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JAK2 phosphorylates histone H3Y41 and excludes HP1α from chromatin

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Abstract

Activation of Janus Kinase 2 (JAK2) by chromosomal translocations or point mutations is a frequent event in haematological malignancies[1-5]. JAK2 is a non-receptor tyrosine kinase that regulates a number of cellular processes by inducing cytoplasmic signalling cascades. Here we show that JAK2 is present in the nucleus of haematopoietic cells and directly phosphorylates Y41 on histone H3. Heterochromatin protein 1 alpha (HP1 □), but not HP1 □, specifically binds to this region of H3 and phosphorylation of H3Y41 by JAK2 prevents this binding. Inhibition of JAK2 activity in leukaemic cells reduces both the expression of the haematopoietic oncogene *Imo2,* and the phosphorylation of H3Y41 at its promoter, whilst simultaneously increasing the binding of HP1 □at the same site. Together, these results identify a previously unrecognised nuclear role for JAK2 in the phosphorylation of H3Y41 and reveal a direct mechanistic link between two genes, *jak2* and *Imo2*, involved in normal haematopoiesis and leukaemia[1-8]

The DNA of most nucleated eukaryotic cells is packaged within chromatin[9]. The core histones within nucleosomes are subject to numerous post-translational modifications including phosphorylation[9]. Only kinases responsible for serine and threonine phosphorylation of non-variant histones have been reported[9], and thus far tyrosine phosphorylation of non-variant histones remains uncharacterised.

Janus kinase 2 (JAK2) is a non-receptor tyrosine kinase that is critical for haematopoiesis, adipogenesis, immune and mammary development[10]. Recently, constitutive activation of JAK2 has been noted as a sentinel event in several different haematological malignancies[3-5,11-14]. The most prevalent gain of function mutation in JAK2 results from a missense mutation in its JH2 autoregulation domain (JAK2 V617F)[11-14]. JAK2 signalling is implicated in diverse biological processes such as cell cycle progression, apoptosis, mitotic recombination, genetic instability and alteration of heterochromatin[15-19]. Mechanistic insights into these potential oncogenic events are elusive but the presiding opinion is that the biological/oncogenic effects of JAK2 are mediated by cytoplasmic signalling pathways [1,2].

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We considered the possibility that the V617F mutation may perturb the subcellular localisation of JAK2. Figure 1A and supplementary figure 1 show that JAK2 is present within the nucleus of three cell lines (HEL, UKE1 and SET2) carrying the JAK2 V617F mutation[20]. However, K562 cells, which express wild-type JAK2, also contain the enzyme in the nucleus. Nuclear JAK2 levels are higher in HEL and SET2 as these lines contain multiple copies of JAK2 (figure 1A and supplementary figure 1)[20]. Nuclear JAK2 was also observed in primary cells positive for the CD34 stem cell antigen obtained from a patient with JAK2 V617F-positive post-polycythaemic myelofibrosis. Transfection of JAK2 into a JAK2 null background, 2A cells,[21] independently confirms the nuclear localisation of JAK2 and serves to validate the specificity of the antibodies used in IF (figure 1B and supplementary figure 2). Finally, subcellular fractionation experiments on HEL cells also demonstrate that JAK2 is indeed in the nucleus (figure 1C). Taken together these results demonstrate that a significant fraction of JAK2 is present in the nucleus of haematopoietic cells irrespective of JAK2 mutation status.

To explore the role of JAK2 within the nucleus we investigated the possibility that histones could be a substrate. Figure 2A and supplementary figure 3 show that recombinant JAK2 (rJAK2) can specifically phosphorylate histone H3 and that this activity is inhibited by the JAK2 inhibitor TG101209[22].

Histone H3 contains three tyrosine residues that are highly conserved. One of these, H3Y41, is positioned at the N-terminus of the first helix of H3 (the N1-helix) where the DNA enters the nucleosome, and is juxtaposed to the major groove of the DNA double helix (figure 2B). Given its position within the nucleosome, we considered that this residue might be the target of JAK2 kinase activity. We therefore raised an antibody to phosphorylated H3Y41 (H3Y41ph) and verified its specificity (supplementary figures 4-6). Using this antibody we show that rJAK2 phosphorylates H3Y41 when core histones, purified histone H3 or recombinant H3 are used as substrates (figure 2C, lanes 1-6). This phosphorylation was markedly reduced by the JAK2 inhibitor TG101209 (figure 2C, lanes 9&10). Mutation of H3Y41 to phenylalanine demonstrates that this tyrosine is a target of JAK2 *in vitro* (figure 2C, lanes 7&8), and it confirms the specificity of the antibody. To ensure that cellular JAK2 can also phosphorylate H3Y41 we immunoprecipitated JAK2 from HEL cells and used it in phosphorylation experiments. Supplementary figure 7 shows that endogenous JAK2 phosphorylates H3Y41 and that the TG101209 inhibitor blocked this activity.

To assess if tyrosine phosphorylation of H3Y41 is present *in vivo*, chromatin preparations from six cell lines were probed with the H3Y41ph antibody. Notably, H3Y41 phosphorylation was more abundant in the cell lines that contain active JAK2 signalling (SET2, HEL, UKE1 and K562)[20,23]. In contrast, H3Y41ph was significantly reduced in HL60 cells and 2A cells, which both lack detectable JAK2[2321] (figure 2D). Cytokine stimulation of K562 cells with leukaemia inhibitory factor (LIF) activates JAK2, as evidenced by JAK2 phosphorylation, and leads to a concomitant increase in H3Y41ph suggesting a role for JAK2 in this pathway (Figure 2E). Similar results were noted with PDGF-BB in K562 cells. A direct role for JAK2 in this pathway is demonstrated by the ability of the JAK2 inhibitor TG101209 to block the PDGF-BB mediated increase in H3Y41ph (supplementary figure 8). Finally, we demonstrate that stimulation of murine BaF3 cells with IL3 (a cytokine that exclusively signals via JAK2 in these cells) also leads to an increase in H3Y41ph (supplementary figure 8). Taken together these data suggest that activation of JAK2 is upstream of H3Y41ph.

Whilst the presence of H3Y41ph in both HL60 and 2A cells indicates that JAK2 is not the only tyrosine kinase responsible for this post-translational modification, transfection of JAK2 into JAK2-null (2A) cells demonstrates that this enzyme is one of the cellular

kinases responsible for this post-translational modification on histone H3 *in vivo* (figure 2F). Finally, to provide further evidence that JAK2 phosphorylates H3Y41 *in vivo*, we used two specific, chemically distinct JAK2 inhibitors, TG101209 and AT9283, that have been extensively characterised[22,24]. Analysis of chromatin preparations from HEL cells grown in the presence or absence of the JAK2 inhibitors demonstrated that H3Y41ph was markedly reduced following four hours of exposure to either of the specific JAK2 inhibitors and that these changes were not a consequence of broad effects on cell cycle or apoptosis (supplementary figure 9A&B). Moreover, JAK2 inhibition leads to a rapid and sustained loss of H3Y41ph. The reduction in H3Y41ph is observed within 15 minutes and by one-hour an 80% decrease is observed (figure 2G-H). The rapidity of this *in vivo* response when coupled to the *in vitro* data strongly suggests that JAK2 directly phosphorylates H3Y41 *in vivo*.

