true incidence of high CIP2A patients in the general CML population is needed. A multivariate analysis of a bigger cohort is also required to establish relationships between the high level of CIP2A and other factors in predicting response to these TKIs and clinical outcome in CML.

Supplementary information is available at Leukemia's website.

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**Authorship**

CML performed experiments, designed the study and wrote the manuscript.

AKH, EM, NC and LJS performed experiments.

FP advised on and performed statistical analysis.

RJH and REC designed the study and wrote the manuscript

**REFERENCES**


1. Ben-Neriah Y, Daley G Q, Mes-Masson A M, Witte O N, Baltimore D. The chronic myelogenous leukemia-specific p210 protein is the product of the BCR/ABL hybrid gene. *Science* 1986; **233:** 212–214.

2. Lucas CM, Wang L, Austin GM, Knight K, Watmough SJ, Shwe KH*, et al.* A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia* 2008; **22**(10)**:** 1963-1966.

3. de Lavallade H, Apperley JF, Khorashad JS, Milojkovic D, Reid AG, Bua M*, et al.* Imatinib for Newly Diagnosed Patients With Chronic Myeloid Leukemia: Incidence of Sustained Responses in an Intention-to-Treat Analysis. *Journal of Clinical Oncology* 2008; **26**(20)**:** 3358-3363.

4. Neviani P, Santhanam R, Trotta R, Notari M, Blaser BW, Liu S*, et al.* The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell* 2005; **8**(5)**:** 355-368.

5. Lucas CM, Harris RJ, Giannoudis A, Copland M, Slupsky JR, Clark RE. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression. *Blood* 2011; **117**(24)**:** 6660-6668.

6. Côme C, Laine A, Chanrion M, Edgren, 4 H, Mattila E, Liu X*, et al.* CIP2A Is Associated with Human Breast Cancer Aggressivity. *Clinical Cancer Research* 2009; **15**(16)**:** 5092-5100.

7. Junttila MR, Puustinen P, Niemela M, Ahola R, Arnold H, Bottzauw T*, et al.* CIP2A inhibits PP2A in human malignancies. *Cell* 2007 Jul 13; **130**(1)**:** 51-62.

8. Laine A, Sihto H, Come C, Rosenfeldt MT, Zwolinska A, Niemela M*, et al.* Senescence sensitivity of breast cancer cells is defined by positive feedback loop between CIP2A and E2F1. *Cancer Discov* 2013; **3**(2)**:** 182-197.

9. Khanna A, Bockelman C, Hemmes A, Junttila MR, Wiksten JP, Lundin M*, et al.* MYC-dependent regulation and prognostic role of CIP2A in gastric cancer. *Journal of the National Cancer Institute* 2009 Jun 3; **101**(11)**:** 793-805.

10. Ma L, Wen Z, Liu Z, Hu Z, Ma J, Chen X-Q*, et al.* Overexpression and Small Molecule-Triggered Downregulation of CIP2A in Lung Cancer. *PLoS ONE* 2011; **6**(5)**:** e20159.

11. Mathiasen DP, Egebjerg C, Andersen SH, Rafn B, Puustinen P, Khanna A*, et al.* Identification of a c-Jun N-terminal kinase-2-dependent signal amplification cascade that regulates c-Myc levels in ras transformation. *Oncogene* 2012; **31**(3)**:** 390-401.

12. Wang J, Li W, Li L, Yu X, Jia J, Chen C. CIP2A is over-expressed in acute myeloid leukaemia and associated with HL60 cells proliferation and differentiation. *International Journal of Laboratory Hematology* 2011; **33**(3)**:** 290-298

13. Yu G, Liu G, Dong J, Jin Y. Clinical implications of CIP2A protein expression in breast cancer. *Med Oncol* 2013; **30**(2)**:** 013-0524.

14. Tseng LM, Liu CY, Chang KC, Chu PY, Shiau CW, Chen KF. CIP2A is a target of bortezomib in human triple negative breast cancer cells. *Breast cancer research : BCR* 2012; **14**(2)**:** R68.

15. Niemela M, Kauko O, Sihto H, Mpindi J, Nicorici D, Pernila P*, et al.* CIP2A signature reveals the MYC dependency of CIP2A-regulated phenotypes and its clinical association with breast cancer subtypes. *Oncogene* 2012.

16. Huang LP, Savoly D, Sidi AA, Adelson ME, Mordechai E, Trama JP. CIP2A protein expression in high-grade, high-stage bladder cancer. *Cancer Med* 2012; **1**(1)**:** 76-81.

17. Pallai R, Bhaskar A, Sodi V, Rice LM. Ets1 and Elk1 transcription factors regulate cancerous inhibitor of protein phosphatase 2A expression in cervical and endometrial carcinoma cells. *Transcription* 2012; **3:** 6.

18. Huang LP, Adelson ME, Mordechai E, Trama JP. CIP2A expression is elevated in cervical cancer. *Cancer Biomark* 2010; **8**(6)**:** 309-317.

19. He H, Wu G, Li W, Cao Y, Liu Y. CIP2A is highly expressed in hepatocellular carcinoma and predicts poor prognosis. *Diagn Mol Pathol* 2012; **21**(3)**:** 143-149.

20. Sung WW, Wang YC, Lin PL, Cheng YW, Chen CY, Wu TC*, et al.* IL-10 Promotes Tumor Aggressiveness via Upregulation of CIP2A Transcription in Lung Adenocarcinoma. *Clin Cancer Res* 2013; **6:** 6.

21. Dong Q-Z, Wang Y, Dong X-J, Li Z-X, Tang Z-P, Cui Q-Z*, et al.* CIP2A is Overexpressed in Non-Small Cell Lung Cancer and Correlates with poor prognosis. *Annals of Surgical Oncology* 2010**:** 1-9.

22. Mukhopadhyay A, Saddoughi SA, Song P, Sultan I, Ponnusamy S, Senkal CE*, et al.* Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *Faseb j* 2009 Mar; **23**(3)**:** 751-763.

23. Sawyers CL, Callahan W, Witte ON. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell* 1992; **70**(6)**:** 901-910.

24. Strnad M, Brajusković G, Strelić N, Zivanović BT, Tukić L, Stamatović D. Expression of Bcl-2 protein and the amplification of c-myc gene in patients with chronic myeloid leukemia. *Vojnosanit Pregl* 2006; **63**(4)**:** 364-369.

25. Prochownik EV, Li Y. The ever expanding role for c-Myc in promoting genomic instability. *Cell Cycle* 2007; **6**(9)**:** 1024-1029.

26. Oudat R, Khan Z, Glassman AB. Detection of Trisomy 8 in Philadelphia Chromosome-Positive CML Patients Using Conventional Cytogenetic and Interphase Fluorescence in situ Hybridization Techniques and its Relation to c-myc Involvement. *Annals of Clinical and Laboratory Science* 2001 January 1, 2001; **31**(1)**:** 68-74.

27. Mohty M, Yong ASM, Szydlo RM, Apperley JF, Melo JV. The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. *Blood* 2007 July 1, 2007; **110**(1)**:** 380-383.

28. Engelmann D, Pützer BM. The Dark Side of E2F1: In Transit beyond Apoptosis. *Cancer Research* 2012 February 1, 2012; **72**(3)**:** 571-575.

29. Stewart MJ, Litz-Jackson S, Burgess GS, Williamson EA, Leibowitz DS, Boswell HS. Role for E2F1 in p210 BCR-ABL downstream regulation of c-myc transcription initiation. Studies in murine myeloid cells. *Leukemia* 1995; **9**(9)**:** 1499-1507.

30. Singh P, Wong SH, Hong W. Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *EMBO J* 1994; **13**(14)**:** 3329-3338.

31. Tsai S-Y, Opavsky R, Sharma N, Wu L, Naidu S, Nolan E*, et al.* Mouse development with a single E2F activator. *Nature* 2008; **454**(7208)**:** 1137-1141.

32. Mundle SD, Saberwal G. Evolving intricacies and implications of E2F1 regulation. *FASEB J* 2003; **17**(6)**:** 569-574.

33. Polager S, Ginsberg D. p53 and E2f: partners in life and death. *Nat Rev Cancer* 2009; **9**(10)**:** 738-748.

34. Larson RA, Hochhaus A, Hughes TP, Clark RE, Etienne G, Kim DW*, et al.* Nilotinib vs imatinib in patients with newly diagnosed Philadelphia chromosome-positive chronic myeloid leukemia in chronic phase: ENESTnd 3-year follow-up. *Leukemia* 2012; **26**(10)**:** 2197-2203.

