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LIF independent JAK signalling to chromatin in embryonic stem cells uncovered from an adult stem cell disease

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Abstract

Activating mutations in the tyrosine kinase JAK2 cause myeloproliferative neoplasms, clonal blood stem cell disorders with a propensity for leukaemic transformation. LIF signalling through JAK-STAT enables ES cell self-renewal. Here we show that mouse ES cells carrying the human JAK2V617F mutation could self-renew in chemically defined conditions without cytokines or small molecule inhibitors independently of JAK signalling through STAT3 or PI3K pathways. Phosphorylation of histone H3Y41 by JAK2 was recently shown to interfere with HP1α binding. Chromatin bound HP1α was lower in JAK2V617F ES cells but increased following JAK2 inhibition, coincident with a global reduction in H3Y41ph. JAK2 inhibition reduced Nanog, with a reduction in H3Y41ph and concomitant increase in HP1α at the Nanog promoter. Furthermore, Nanog was required for factor-independence of JAK2V617F ES cells. Taken together, these results uncover a previously unrecognised role for direct signalling to chromatin by JAK2 as an important mediator of ES cell self-renewal.

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Introduction

The formation of mature blood cells from haematopoietic stem cells (HSCs) represents the best characterized adult stem cell system. More than 10 distinct mature lineages are generated from the multipotent HSC via a plethora of oligo- and unipotent progenitors, all of which can be identified on the basis of cell surface marker expression. Haematopoietic malignancies are caused by acquired mutations that perturb the balance between proliferation and differentiation of blood stem and/or progenitor cells. The myeloproliferative neoplasms (MPNs) are characterised by an overproduction of cells of one or more myeloid lineages and arise as a consequence of somatically acquired mutations in haematopoietic stem or progenitor cells[1,2]. Activating mutations of the non-receptor tyrosine kinase JAK2 occur in the vast majority of polycythaemia vera patients, an MPN characterised by overproduction of erythroid cells [3-6]. The mutant JAK2V617F allele is the result of a point mutation within the JH2 pseudo-kinase domain of JAK2 which results in activation of downstream signalling pathways in the absence of relevant cytokines [3,4].

Murine embryonic stem (ES) cells are derived from the inner cell mass of the developing mouse blastocyst. They can be maintained in culture indefinitely, while retaining the ability to differentiate into all somatic cell types. ES cells are commonly isolated and maintained using a combination of the interleukin class 6 cytokine leukaemia inhibitory factor (LIF) and foetal calf serum (FCS)[7-9]. LIF signals via JAK kinases and involves activation of STAT3[10], which is essential for LIF dependent ES cell self-renewal[11]. FCS can be replaced by the addition of Bone Morphogenetic Protein (BMP) thus permitting ES cell culture in chemically defined conditions[12]. More recently it has been demonstrated that both LIF and BMP can be replaced by two small molecule inhibitors of ERK and GSK3 kinase pathways known as 2i growth conditions[13].

JAK signalling therefore controls the balance between self-renewal and differentiation of both HSCs and ES cells. To gain new insights into the underlying processes, we examined molecular consequences of the JAK2V617F mutation in the context of ES cell self-renewal. ES cells engineered to contain the JAK2V617F mutant allele were able to self-renew in chemically defined conditions without any cytokines or small molecule inhibitors. Moreover, cytokine independent growth did not require STAT3 function but was sensitive to the level of the pluripotency regulator Nanog. We have recently shown that JAK2 can phosphorylate tyrosine 41 of histone H3 (H3Y41ph) and thus interfere with HP1α binding[14]. Here we show that inhibition of JAK2 signalling reduced Nanog expression, which was coupled to a decrease in H3Y41ph and concomitant increase in HP1α at the Nanog promoter. Our results are therefore consistent with a new LIF-independent role for JAK proteins in ES cell self-renewal, whereby direct JAK signalling to chromatin contributes to the regulation of genes important for pluripotency.