JAK2 has recently been implicated in the DNA damage response[15,19] and the alteration of heterochromatin[16]. Analysis of histones from cells that were subject to ionising radiation indicates that H3Y41ph is not responsive to DNA double strand breaks (supplementary figure 6). Given the connection between HP1 and the JAK pathway in *Drosophila*,[16] we next investigated whether increased JAK2 activity in haematopoietic cells affects heterochromatin by regulating the binding of heterochromatin protein 1 (HP1). When we compared the binding of HP1 □and □to chromatin in permeabilised nuclei from haematopoietic cells containing active JAK2 (HEL cells) we find that there is significant amount of soluble, non chromatin bound HP1 □present in HEL cells whereas HP1 □is essentially all chromatin bound (Figure 3A). Since the binding of HP1 □and HP1 □were analysed within the same population of cells, the difference in their apparent chromatin binding affinities cannot be attributed to H3K9me. Our results therefore raised the possibility that in HEL cells JAK2 signalling may weaken HP1 □binding and/or stabilise the binding of HP1 □

Given our findings we searched whether an additional binding site for HP1 □or HP1 □lies around H3Y41, the site phosphorylated by JAK2. Figure 3B and supplementary figure 10 A & B show that HP1 □binds specifically to an unmodified H3 peptide encompassing amino acids 31-56, and this binding is markedly reduced when the peptide is phosphorylated at H3Y41. In contrast, HP1 □binds neither the unmodified nor the modified peptide. The integrity of the H3Y41ph peptide is demonstrated by the fact that the H3Y41ph antibody binds only the modified peptide. Thus, these *in vitro* data demonstrate that phosphorylation of H3Y41 selectively destabilises the binding of HP1 □from this region of H3. Furthermore, the binding of HP1 □to the Y41 region of H3 is specifically mediated by its chromoshadow domain (CSD), therefore utilising an alternative binding domain to its interaction with H3K9me (figure 3C)[25]. Indeed, H3K9me peptides *in trans* neither cooperate with nor compete for binding of HP1 □to the Y41 region of H3 (supplementary figure 10C). Importantly, the interaction between the CSD of HP1 □and H3 is inhibited by the presence of H3Y41ph (figure 3D).

To provide further evidence that HP1 □recognises chromatin via a second H3 binding site (other than H3K9me), we used a peptide competition assay. This assay was previously used to demonstrate binding of HP1 □to H3K9me[25]. Figure 3E and F show that an H3K9me3 peptide displaces HP1 □from nuclear heterochromatic speckles and a peptide spanning H3 residues 31-56 displaces HP1 □to a similar extent. In contrast, the H3(31-56) peptide phosphorylated at Y41 (H3Y41ph) is unable to displace HP1 □from heterochromatin, consistent with our *in vitro* experiments showing that HP1 □is unable to bind H3 phosphorylated at Y41.

These results indicate that HP1 has a second binding site on H3 that tethers its CSD to chromatin and that this contact is disrupted by phosphorylation of Y41. We next asked whether binding of HP1 is modulated by JAK2 signalling *in vivo*. Permeabilised nuclei were prepared from HEL cells cultured with or without JAK2 inhibitors. Figure 3G demonstrates that inhibition of JAK2 results in retention of chromatin-bound HP1 and prevents its displacement into the soluble fraction (compare lanes 2 and 3 to 1). Notably, in contrast to H3Y41ph (figure 2G), the level of H3K9me3 is unaffected by JAK2 inhibition consistent with a role for JAK2 kinase in the release of HP1 from chromatin as a consequence of H3Y41 phosphorylation.

To investigate the biological function of the interplay between H3Y41ph and HP1 we performed genome wide expression profiling of HEL cells grown with or without the JAK2 inhibitor TG101209 for 4 hours to identify genes regulated by JAK2. Figure 4 and supplementary figure 11 A&B show the genes whose expression is most reduced by inhibition of JAK2. A number of these genes (e.g *Pim1, Bcl-x$_L$, CyclinD)* have previously been identified as transcriptional targets of JAK2, as part of the canonical JAK2/STAT5 pathway, which is a major pathway operational in normal and dysregulated erythroid cells [17,26]. These genes and many others in our profiling contain canonical STAT5 binding sites. In addition, our profiling approach also identified several JAK2 regulated genes which do not contain predicted STAT5 binding sites[27].

One of the top 0.5% of genes down-regulated by JAK2 inhibition is the *lmo2* oncogene, which has been linked to JAK2 signalling.[28] The connect between *lmo2* expression and JAK2 inhibition was independently confirmed with a second JAK2 inhibitor, AT9283 (supplementary figure 11C). *Lmo2* is essential for normal haematopoietic development and has been implicated in leukemogenesis[6,8]. We therefore employed chromatin immunoprecipitation to assess changes to the chromatin structure of the *lmo2* gene following JAK2 inhibition. Down-regulation of *lmo2* expression (corroborated by reduced levels of H3K4me3) was accompanied by reduced levels of H3Y41ph together with a reciprocal increase in the binding of HP1 (figure 4B and supplementary figure 12) at sites surrounding the *lmo2* transcriptional start site. In contrast, HP1 did not increase on the *lmo2* promoter following JAK2-inhibition (data not shown) and the promoter of 2microglobulin, a housekeeping gene, and two sites upstream of the *lmo2* promoter showed no change in H3K4me3, H3Y41ph or in HP1 binding (figure 4B and supplementary figure 13). Collectively, these results demonstrate that JAK2 signalling results in H3Y41 phosphorylation and the exclusion of HP1 at the *lmo2* promoter.

The data presented here demonstrate a novel nuclear role for JAK2 outside its established involvement as an initiator of cytoplasmic signalling cascades. In the nucleus, JAK2 mediates the phosphorylation of H3 and displaces HP1 from a novel binding site surrounding H3Y41. Since HP1 can also associate with methylated H3K9, binding to the H3Y41 region via its CSD may allow HP1 to further stabilise its association with chromatin. Alternatively, HP1 may utilise this new binding site on H3 to localise at distinct loci, where H3K9 methylation is absent. In either scenario, phosphorylation of H3Y41 by JAK2 would destabilise the binding of HP1 , but not HP1 , to chromatin.

The displacement of HP1 by JAK2 is likely to be tightly regulated in normal cells. However, in malignancies driven by constitutive activation of JAK2, unregulated displacement of chromatin-bound HP1 may override potential tumour suppressive functions of HP1 [29,30]. This suggestion is supported by the fact that enforced over-expression of HP1 ameliorates the leukaemic phenotype of overactive JAK signalling in *Drosophila melanogaster*[16]. The data presented here provide a direct mechanistic explanation for the regulation of HP1 by the JAK2 pathway and identify the euchromatic

oncogene *Imo2* as a direct target for JAK2. Interestingly, the *Imo2* locus lacks a predicted STAT5 binding site. Moreover, chromatin immunoprecipitation (ChIP) analyses indicates that STAT5 does not bind the Lmo2 locus (A. Wood and B. Göttgens, manuscript submitted) and manipulation of STAT5 in haematopoietic progenitors does not alter the expression of *Imo2*[31] (Jan Jacob Schuringa personal communication). Together these observations raise the possibility that regulation of *Imo2* by JAK2 may not require STAT5.