35. Kantarjian HM, Shah NP, Cortes JE, Baccarani M, Agarwal MB, Undurraga MS*, et al.* Dasatinib or imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: 2-year follow-up from a randomized phase 3 trial (DASISION). *Blood* 2012; **119**(5)**:** 1123-1129.

36. Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. *British Journal of Haematology* 2010; **149**(3)**:** 458-460.

37. Wang L, Pearson K, Ferguson JE, Clark RE. The early molecular response to imatinib predicts cytogenetic and clinical outcome in chronic myeloid leukaemia. *British Journal of Haematology* 2003; **120:** 990-999.

38. Marin D, Ibrahim AR, Lucas C, Gerrard G, Wang L, Szydlo RM*, et al.* Assessment of BCR-ABL1 transcript levels at 3 months is the only requirement for predicting outcome for patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors. *Journal of Clinical Oncology* 2012 Jan 20; **30**(3)**:** 232-238.

39. Neelakantan P, Gerrard G, Lucas C, Milojkovic D, May P, Wang L*, et al.* Combining BCR-ABL1 transcript levels at 3 and 6 months in chronic myeloid leukemia: implications for early intervention strategies. *Blood* 2013; **121**(14)**:** 2739-2742.

40. Hamilton A, Elrick L, Myssina S, Copland M, Jorgensen H, Melo JV*, et al.* BCR-ABL activity and its response to drugs can be determined in CD34+ CML stem cells by CrkL phosphorylation status using flow cytometry. *Leukemia* 2006; **20:** 1035-1039.

41. White DL, Saunders V, Lyons AB, Branford S, Grigg A, To LB*, et al.* In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. *Blood* 2005 1st October 2005; **106**(7)**:** 2520-2526.

42. Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood* 2004 June 1, 2004; **103**(11)**:** 4010-4022.

43. Feinstein E, Cimino G, Gale RP, Alimena G, Berthier R, Kishi K*, et al.* p53 in chronic myelogenous leukemia in acute phase. *Proceedings of the National Academy of Sciences* 1991 July 15, 1991; **88**(14)**:** 6293-6297.

44. Ahuja H, Bar-Eli M, Advani SH, Benchimol S, Cline MJ. Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia. *PNAS* 1989 September 1, 1989; **86**(17)**:** 6783-6787.

45. Neviani P, Santhanam R, Oaks J, Eiring A, Notari M, Blaser B*, et al.* FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *Journal of Clinical Investigation* 2007; **117**(9)**:** 2408–2421.

46. Saddoughi SA, Gencer S, Peterson YK, Ward KE, Mukhopadhyay A, Oaks J*, et al.* Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumour suppression via activation of PP2A-RIPK1-dependent necroptosis. *EMBO Molecular Medicine* 2013; **5**(1)**:** 105-121.

47. Neviani P, Harb JG, Oaks JJ, Santhanam R, Walker CJ, Ellis JJ*, et al.* PP2A-activating drugs selectively eradicate TKI-resistant chronic myeloid leukemic stem cells. *J Clin Invest* 2013 Oct 1; **123**(10)**:** 4144-4157.

48. Kerosuo L, Fox H, Perälä N, Ahlqvist K, Suomalainen A, Westermarck J*, et al.* CIP2A increases self-renewal and is linked to Myc in neural progenitor cells. *Differentiation* 2010; **80**(1)**:** 68-77.

49. Amin HM, Hoshino K, Yang H, Lin Q, Lai R, Garcia-Manero G. Decreased expression level of SH2 domain-containing protein tyrosine phosphatase-1 (Shp1) is associated with progression of chronic myeloid leukaemia. *J Pathol* 2007; **212**(4)**:** 402-410.

50. Birchenall-Roberts MC, Yoo YD, Bertolette DC, 3rd, Lee KH, Turley JM, Bang OS*, et al.* The p120-v-Abl protein interacts with E2F-1 and regulates E2F-1 transcriptional activity. *J Biol Chem* 1997; **272**(14)**:** 8905-8911.

51. Eiring AM, Neviani P, Santhanam R, Oaks JJ, Chang JS, Notari M*, et al.* Identification of novel posttranscriptional targets of the BCR/ABL oncoprotein by ribonomics: requirement of E2F3 for BCR/ABL leukemogenesis. *Blood* 2008 Jan 15; **111**(2)**:** 816-828.

52. Bilousova G, Marusyk A, Porter CC, Cardiff RD, DeGregori J. Impaired DNA replication within progenitor cell pools promotes leukemogenesis. *PLoS biology* 2005 Dec; **3**(12)**:** e401.

53. Lucas CM, Harris RJ, Giannoudis A, Clark RE. c-Myc inhibition decreases CIP2A and reduces BCR-ABL1 tyrosine kinase activity in chronic myeloid leukemia. *Haematologica* 2015 2015-01-01 00:00:00; **Ahead of print**.

54. Jeong AL, Lee S, Park JS, Han S, Jang C-Y, Lim J-S*, et al.* Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A) Protein Is Involved in Centrosome Separation through the Regulation of NIMA (Never In Mitosis Gene A)-related Kinase 2 (NEK2) Protein Activity. *Journal of Biological Chemistry* 2014 January 3, 2014; **289**(1)**:** 28-40.

**FIGURE LEGENDS**

**Table 1. Patient characteristics.**

**Figure 1. High CIP2A expressing patients do not progress if treated with a second generation TKI.**
All 74 patients involved in the study are included in the Kaplan-Meier estimates of overall survival and progression free survival plots. Panel A. Overall survival, Panel B. Progression-free survival, the number of assessable cases are shown below each plot and the survival probability along with confidence internals (CI) are shown in the table directly below each plot. The Log rank test was used.

**Figure 2. High CIP2A expressing patients can achieve a deep molecular response if treated with a second generation TKI.**
Cumulative Incidence functions for time to CCR, MMR and MR4 adjusted for the competing risk of death/progression. All 74 patients involved in the study are included in the analysis. Panel A. Time to CCR, Panel B. Time to MMR, Panel C. Time to MR4 and the number of assessable cases are shown below each plot and the probability of achieving CCR, MMR and MR4 along with confidence internals (CI) are shown in the table directly below each plot. Gray's test was used to analyse panels A-C and Fisher's exact test in panel C.

**Figure 3. 2G TKIs decrease CIP2A and c-Myc with reactivation of PP2A.**
MNC from chronic phase newly diagnosed CML patients were treated with 5μM imatinib, 150nM dasatinib or 5μM nilotinib for 24 hours. Patients were stratified into high CIP2A (left hand side of the Figure) and low CIP2A (right hand side of the Figure). Panel A show the effects of TKIs on CIP2A protein as assessed by flow cytometry. Panels B and C, show the effect on c-Myc and c-Myc$^{S62}$ levels expressed as absorbance units by an ELISA. Panel D, the effect on PP2A$^{Y307}$ protein level assessed by flow cytometry. Panel E the effect on BCR-ABL tyrosine kinase activity, determined using the pCrKL/CrKL ratio assessed by flow cytometry (n=20 total, low CIP2A n=9 and high CIP2A n=11). p values in each case relate to the comparison with untreated control.; for comparison of imatinib vs 2G TKI see text. Statistical analysis was calculated using Mann-Whitney test. Panel F shows the effects on PP2A activity in K562 cells. Error bars are standard error of mean (SEM)

**Figure 4. The transcription factor E2F1 is elevated in high CIP2A patients and is suppressed by 2G TKIs but not imatinib.**

Panel A. E2F1 levels were assessed by flow cytometry using diagnostic chronic phase MNC from low and high CIP2A patients (n=21). Panel B. E2F1 levels in high CIP2A patients at diagnosis and following 1 month of clinical imatinib (n=5) or 2G TKI treatment (n=5). Panel C. CIP2A mRNA expression decreases following 24 hour treatment with 2G TKI but not imatinib (n=18). Panel D. effects of 24 hours treatment with the PP2A activator 2.5μM FTY720 on PP2A activity in K562 cells (n=7). Panel E. CIP2A protein following treatment with 2.5μM FTY720 (n=10). Panel F-G. FTY720 treatment decreases both E2F1 and E2F1$^{S364}$ in K562 cells (n=6). All values in the panels are means -/+ standard error of mean (SEM).Panel H. A suggested model for the CIP2A/c-Myc/E2F1 pathway. Arrows denote activation, blunt ended arrows indicated inhibition. Phosphorylated E2F1 can transcribe both CIP2A and c-Myc, which stabilise each other and cause PP2A inhibition. Inactive PP2A cannot dephosphorylate E2F1 and thus E2F1 remains active; creating a positive feedback loop. Statistical analysis was performed using Mann-Whitney test.

**Figure 5. Inhibition of CIP2A, c-Myc and E2F1 in CD34+ cells**

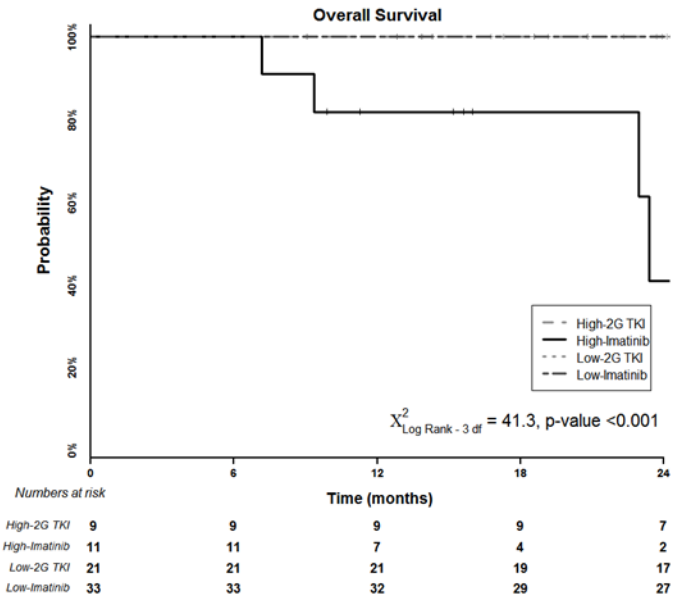
CD34+ selected CML cells from five patients were treated with each siRNA for 72 hours prior to analysis. With CIP2A siRNA a decrease in CIP2A, c-Myc and E2F1 was observed (panels A-C). With c-Myc siRNA a decrease in CIP2A, c-Myc and E2F1 was observed (panels D-F). With E2F1 siRNA a decrease in CIP2A, c-Myc and E2F1 was observed (panels G-I). Statistical analysis was performed using Mann-Whitney test. Error bars are standard error of mean (SEM).

**Figure 6. CIP2A alters the rate of proliferation**

The effects of CIP2A and TKI treatment on proliferation rate was assessed in K562 cells using a BrdU assay each assay was performed in triplicate and the experiment was perform a minimum of three times. Panel A. Inhibition of CIP2A decreases proliferation (n=4). Panel B. Overexpression of CIP2A using the pCMV8-CIP2A-GFP plasmid increases proliferation (n=5). Panel C. FTY720 reduces proliferation (n=4). Panel D. All three TKIs reduce proliferation rates significantly (n=4). Panel E. CIP2A was over expressed using the pCMV8-CIP2A-GFP and subsequently treated with imatinib, dasatinib and nilotinib. Only dasatinib and nilotinib reduced the rate of proliferation (n=5). Statistical analysis was performed using Mann-Whitney test. Error bars are standard error of mean (SEM)

**Figure 1**

**A**

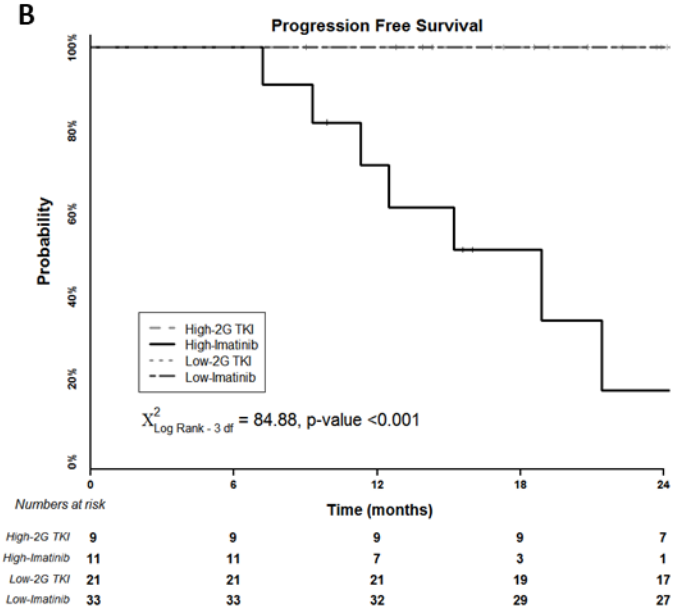



| Group | No. treated | No. of events [1] |
|---|---|---|
| High CIP2A – 2G TKI | 9 | 0 |
| High CIP2A - Imatinib | 11 | 5 |
| Low CIP2A – 2G TKI | 21 | 0 |
| Low CIP2A - Imatinib | 33 | 1 |

1. Death from any cause

| Overall Survival estimates for High CIP2A – Imatinib | | |
|---|---|---|
| Time (months) | Survival Probability | 95% CI (log-log) |
| 6 | 100% | - |
| 12 | 82% | 45% - 95% |
| 18 | 82% | 45% - 95% |
| 24 | 41% | 6% - 75% |

**B**



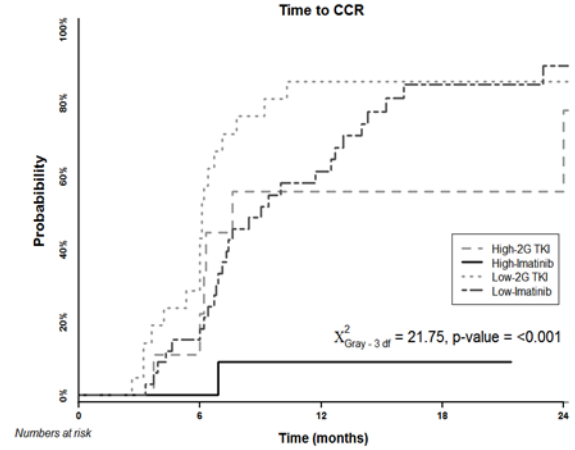

| Group | No. treated | No. of events [2] |
|---|---|---|
| High CIP2A – 2G TKI | 9 | 0 |
| High CIP2A - Imatinib | 11 | 8 |
| Low CIP2A – 2G TKI | 31 | 0 |
| Low CIP2A - Imatinib | 33 | 1 |

2. Progression or death from any cause (whichever occurred first)

| Progression free Survival estimates for High CIP2A – Imatinib | | |
|---|---|---|
| Time (months) | Survival Probability | 95% CI (log-log) |
| 6 | 100% | - |
| 12 | 72% | 35% - 90% |
| 18 | 51% | 19% - 76% |
| 24 | 17% | 1% - 51% |

## Figure 2

**A**



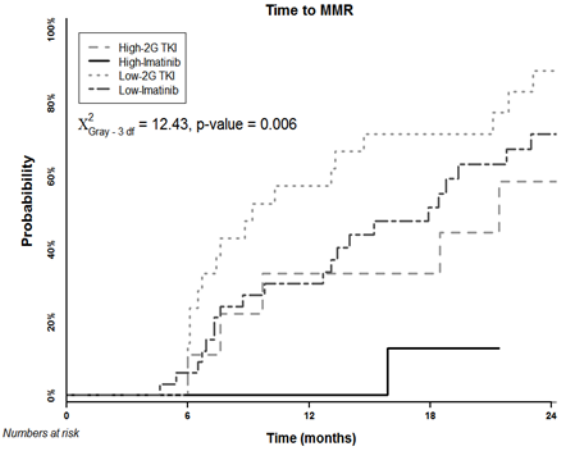

Numbers at risk

| | | | | | |
|---|---|---|---|---|---|
| High-2G TKI | 9 | 8 | 4 | 4 | 2 |
| High-Imatinib | 11 | 11 | 6 | 2 | 0 |
| Low-2G TKI | 21 | 15 | 3 | 2 | 1 |
| Low-Imatinib | 33 | 28 | 12 | 4 | 2 |

| Group | No. treated | No. of competing events [1] | No. of CCRs | Cumulative Incidence estimates for CCR Probability at 18 months | |
|---|---|---|---|---|---|
| | | | | CCR % | 95% CI (log-log) |
| High CIP2A – 2G TKI | 9 | 0 | 7 | 56% | 28% - 86% |
| High CIP2A - Imatinib | 11 | 7 | 1 | 9% | 1% - 49% |
| Low CIP2A – 2G TKI | 21 | 0 | 19 | 86% | 68% - 96% |
| Low CIP2A - Imatinib | 33 | 0 | 29 | 85% | 70% - 95% |