In addition to transcriptional regulation of *Imo2* it is possible that dysregulated displacement of HP1 by H3Y41 phosphorylation may have other oncogenic consequences (Figure 4C). HP1 is recognised to reduce mitotic recombination[29], repress transcription of heterochromatic genes[32] and preserve centromeric architecture leading to faithful sister chromatid segregation[30]. Indeed, the phenotypic consequences of constitutive JAK2 activation in haematopoietic malignancies (increased gene expression, mitotic recombination and genetic instability)[1,2,15] are consistent with reversal of these functions.

Methods Summary

Cell culture and isolation of peripheral blood stem cells were performed using standard methodology[15]. Immunofluorescence images were captured with an Olympus Fluoview FV1000 microscope and cells were prepared and stained as previously described[15,25]. Cell fractionation, immunoprecipitation, western blotting and kinase assays were performed using standard methodology[25]. Peptides (supplementary table 1) were synthesized by Almac Sciences and used for binding/competition assays as previously described[25]. Information about parameters for STAT5 binding site analysis and antibodies used is available in full methods summary (supplementary information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Campbell PJ, Green AR. N Engl J Med. 2006; 355(23):2452. [PubMed: 17151367]
2. Levine RL, Pardanani A, Tefferi A, et al. Nat Rev Cancer. 2007; 7(9):673. [PubMed: 17721432]
3. Lacronique V, Boureux A, Valle VD, et al. Science. 1997; 278(5341):1309. [PubMed: 9360930]
4. Scott LM, Tong W, Levine RL, et al. N Engl J Med. 2007; 356(5):459. [PubMed: 17267906]
5. Bercovich D, Ganmore I, Scott LM, et al. Lancet. 2008
6. McCormack MP, Rabbitts TH. N Engl J Med. 2004; 350(9):913. [PubMed: 14985489]
7. Neubauer H, Cumano A, Muller M, et al. Cell. 1998; 93(3):397. [PubMed: 9590174]
8. Yamada Y, Warren AJ, Dobson C, et al. Proc Natl Acad Sci U S A. 1998; 95(7):3890. [PubMed: 9520463]
9. Kouzarides T. Cell. 2007; 128(4):693. [PubMed: 17320507]

10. O'Shea JJ, Gadina M, Schreiber RD. Cell. 2002; 109(Suppl):S121. [PubMed: 11983158]
11. Baxter EJ, Scott LM, Campbell PJ, et al. Lancet. 2005; 365(9464):1054. [PubMed: 15781101]
12. James C, Ugo V, Le Couedic JP, et al. Nature. 2005; 434(7037):1144. [PubMed: 15793561]
13. Kralovics R, Passamonti F, Buser AS, et al. N Engl J Med. 2005; 352(17):1779. [PubMed: 15858187]
14. Levine RL, Wadleigh M, Cools J, et al. Cancer Cell. 2005; 7(4):387. [PubMed: 15837627]
15. Plo I, Nakatake M, Malivert L, et al. Blood. 2008
16. Shi S, Calhoun HC, Xia F, et al. Nat Genet. 2006; 38(9):1071. [PubMed: 16892059]
17. Socolovsky M, Fallon AE, Wang S, et al. Cell. 1999; 98(2):181. [PubMed: 10428030]
18. Walz C, Crowley BJ, Hudon HE, et al. J Biol Chem. 2006; 281(26):18177. [PubMed: 16632470]
19. Slupianek A, Hoser G, Majsterek I, et al. Mol Cell Biol. 2002; 22(12):4189. [PubMed: 12024032]
20. Quentmeier H, MacLeod RA, Zaborski M, et al. Leukemia. 2006; 20(3):471. [PubMed: 16408098]
21. Watling D, Guschin D, Muller M, et al. Nature. 1993; 366(6451):166. [PubMed: 7901766]
22. Pardanani A, Hood J, Lasho T, et al. Leukemia. 2007; 21(8):1658. [PubMed: 17541402]
23. Xie S, Wang Y, Liu J, et al. Oncogene. 2001; 20(43):6188. [PubMed: 11593427]
24. Squires MS, Curry JE, Dawson MA, et al. Blood. 2007; 110:3537.
25. Bannister AJ, Zegerman P, Partridge JF, et al. Nature. 2001; 410(6824):120. [PubMed: 11242054]
26. Garcon L, Rivat C, James C, et al. Blood. 2006; 108(5):1551. [PubMed: 16684963]
27. Donaldson IJ, Chapman M, Gottgens B. Bioinformatics. 2005; 21(13):3058. [PubMed: 15855248]
28. Ma AC, Ward AC, Liang R, et al. Blood. 2007; 110(6):1824. [PubMed: 17545503]
29. Cummings WJ, Yabuki M, Ordinario EC, et al. PLoS Biol. 2007; 5(10):e246. [PubMed: 17880262]
30. Yamagishi Y, Sakuno T, Shimura M, et al. Nature. 2008; 455(7210):251. [PubMed: 18716626]
31. Schuringa JJ, Schepers H. Methods Mol Biol. 2009; 538:1. [PubMed: 19277592]
32. Panteleeva I, Boutillier S, See V, et al. Embo J. 2007; 26(15):3616. [PubMed: 17627279]

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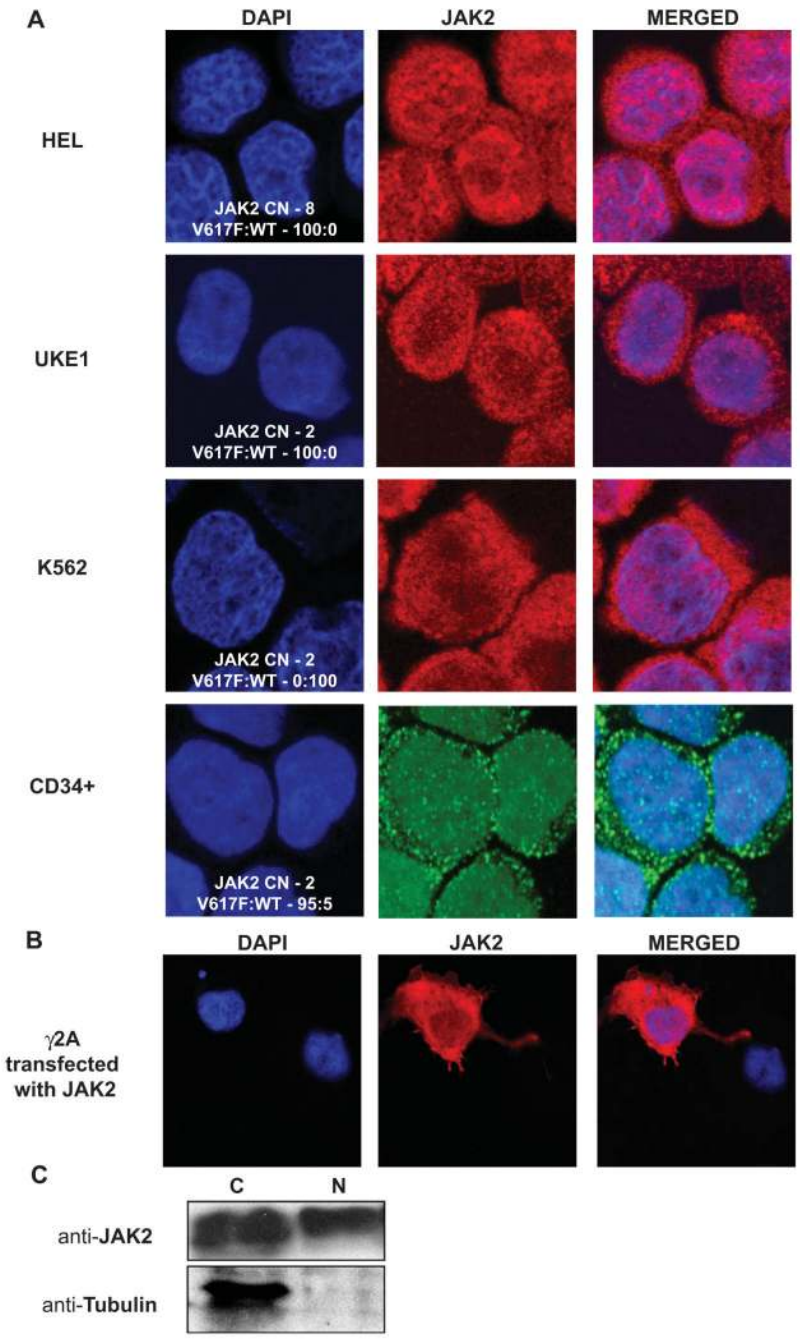