1. Progression or death from any cause (whichever occurred first) before CCR.

**B**



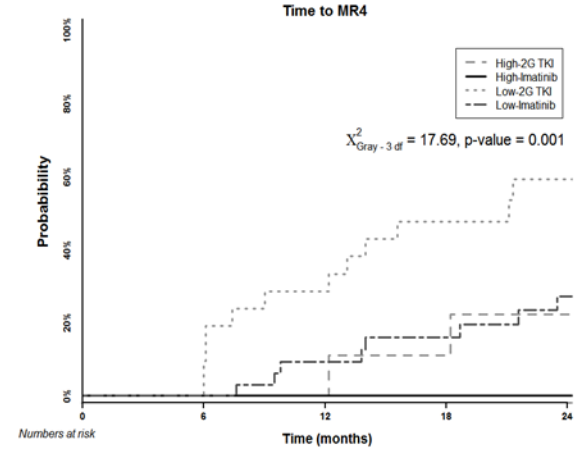

Numbers at risk

| | | | | | |
|---|---|---|---|---|---|
| High-2G TKI | 9 | 9 | 6 | 6 | 2 |
| High-Imatinib | 11 | 11 | 7 | 2 | 0 |
| Low-2G TKI | 21 | 21 | 9 | 5 | 1 |
| Low-Imatinib | 33 | 31 | 22 | 13 | 7 |

| Group | No. treated | No. of competing events [1] | No. of MMRs | Cumulative Incidence estimates for MMR Probability at 18 months | |
|---|---|---|---|---|---|
| | | | | MMR % | 95% CI (log-log) |
| High CIP2A – 2G TKI | 9 | 0 | 7 | 33% | 12% - 72 % |
| High CIP2A - Imatinib | 11 | 7 | 1 | 13% | 1% - 51% |
| Low CIP2A – 2G TKI | 21 | 0 | 18 | 71% | 52% - 88% |
| Low CIP2A - Imatinib | 33 | 0 | 26 | 51% | 35% - 70% |

1. Progression or death from any cause (whichever occurred first) before MMR.

**C**



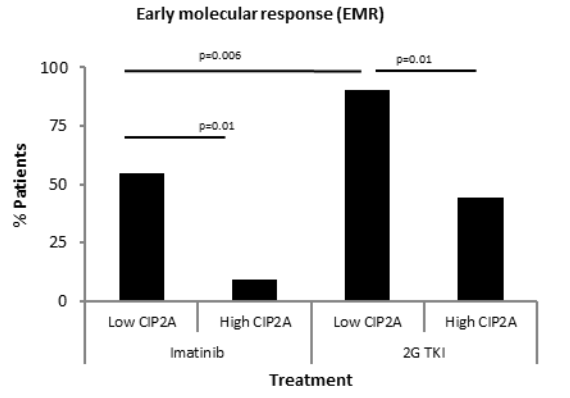

Numbers at risk

| | | | | | |
|---|---|---|---|---|---|
| High-2G TKI | 9 | 9 | 9 | 8 | 5 |
| High-Imatinib | 11 | 11 | 7 | 3 | 1 |
| Low-2G TKI | 21 | 21 | 15 | 9 | 6 |
| Low-Imatinib | 33 | 33 | 29 | 24 | 19 |

| Group | No. treated | No. of competing events [1] | No. of MR4s | Cumulative Incidence estimates for MR4 Probability at 18 months | |
|---|---|---|---|---|---|
| | | | | MR4 % | 95% CI (log-log) |
| High CIP2A – 2G TKI | 9 | 0 | 3 | 11% | 2% - 57% |
| High CIP2A - Imatinib | 11 | 8 | 0 | 0% | - |
| Low CIP2A – 2G TKI | 21 | 0 | 16 | 48% | 29% - 70% |
| Low CIP2A - Imatinib | 33 | 0 | 18 | 16% | 7% - 34% |

1. Progression or death from any cause (whichever occurred first) before MR4.

**D**




Two sided Fisher's exact test p-values

| Comparison | | No. treated | No. of EMRs (%) | Relative Risk [1] | 95% Confidence Interval (RR) [1] |
|---|---|---|---|---|---|
| 2G TKI | Low CIP2A | 21 | 19 (90%) | 2.04 | 0.97 – 4.28 |
| | High CIP2A | 9 | 4 (44%) | | |
| Imatinib | Low CIP2A | 33 | 18 (55%) | 6.00 | 0.90-39.90 |
| | High CIP2A | 11 | 1 (9%) | | |
| High CIP2A | 2G TKI | 9 | 4 (4%) | 4.89 | 0.66 – 36.36 |
| | Imatinib | 11 | 1 (9%) | | |

1. Unconditional normal approximation (Wald)

**Figure 3.**

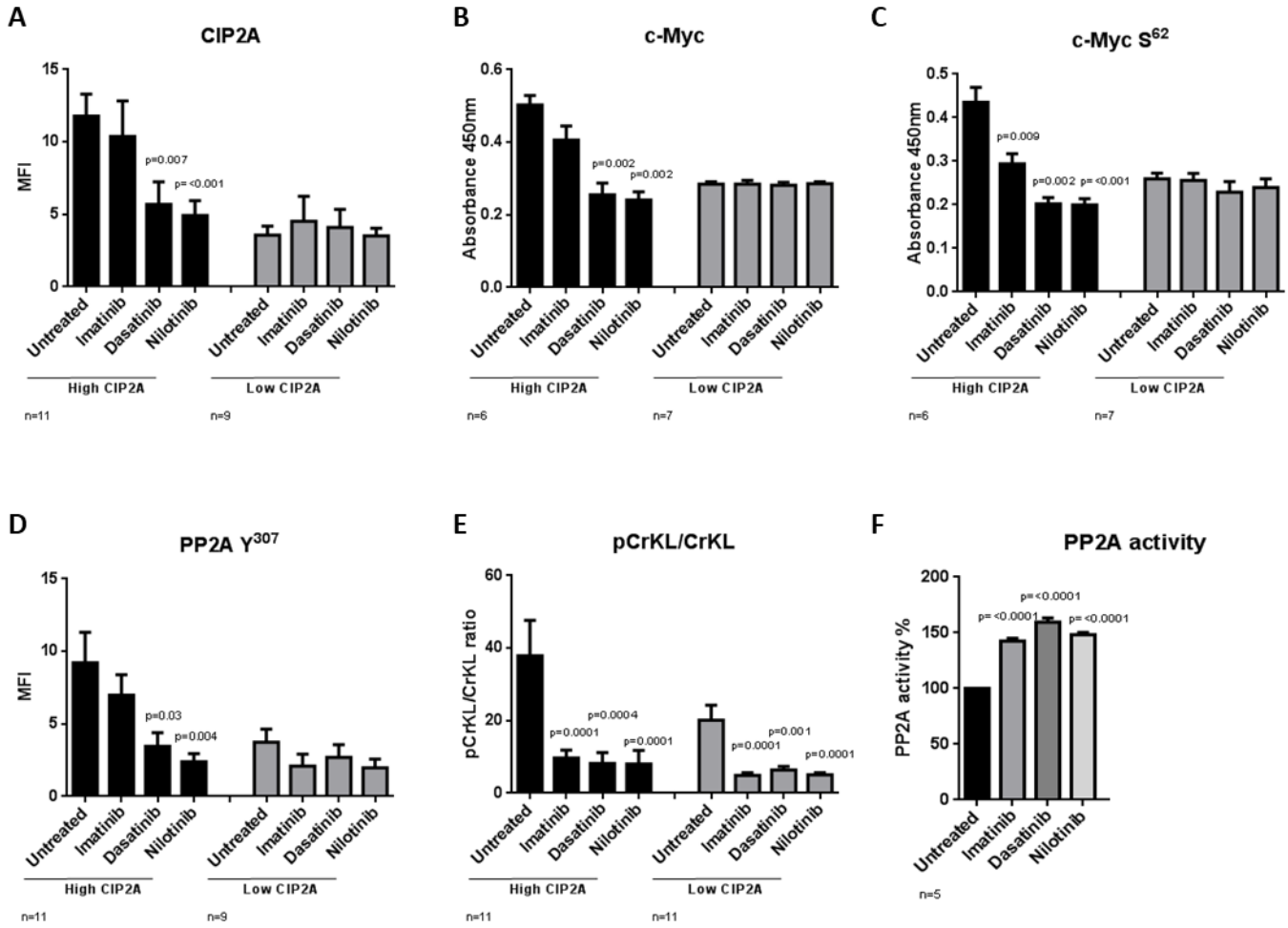

A
CIP2A
MFI
p=0.007
p= <0.001
Untreated
Imatinib
Dasatinib
Nilotinib
High CIP2A
Low CIP2A
n=11
n=9
B
c-Myc
Absorbance 450nm
p=0.002
p=0.002
n=6
n=7
C
c-Myc S62
Absorbance 450nm
p=0.009
p=0.002
p= <0.001
n=6
n=7
D
PP2A Y307
MFI
p=0.03
p=0.004
n=11
n=9
E
pCrKL/CrKL
pCrKL/CrKL ratio
p=0.0001
p=0.0004
p=0.0001
p=0.0001
p=0.001
p=0.0001
n=11
n=11
F
PP2A activity
PP2A activity %
p= <0.0001
p= <0.0001
p= <0.0001
n=5