Figure 1. JAK2 is present in the nucleus of haematopoietic cells

(A) Confocal laser scanned images of asynchronous cells demonstrate that JAK2 has a nuclear subcellular localisation in haematopoietic cell lines and primary CD34+ peripheral blood stem cells (CD34+). CN indicates copy number of JAK2 and V617F:WT represents the ratio of JAK2 V617F to JAK2 wildtype for each cell type. Two primary anti-JAK2 antibodies were used for the IF images (Cell Signalling, (cat. no. D2E12 #3230) shown in red, and Imgenex (cat. no IMG-3007) shown in green). The two JAK2 antibodies recognize different JAK2 epitopes (detailed in Materials and Methods and supplementary figure 2C). Representative images from experiments performed on three occasions are shown. Images were captured with a X40 oil immersion lens. (B) Confocal laser scanned images of JAK2-

null (2A) cells transfected with JAK2. The field of view shown was chosen because it contains a JAK2 transfected cell and an untransfected cell highlighting the specificity of the JAK2 antibody as JAK2 is only detected in transfected cells. Again, JAK2 is detected in the nucleus. (C) Biochemical evidence of nuclear JAK2 in HEL cells. Cells were fractionated into cytoplasmic (C) and nuclear extracts (N). Western blotting analysis demonstrates that JAK2 is present in both cellular compartments, however tubulin (anti-Tubulin) is confined to the cytoplasmic fraction and testifies to the purity of the cell fractionation.

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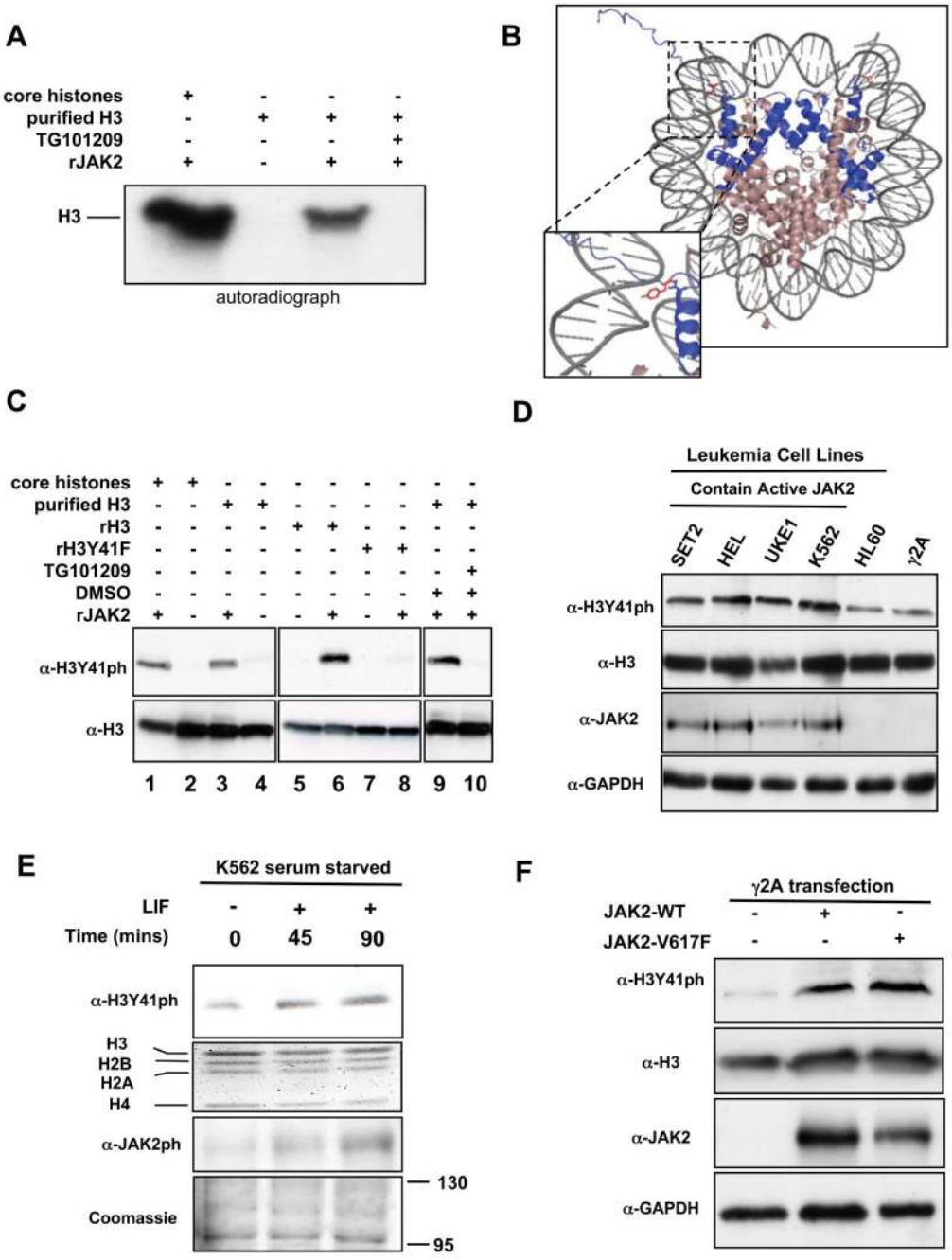

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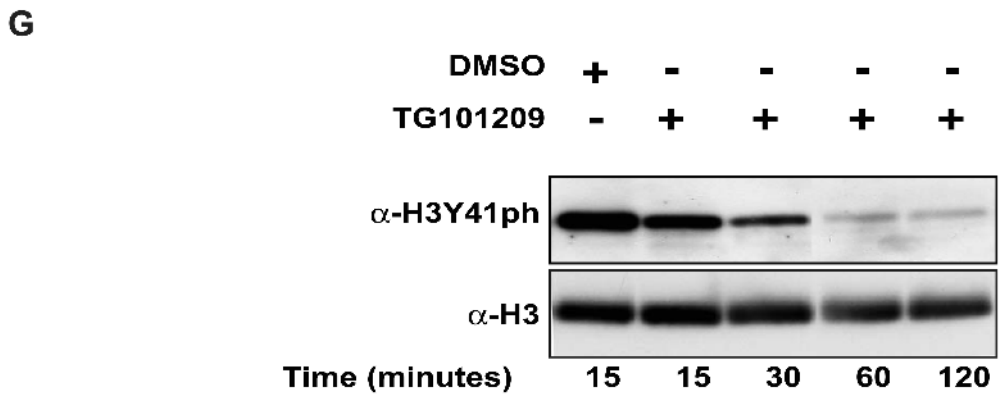


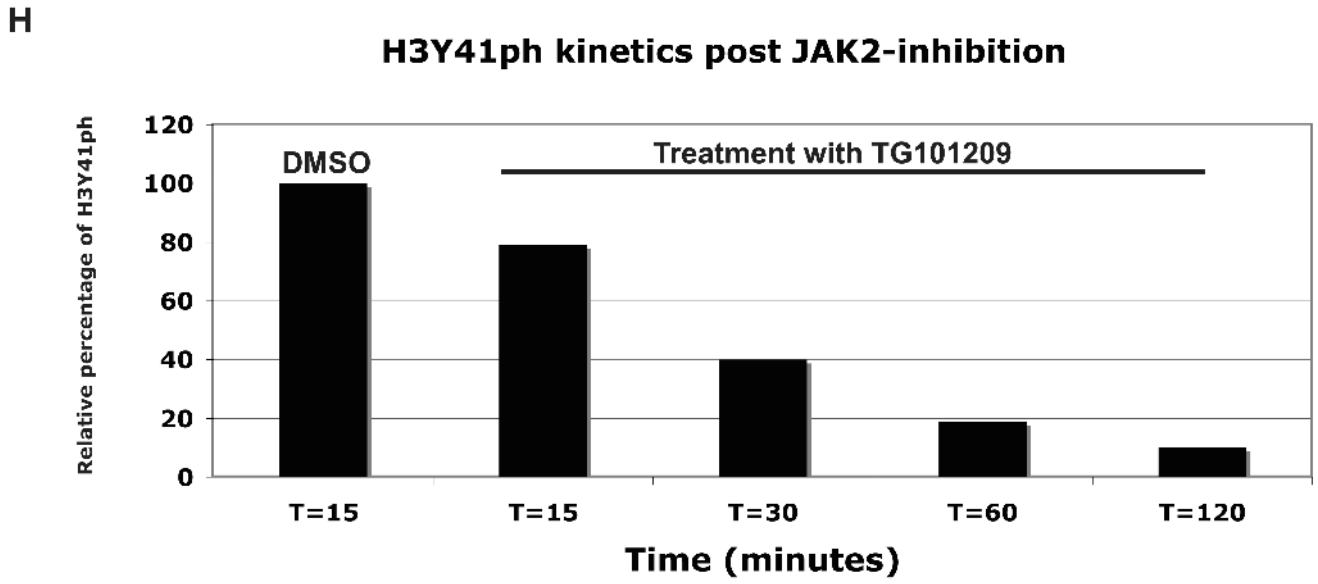
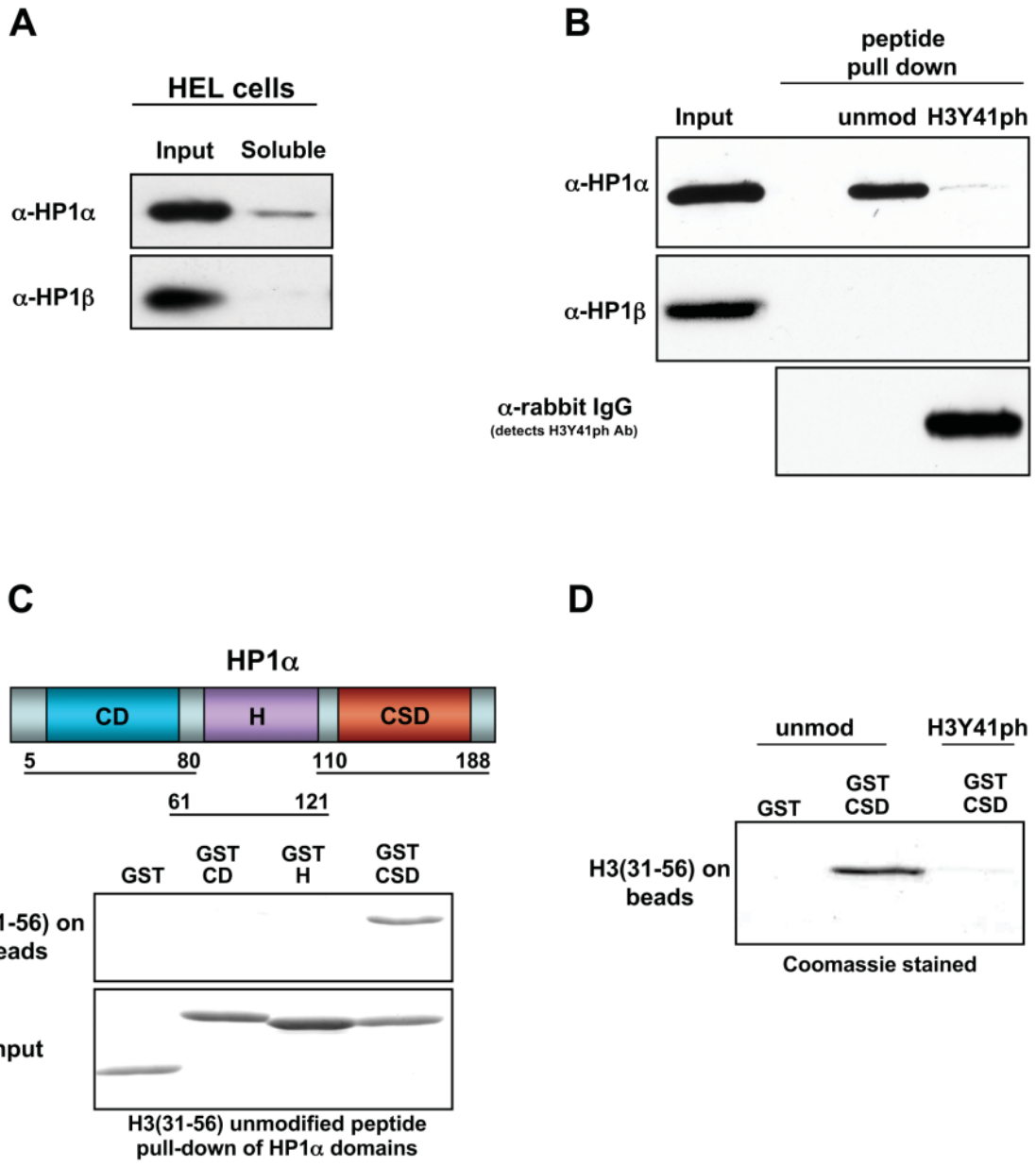