**Figure 4.**

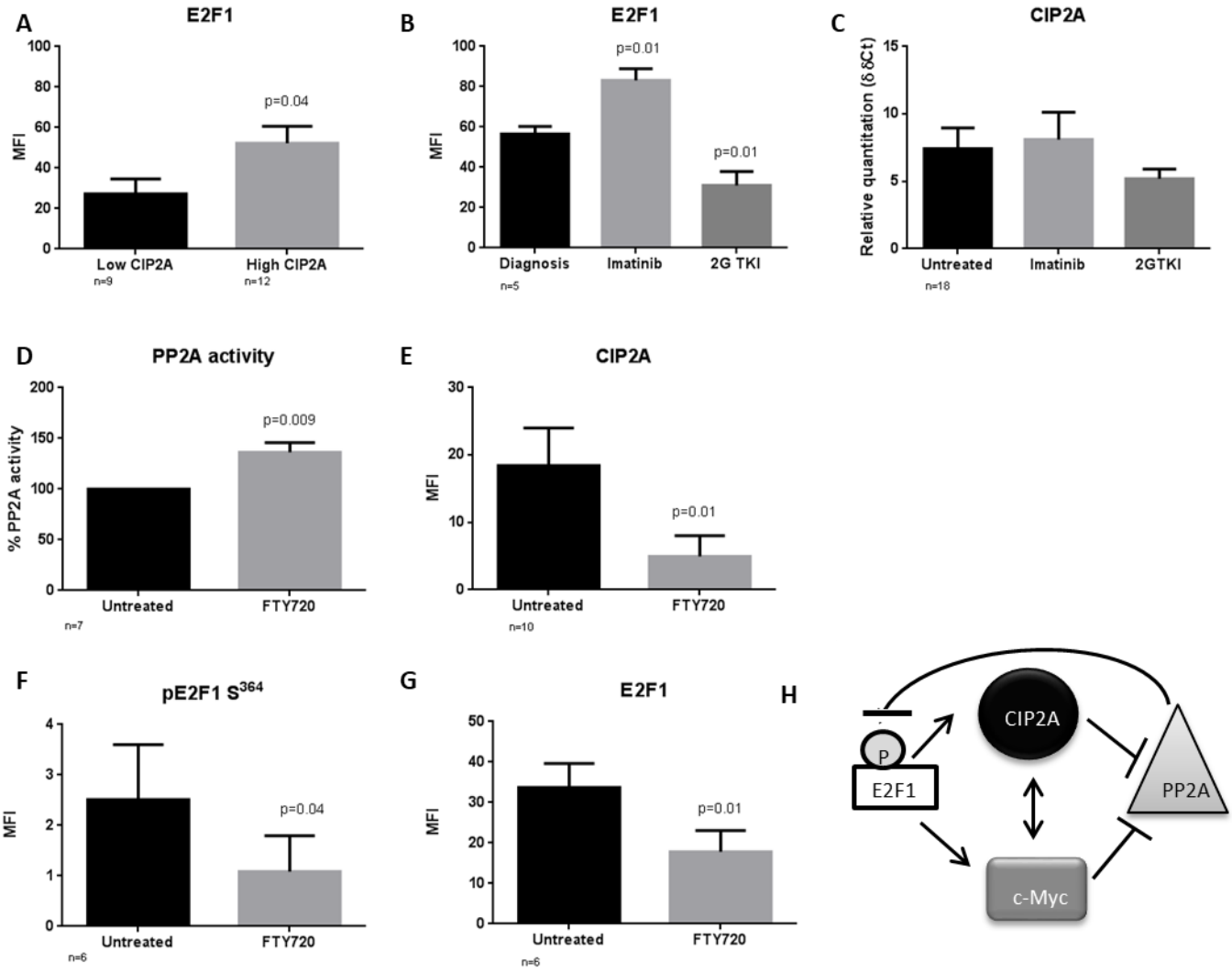

A
E2F1
MFI
p=0.04
Low CIP2A
High CIP2A
n=9
n=12
B
E2F1
MFI
p=0.01
p=0.01
Diagnosis
Imatinib
2G TKI
n=5
C
CIP2A
Relative quantitation (δ δCt)
Untreated
Imatinib
2GTKI
n=18
D
PP2A activity
% PP2A activity
p=0.009
Untreated
FTY720
n=7
E
CIP2A
MFI
p=0.01
Untreated
FTY720
n=10
F
pE2F1 S364
MFI
p=0.04
Untreated
FTY720
n=6
G
E2F1
MFI
p=0.01
Untreated
FTY720
n=6
H
P
E2F1
CIP2A
PP2A
c-Myc

**Figure 5.**

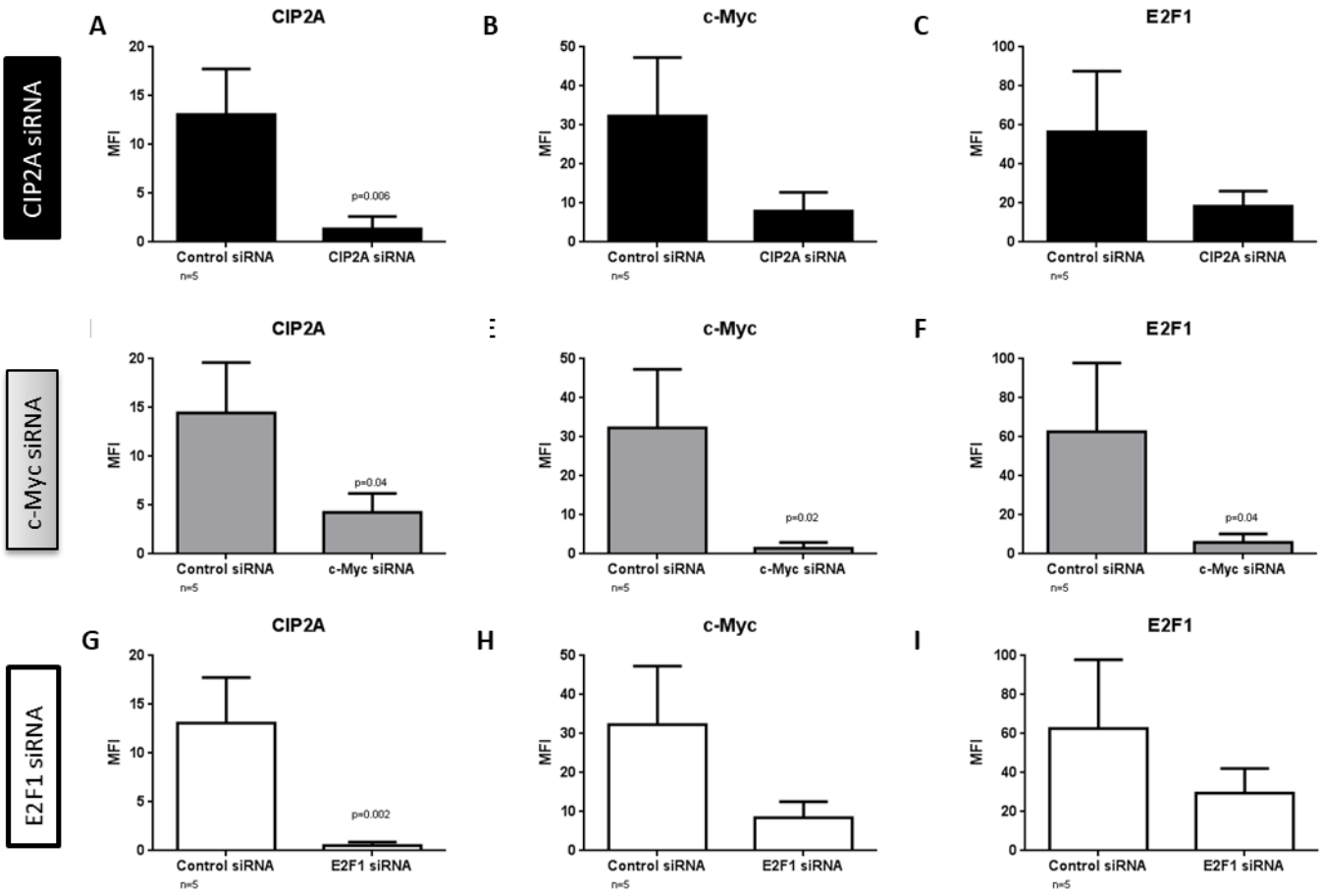

A
CIP2A
B
c-Myc
C
E2F1
CIP2A siRNA
c-Myc siRNA
E2F1 siRNA
MFI
p=0.006
p=0.04
p=0.02
p=0.002
Control siRNA
CIP2A siRNA
c-Myc siRNA
E2F1 siRNA
n=5
F
G
H
I