Figure 2. JAK2 phosphorylates H3Y41 *in vitro* and *in vivo*

(A) A mixture of core histones or purified histone H3 from calf thymus were used as substrate in an *in vitro* kinase assay using γ-^{32}P-ATP and recombinant JAK2 (rJAK2). In this assay rJAK2 specifically phosphorylates H3 and its activity is annulled by 10 nM of the JAK2 inhibitor TG101209. (B) Histone H3 (blue) is shown in the nucleosome. The inset image highlights that H3Y41 is positioned at the N-terminus of the first helix of H3 (the αN1-helix) where the DNA enters the nucleosome, and it is juxtaposed to the major groove of the DNA double helix. Images were constructed using Macpymol software. (C) *In vitro* kinase assay followed by western blot analysis using the H3Y41ph antibody demonstrates that the antibody detects the site of phosphorylation by rJAK2 on core histones, purified H3 and bacterially synthesised recombinant H3 (rH3). Notably, the signal is virtually absent when recombinant H3Y41F mutant (rH3Y41F) is used as the substrate. The kinase activity is markedly attenuated by 10 nM of TG101209 diluted in dimethyl sulfoxide (DMSO). In contrast, an equal quantity of DMSO alone has no inhibitory activity on rJAK2. As a loading control, H3 is shown following western blotting of the reactions with an anti-H3 antibody

(-H3). (D) Chromatin was prepared from the indicated cell lines and the level of H3Y41ph was determined by western blot. The H3Y41ph antibody detects higher levels of H3Y41 phosphorylation in the cell lines containing active JAK2. Equal loading of histones was confirmed by western blotting for histone H3 (-H3). JAK2 protein expression was analysed in whole cell extracts from the above cell lines confirming its absence in HL60 and 2a cells (-JAK2), and equal loading of the whole cell extracts was confirmed by western blotting for GAPDH (-GAPDH). (E) Following serum starvation for 72 hours, K562 cells were stimulated with LIF for up to 90 minutes. The levels of H3Y41ph and phospho-JAK2 (-JAK2ph) were determined by western blot analyses of whole cell extracts. The H3Y41ph and phospho-JAK2 antibodies demonstrate higher levels of phosphorylation following cytokine induction with LIF at both 45 and 90 minutes. Coomassie-blue staining of the probed extracts demonstrates equal loading of both histones and higher molecular weight proteins. (F) Chromatin was prepared from 2A cells that were transfected with JAK2 WT, JAK2 V617F or empty vector and the level of H3Y41ph was determined by western blot analysis. H3Y41 phosphorylation is higher in 2A cells transfected with JAK2 (wild type and V617F mutant). Equal loading of histones was confirmed by western blotting for histone H3 (-H3). JAK2 protein (-JAK2) was monitored in whole cell extracts from the same cells and its expression was detected only in JAK2 transfected 2a cells. Equal loading of the whole cell extracts was confirmed by western blotting for GAPDH (-GAPDH). (G) HEL cells were grown in the presence of a specific JAK2 inhibitor (TG101209) or DMSO (vehicle control) for the indicated times. Chromatin extracts were prepared and analysed by western blot analysis. These data demonstrate that H3Y41 phosphorylation (-H3Y41ph) is markedly reduced within minutes of JAK2 inhibition. Equal loading of the extracts was demonstrated using an anti-histone H3 antibody (-H3). (H) The changes observed above were quantitated using Image J (National Institutes of Health, USA) software. Similar results were noted with a second specific JAK2 inhibitor (AT9283), data not shown. All blots shown are representative images of experiments conducted on at least 3 occasions.



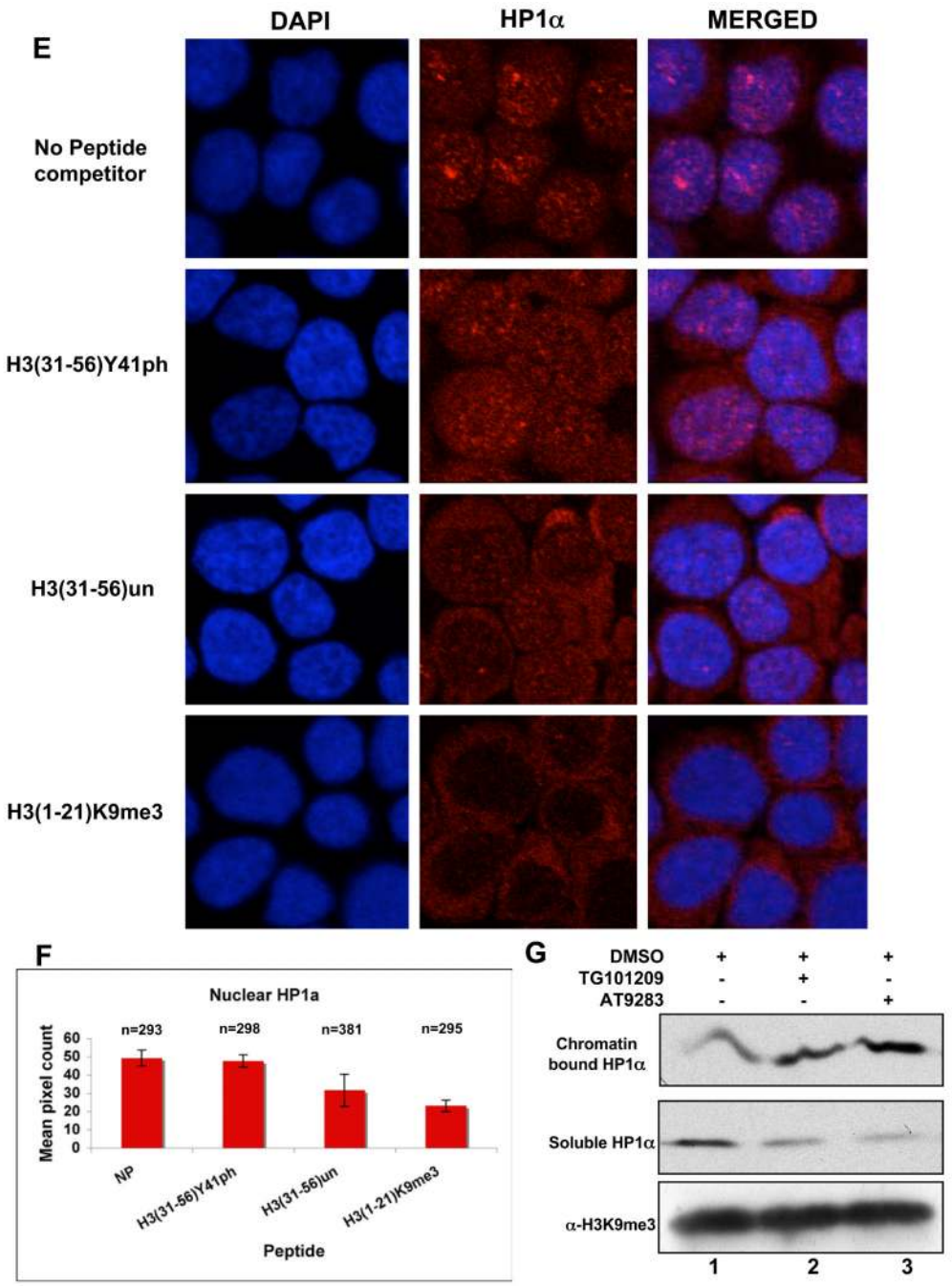


Figure 3. HP1 binds the Y41 region of H3 in a phosphorylation-dependent manner

(A) Permeabilised nuclei were prepared from HEL cells and separated into soluble and chromatin bound fractions. The soluble fraction was analysed by western blotting for HP1 and HP1 As identical fractions were analysed for both HP1 and HP1 this provides an internal control for any variation in loading. These data demonstrate that a higher percentage of HP1 is present in the soluble phase compared to HP1 Input represents the total HP1 (chromatin bound and soluble fraction) present in an identical amount of nuclei prior to fractionation. (B) HP1 HP1 and H3Y41ph antibody were tested for their ability to bind either the unmodified H3(31-56) peptides (unmod) or identical peptides phosphorylated at H3Y41 (H3Y41ph) immobilized on sepharose beads. Following binding, the products were

resolved by SDS-PAGE, western blotted and probed for HP1 , HP1 and rabbit IgG (to detect H3Y41ph antibody). The data show that HP1 , but not HP1 , binds the unmodified region of H3(31-56) and that this binding is severely abrogated by phosphorylation of H3Y41. The binding of the H3Y41ph antibody to only the modified peptide demonstrates the integrity of this affinity matrix and the specificity of the assay. (C) A schematic diagram of HP1 shows three characterized domains; chromodomain (CD), Hinge (H) and chromoshadow domain (CSD) of HP1 . Also shown are the amino acid (numbered) boundaries of each domain. The indicated regions, encompassing each domain, were cloned separately into pGex vector to allow expression of the three HP1 domains as separate GST-fusions. An equal amount of GST, GST-CD, GST-H and GST-CSD were tested for their ability to bind unmodified H3(31-56) peptides (unmod) immobilized on beads. Following binding, the products were resolved by SDS-PAGE. The resulting Coomassie Blue stained gel is shown. Input shows the quantity of GST-fusions used in the pull-down assay. The data show that only the CSD of HP1 binds the unmodified region of H3 (31-56). (D) Unmodified H3(31-56) peptides (unmod) or identical peptides phosphorylated at H3Y41 (H3Y41ph) were immobilized on beads and used to test the binding of the HP1 CSD to this region of H3. The data show that the HP1 CSD binding to H3(31-56) is severely abrogated by phosphorylation of H3Y41. (E) HL60 cells were permeabilised and HP1 binding to chromatin was challenged by competition with the indicated peptides. HP1 localization was then visualized using standard immmunofluorescence methodology. Peptides used were; unmodified H3(31-56) [H3(31-56)un], the same peptide but phosphorylated at Y41 (H3(31-56)Y41ph), and a H3 peptide trimethylated at H3K9 (H3K9me3(1-21)). HP1 is displaced from heterochromatic speckles by the H3(31-56) and H3K9me3 peptides suggesting that HP1 can bind both these regions of H3 independently. In contrast, challenge with H3Y41ph peptide [H3Y41ph(31-56)] failed to show significant disruption of HP1 when compared to unchallenged cells. (F) The degree of HP1 displacement observed in panel E was quantitated according to the mean pixel count of the red fluorochrome (representing HP1) present within the nuclei of cells following peptide challenge. The mean pixel count, standard deviation of the mean pixel count (error bars) and the number of cells counted (n = X) are plotted demonstrating that in comparison to unchallenged cells only the H3(31-56) unmodified peptide and the H3K9me3 peptide significantly displace the nuclear localization of HP1 (G) HEL cells were treated with vehicle alone (DMSO) or a specific JAK2 inhibitor (TG101209 or AT9283 at their cellular IC50s). Permeabilised nuclei were then prepared and challenged with 0.75 ng/ml of the H3K9me3 peptide, an amount sufficient to disassociate a relatively small percentage of HP1 form chromatin. Chromatin and soluble fractions were then subjected to SDS-PAGE, western blotted and probed for HP1 and H3K9me. The data demonstrate that HP1 is bound more avidly to chromatin following JAK2 inhibition and that this increased avidity for chromatin is not accounted for by detectable changes in H3K9 methylation. All data demonstrated are representative of experiments performed on at least three occasions.

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40 Most Down-regulated Genes of 18,164 tiled transcripts

Gene ID	LOG2 Fold Change	# STAT5 Sites
STS-1	-2.12	1
ID1	-2.11	1
IGFBP5	-1.93	2
FLJ11795	-1.92	4
PIM1	-1.87	1
HSPA5	-1.70	3
LOC317671	-1.65	1
DARC	-1.59	1
PIM2	-1.58	2
HSPC111	-1.56	0
TUBAL3	-1.51	1
PLVAP	-1.49	1
BCL2L1	-1.46	3
GDF3	-1.46	1
RAB3IL1	-1.45	3
HMBS	-1.43	0
SDF2L1	-1.41	1
SLCO4A1	-1.41	0
NME1	-1.39	0
KCNH2	-1.39	0
PCOLCE2	-1.35	1
HBBP1	-1.33	1
ISG20L1	-1.33	0
PSKH2	-1.32	0
LOC201164	-1.31	0
LMO2	-1.30	0
NOLA1	-1.29	1
GPR56	-1.29	1
C1ORF33	-1.28	0
EGR1	-1.27	1
FLJ43339	-1.27	3
C1ORF186	-1.25	2
RRS1	-1.24	1
CCDC58	-1.24	0
RGS19	-1.23	2
XTP3TPA	-1.22	0
TMC6	-1.21	1
SLA2	-1.18	0
KCNN4	-1.18	1
AGTRL1	-1.17	0

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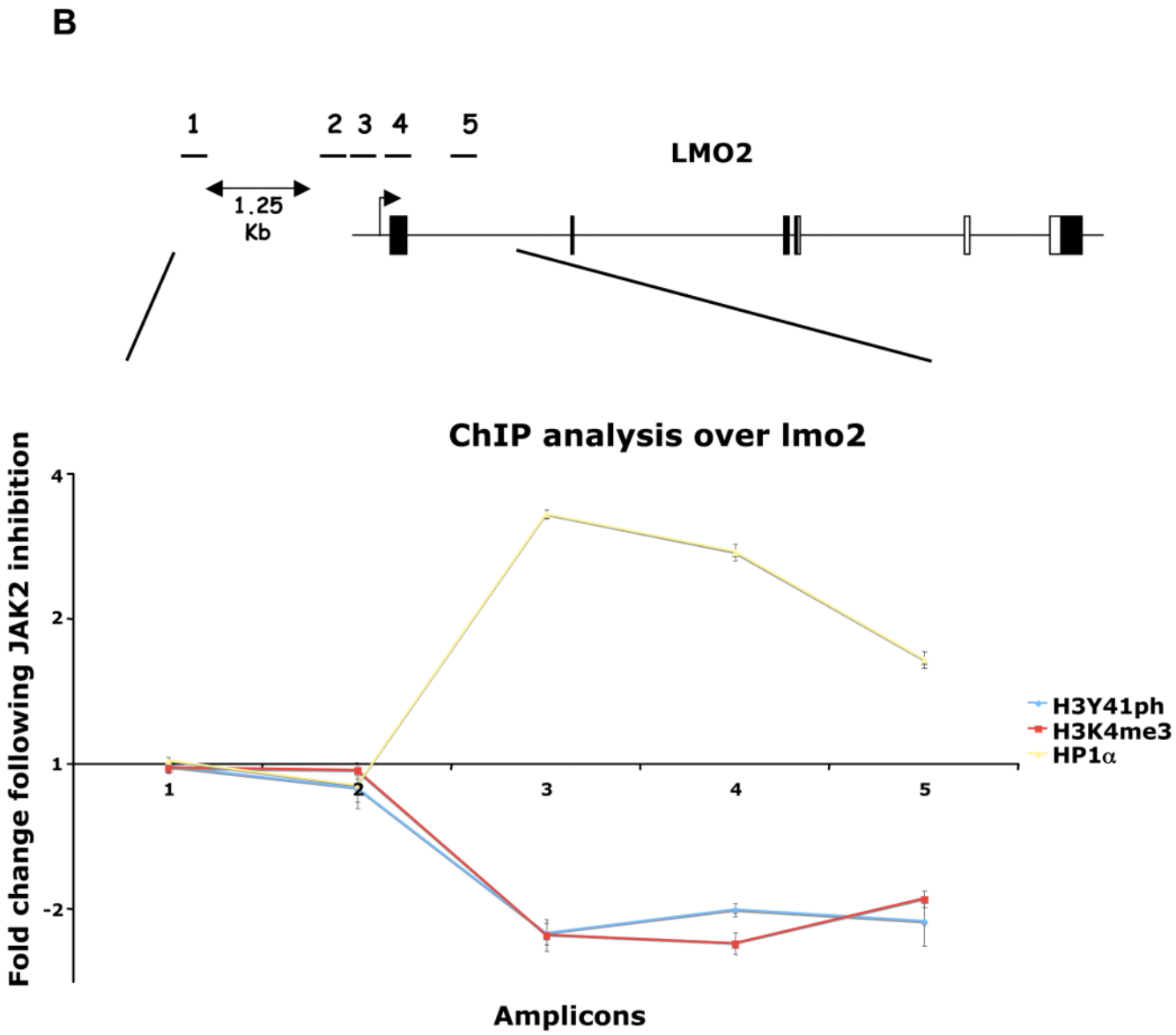