**Figure 6.**

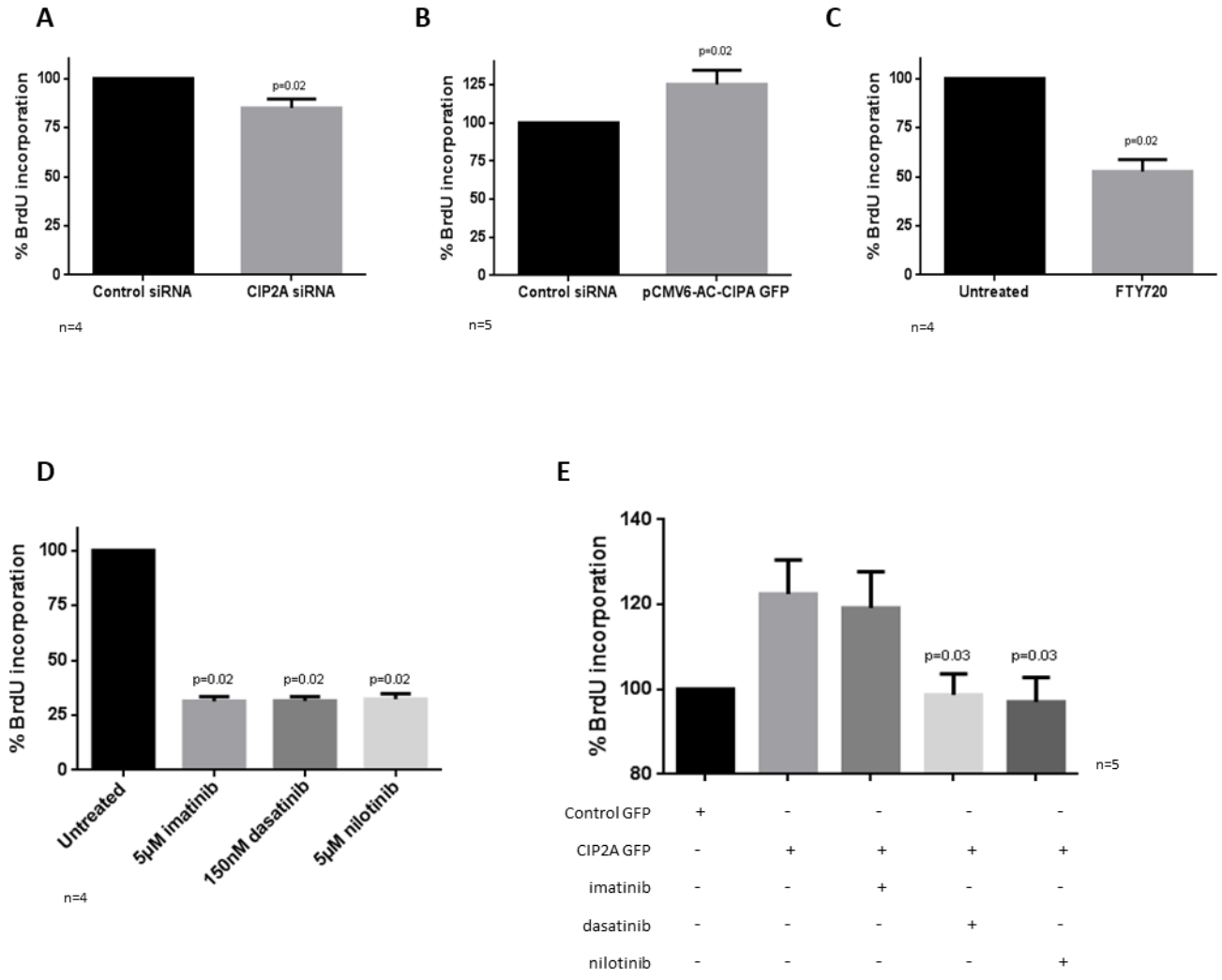
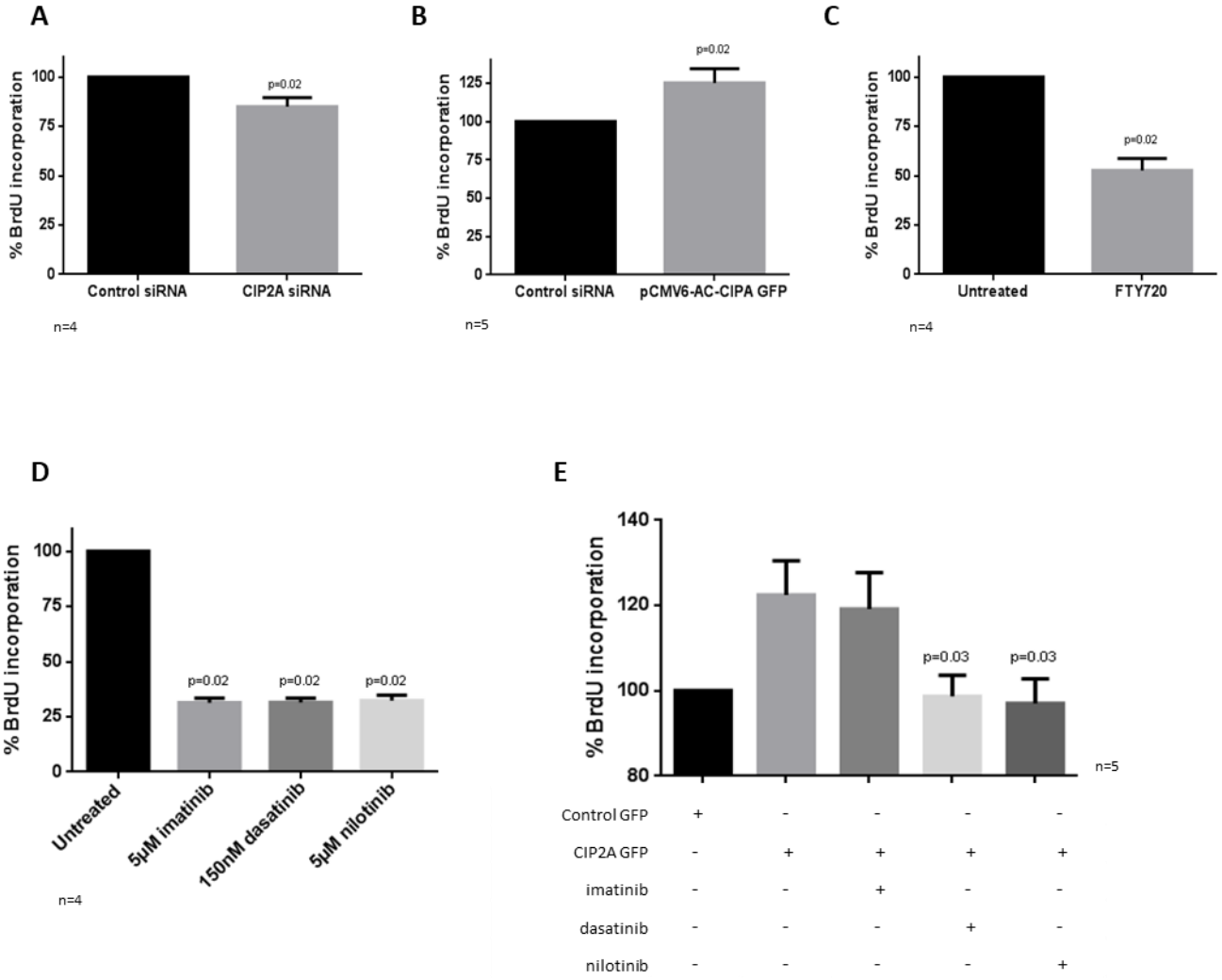

A
% BrdU incorporation
100
75
50
25
0
p=0.02
Control siRNA
CIP2A siRNA
n=4
B
% BrdU incorporation
125
100
75
50
25
0
p=0.02
Control siRNA
pCMV6-AC-CIPA GFP
n=5
C
% BrdU incorporation
100
75
50
25
0
p=0.02
Untreated
FTY720
n=4
D
% BrdU incorporation
100
75
50
25
0
p=0.02
p=0.02
p=0.02
Untreated
5μM imatinib
150nM dasatinib
5μM nilotinib
n=4
E
% BrdU incorporation
140
120
100
80
p=0.03
p=0.03
n=5
Control GFP + - - - -
CIP2A GFP - + + + +
imatinib - - + - -
dasatinib - - - + -
nilotinib - - - - +