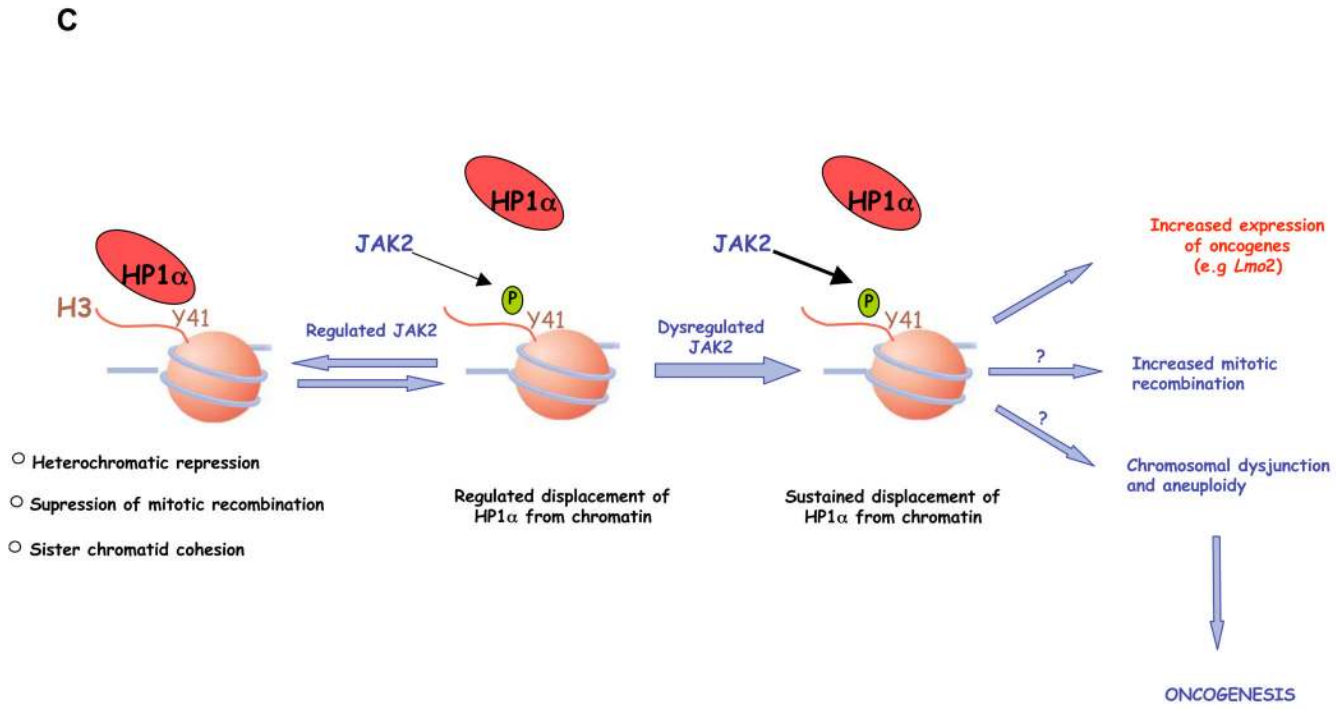

Figure 4. JAK2 signaling regulates the expression of the *lmo2* oncogene

(A) HEL cells were treated for four hours with either TG101209 JAK2 inhibitor or DMSO (vehicle) alone. From these cells, mRNA and chromatin (used in B, below) were prepared. The mRNA from two biological replicates was used to generate a gene expression profile. The forty most down-regulated genes are illustrated. *Lmo*2 is highlighted as a major target of JAK2 signaling. The number of potential STAT5 DNA binding sites in each locus is indicated. These were determined using an algorithm that searches at low stringency for STAT5 binding sites. (B) Chromatin prepared from the cells used to generate the gene expression profile (panel A) was used in chromatin immunoprecipitation (ChIP) analyses followed by real-time PCR analysis. Five regions within the *lmo2* locus were investigated (amplicons 1 to 5; see schematic representation of *lmo2* locus) using antibodies against HP1 , H3Y41ph and H3K4me3 (a histone modification associated with gene activity). Two regions, one spanning the *lmo2* promoter and one spanning the transcriptional start site (amplicons 3 to 5) show a decrease in H3Y41ph and H3K4me3 following JAK2 inhibition. Importantly, these changes are associated with a reciprocal and significant increase in HP1 . In contrast, there are no changes seen at a region 1.25kb upstream to the *lmo2* promoter (amplicon 1) at a site not known to be involved with the transcriptional control of the *lmo2* gene. The data have been normalized for H3 occupancy (by performing an anti-H3 ChIP) and is displayed as the fold change observed after JAK2-inhibition with TG101209 for 4 hours. A representative example of a ChIP analysis performed on at least three biological replicates is shown. Each amplicon was analysed in duplicate each time and error bars represent the standard deviation for each amplicon. (C) Schematic model depicting the reduction in HP1 binding to chromatin following phosphorylation of H3Y41 by JAK2. On the left are the known functions of HP1 whilst on the right are the known consequences of dysregulated JAK2 seen as a feature in JAK2 mediated haematological malignancies. Our model suggests a role in the regulation of oncogene expression (*lmo2*) and raises the possibility that other phenotypic consequences of JAK2 associated malignancies such as mitotic recombination and chromosomal dysjunction may be accounted for by constitutively active JAK2 phosphorylating H3Y41 leading to the uncontrolled displacement of HP1