**Figure 6.**


A
% BrdU incorporation
100
75
50
25
0
p=0.02
Control siRNA
CIP2A siRNA
n=4
B
% BrdU incorporation
125
100
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50
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p=0.02
Control siRNA
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C
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100
75
50
25
0
p=0.02
Untreated
FTY720
n=4
D
% BrdU incorporation
100
75
50
25
0
p=0.02
p=0.02
p=0.02
Untreated
5μM imatinib
150nM dasatinib
5μM nilotinib
n=4
E
% BrdU incorporation
140
120
100
80
p=0.03
p=0.03
n=5
Control GFP + - - - -
CIP2A GFP - + + + +
imatinib - - + - -
dasatinib - - - + -
nilotinib - - - - +

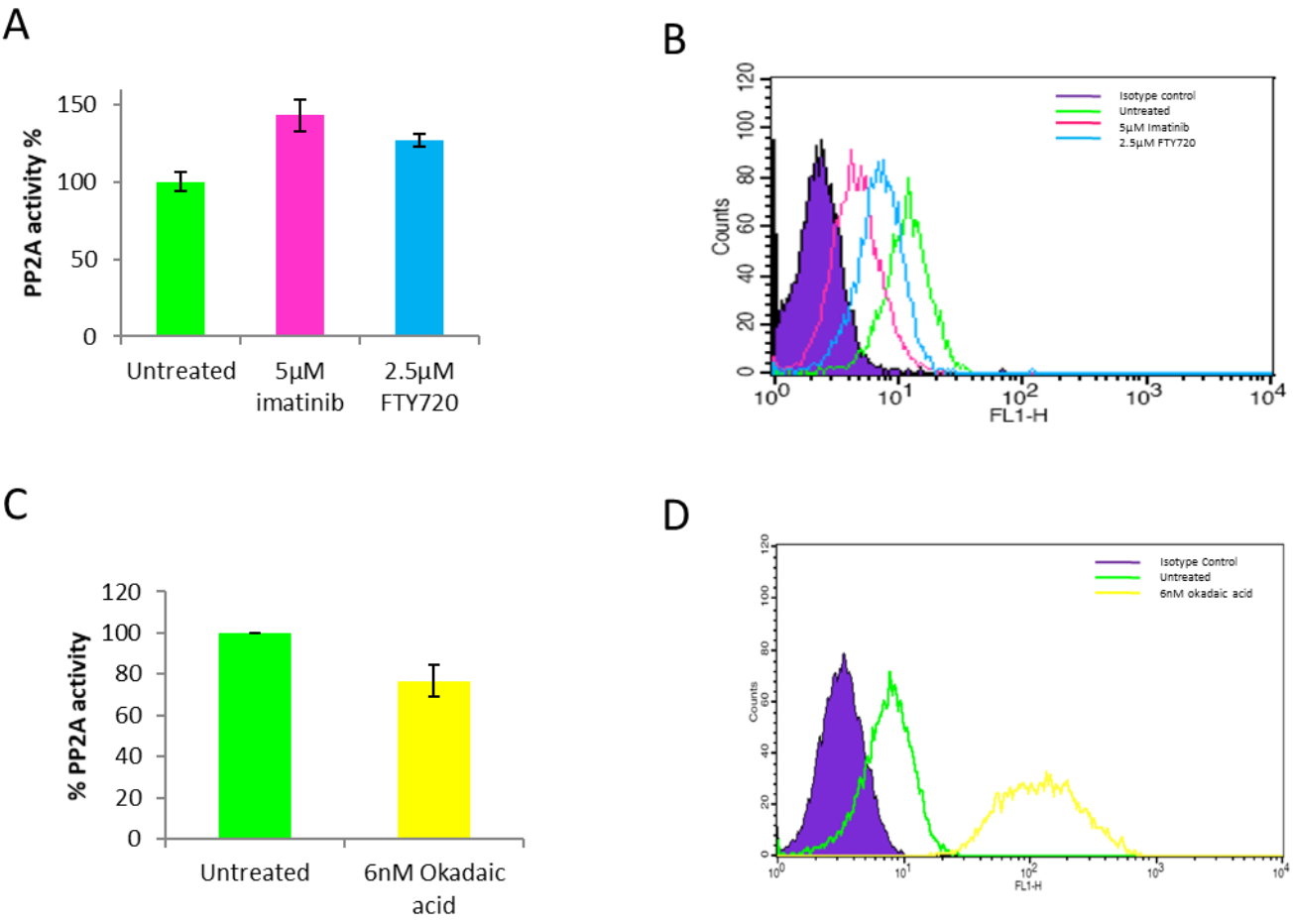
**Supplementary Table 1. Defining CIP2A status**

The table below shows the diagnostic CIP2A level (as mean fluorescence intensity; MFI)for all 74 patients. The range was 0-52, the interquartile range was 0.48 – 8.65, the median was 2.85 and the mean was 6.5. High CIP2A patients are defined as those patients with a CIP2A level greater than or equal to 7.3. The cut-off value was derived using ROC curve analysis for the prediction of BC based on the diagnostic CIP2A protein level; minimisation of the Euclidian distance between the ROC curve and the corner (0, 1) was the criterion used. The optimal cut-off value produced an $AUC_{ROC}$ = 0.902 (95% CI: 0.832, 0.973).

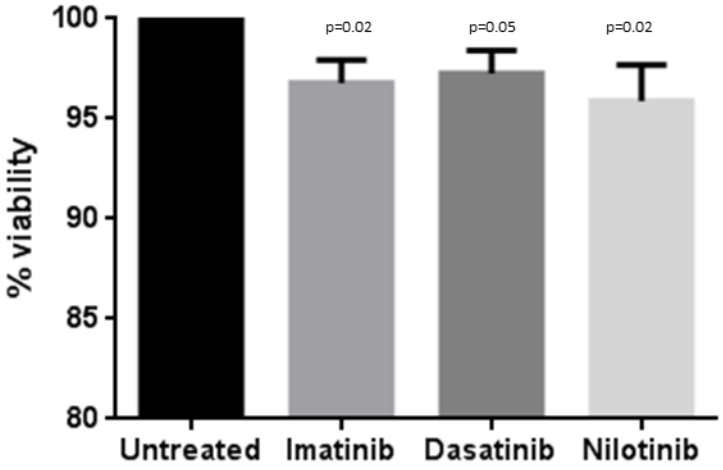
| Patient | First line treatment | CIP2A Status | CIP2A (MFI) |
|---|---|---|---|
| 1 | imatinib | Low | 0.0 |
| 2 | imatinib | Low | 0.0 |
| 3 | imatinib | Low | 0.0 |
| 4 | imatinib | Low | 0.0 |
| 5 | imatinib | Low | 0.0 |
| 6 | imatinib | Low | 0.0 |
| 7 | imatinib | Low | 0.0 |
| 8 | imatinib | Low | 0.0 |
| 9 | imatinib | Low | 0.0 |
| 10 | imatinib | Low | 0.0 |
| 11 | imatinib | Low | 0.0 |
| 12 | imatinib | Low | 0.0 |
| 13 | imatinib | Low | 0.3 |
| 14 | imatinib | Low | 0.4 |
| 15 | imatinib | Low | 0.7 |
| 16 | imatinib | Low | 0.9 |
| 17 | imatinib | Low | 0.9 |
| 18 | imatinib | Low | 1.2 |
| 19 | imatinib | Low | 1.3 |
| 20 | imatinib | Low | 1.6 |
| 21 | imatinib | low | 1.7 |
| 22 | imatinib | Low | 2.0 |
| 23 | imatinib | Low | 2.0 |
| 24 | imatinib | Low | 2.2 |
| 25 | imatinib | Low | 2.8 |
| 26 | imatinib | Low | 3.7 |
| 27 | imatinib | Low | 4.5 |
| 28 | imatinib | low | 4.9 |
| 29 | imatinib | Low | 5.0 |
| 30 | imatinib | Low | 5.7 |
| 31 | imatinib | Low | 6.1 |
| 32 | imatinib | Low | 6.2 |
| 33 | imatinib | Low | 6.3 |
| 34 | imatinib | high | 7.3 |
| 35 | imatinib | high | 9.1 |
| 36 | imatinib | high | 9.9 |
| 37 | imatinib | high | 12.9 |
| 38 | imatinib | high | 15.0 |
| 39 | imatinib | high | 16.4 |
| 40 | imatinib | high | 18.0 |
| 41 | imatinib | high | 19.7 |
| 42 | imatinib | high | 25.9 |
| 43 | imatinib | high | 26.3 |
| 44 | imatinib | high | 35.3 |
| 45 | 2G TKI | Low | 0.0 |
| 46 | 2G TKI | Low | 0.0 |
| 47 | 2G TKI | Low | 0.0 |
| 48 | 2G TKI | Low | 0.0 |
| 49 | 2G TKI | Low | 0.4 |
| 50 | 2G TKI | Low | 0.9 |
| 51 | 2G TKI | Low | 1.2 |
| 52 | 2G TKI | Low | 1.2 |
| 53 | 2G TKI | Low | 2.2 |
| 54 | 2G TKI | low | 2.2 |
| 55 | 2G TKI | low | 2.5 |
| 56 | 2G TKI | Low | 2.6 |
| 57 | 2G TKI | Low | 2.9 |
| 58 | 2G TKI | low | 3.0 |
| 59 | 2G TKI | Low | 3.5 |
| 60 | 2G TKI | Low | 3.7 |
| 61 | 2G TKI | low | 4.0 |
| 62 | 2G TKI | Low | 4.9 |
| 63 | 2G TKI | Low | 5.5 |
| 64 | 2G TKI | Low | 6.7 |
| 65 | 2G TKI | Low | 7.2 |
| 66 | 2G TKI | high | 9.1 |
| 67 | 2G TKI | high | 19.0 |
| 68 | 2G TKI | high | 10.1 |
| 69 | 2G TKI | high | 10.7 |
| 70 | 2G TKI | high | 11.5 |
| 71 | 2G TKI | high | 13.6 |
| 72 | 2G TKI | high | 17.0 |
| 73 | 2G TKI | high | 18.3 |
| 74 | 2G TKI | high | 52.0 |
| | | | |
| | | Average | 6.4 |

**Supplementary Figure 1. Assessment of PP2A activity in K562 cells.**

PP2A activity was assessed by two methods, the PP2A phosphatase immunoprecipitation activity assay and PP2A $Y^{307}$ inactive protein level by flow cytometry. On PP2A activation either by FTY720 or imatinib, both activity increases and PP2A $Y^{307}$ decreases (panels A and B). On PP2A inhibition by okadaic acid, the opposite is seen (panels C and D).

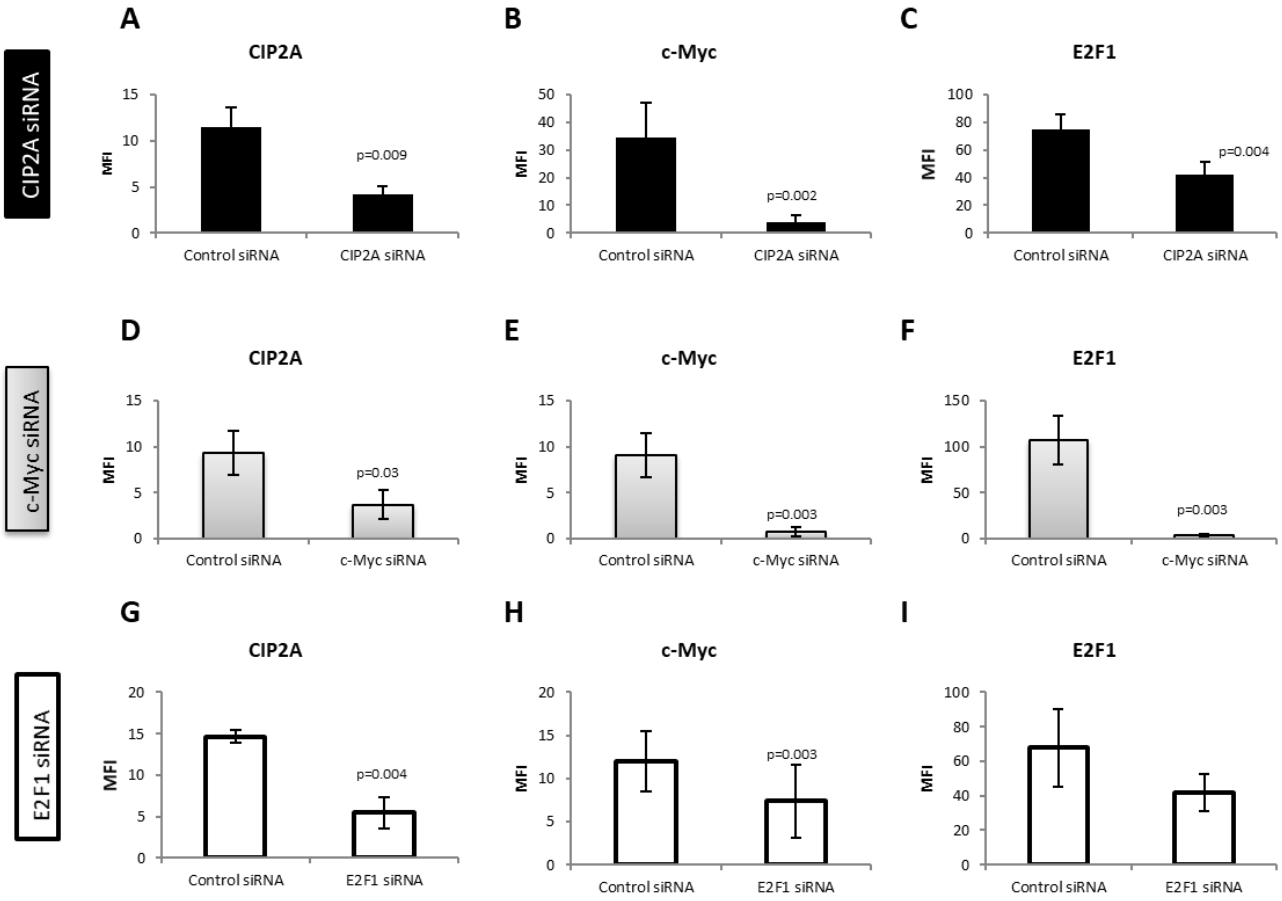


**Supplementary Figure 2. Viability post TKI treatment**
CML patient cells were incubated with 5µM imatinib, 150nM dasatinib and 5µM nilotinib for 24 hours. Viability was assessed by propidium iodide staining (PI)n=10.



**Supplementary Figure 3. siRNA mediated inhibition of the CIP2A/c-Myc/E2F1 pathway in K562 cells**

K562 cells were treated with CIP2A siRNA Panels A-C, c-Myc siRNA Panels D-F and E2F1 siRNA Panels G-I (n=4) for 72 hours.



## Supplementary methods

**Measurement of CIP2A, PP2Ac, phosphorylated PP2A, c-Myc and E2F1.**

Flow cytometry methodology has been previously described[1, 2] and was used for the detection of PP2Ac, PP2A $Y^{307}$, CIP2A, c-Myc, c-Myc $S^{62}$, E2F1 and E2F1 $S^{364}$. Briefly, cells (~$1x10^5$) were fixed by resuspending in 500µl of 2% paraformaldehyde at pH 7.2 (VWR, UK) for 10 minutes at 37°C and then centrifuged at 770g for 3 minutes. 500µl of 90% methanol (Fisher Scientific, UK) was added to the cell pellet and the cells were vortexed and then incubated on ice for 30 minutes. Cells were then washed (throughout with 1ml incubation buffer containing PBS and 0.5% bovine serum albumin (BSA) (Sigma-Aldrich)), and centrifuged at 770g for 3 minutes. Cells were resuspended in 25µl incubation buffer. Appropriate antibody and controls were added Anti-PP2A catalytic subunit (Merck Millipore, UK), PP2A $Y^{307}$ (Epitomics, USA), CIP2A, E2F1 and E2F1 $S^{364}$ (Santa Cruz Biotechnology, USA), c-Myc (New England Biolabs, UK), c-Myc $S^{62}$ (Abcam, UK), anti-mouse and anti-rabbit Alex fluor 488 (Invitrogen, UK). Mouse IgG (BD, UK) and normal rabbit IgG (R&D systems, UK) were used as controls Cells were vortexed and incubated at room temperature for 40 minutes. Cells were then washed twice and resuspended in 100µl incubation buffer containing 10µg/ml goat anti rabbit/mouse second Alexa Fluor 488 antibody (Invitrogen), incubated at room temperature in the dark for 30 minutes, then washed twice and analysed using flow cytometry (FACScalibur), with Cellquest Pro software (BD) for data analysis. The mean fluorescence intensity (MFI) of the protein was determined as the MFI for the given protein minus that of the appropriate control. Levels of pCrKL and CrKL were used as an assay of BCR-ABL activity, measured by flow cytometry as previously described.[2]

1. Lucas CM, Harris RJ, Giannoudis A, Copland M, Slupsky JR, Clark RE. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression. *Blood* 2011; **117**(24)**:** 6660-6668.

2. Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. *British Journal of Haematology* 2010; **149**(3)**:** 458-460.