Results

JAK2V617F enables factor-independent ES cell self-renewal

To gain new insights into the molecular consequences of the JAK2V617F mutation, a human JAK2 cDNA containing the V617F mutation was introduced by homologous recombination into the *jak2* locus of murine embryonic stem (ES) cells (fig 1a). The mutant cDNA was under the normal regulatory control of endogenous *jak2* and the JAK2V617F allele was expressed at an equal level to the wild type allele[15]. ES cells can be maintained in chemically defined media with two small molecule inhibitors of ERK and GSK3 signalling; known as 2i[13]. JAK signalling in this context was thought to be unimportant because 2i obviates the requirement for STAT3 phosphorylation by JAK kinases[13]. However, when JAK2V617F ES cells were grown in 2i conditions at clonal density, there was a substantial

increase in the number of ES cell colonies compared to wild type ES cells. This observation lead us to hypothesise that there may be a previously unknown requirement for Janus kinase signalling in ES cells.

A hallmark of MPNs is the ability to form erythroid colonies from patients' bone marrow without exogenous erythropoietin (Epo)[16]. ES cells are usually supplemented with LIF to promote self-renewal. Because LIF and Epo both signal through Janus kinases associated with their respective receptor[10],[17], we tested whether JAK2V617F ES cells could be maintained without the need for supplementary LIF. Unlike wild type ES cells, JAK2V617F ES cells could grow for multiple passages either in serum-containing medium or in chemically defined media (N2B27) supplemented with BMP4, remaining in an undifferentiated state without the need for LIF supplementation (Supplementary figure 1). Surprisingly, when BMP4 was also withdrawn from the culture medium, JAK2V617F ES cells remained in an undifferentiated state (fig 1b) and could be maintained in chemically defined media for over 50 passages without the addition of cytokines or small molecule inhibitors. Factor-independent JAK2V617F ES cells retained a stable diploid karyotype and were morphologically indistinguishable from the parental ES cells (fig 1b, Supplementary figure 1). JAK2V617F ES cells converted to factor-independent growth after undergoing a crisis in which they detached from the gelatine substrate and formed spheres, but when replated in fresh N2B27, reattached and continued to grow as a monolayer (fig 1b). This conversion was required for clonal expansion of factor-independent JAK2V617F ES cells (fig 1c). Factor-independent JAK2V617F ES cells formed ES cell colonies with similar efficiency to wild type ES cells in serum and LIF or in LIF and BMP4, but were more efficient in 2i (fig 1c). Factor-independent growth was abolished when ES cells were grown in N2B27 in the presence of a small molecule inhibitor of JAK (AG490)[18] (fig 1c). To confirm that acquisition of cytokine-independence was not an isolated event, 48 JAK2V617F positive clones were picked, 45 of which were successfully expanded and all 45 clones could be maintained in N2B27 alone for at least 5 passages (Supplementary figure 1). Factor-independent JAK2V617F ES cells expressed ES cells markers, Oct4 and Nanog, similarly to parental ES cells grown in N2B27 plus LIF and BMP4 (fig 1d). Genome-wide expression profiling was performed using parental ES cells, JAK2V617F ES cells maintained in N2B27 plus LIF and BMP4 and JAK2V617F ES cells grown in N2B27. The majority of genes were expressed at similar levels in all three samples, and there was a strong correlation coefficient for all two-way comparisons (fig 1e). Known regulators of ES cell identity were expressed at similar (e.g. Oct4, Sox2) or slightly elevated levels in JAK2V617F ES cells (e.g. Nanog), and there was no up-regulation of genes characteristic of more committed cell types (fig 1f). Expression profiling therefore confirmed that the transcriptome of factor-independent JAK2V617F ES cells was highly similar to parental wild type ES cells.

Factor-independent JAK2V617F ES cells were not permanently locked into an undifferentiated state; they could differentiate *in vitro* into somatic cell types including erythrocytes and neurons (fig 2a), however, *in vitro* differentiation was less efficient. Haematopoietic differentiation of factor-independent JAK2V617F ES cells resulted in fewer Flk-1 positive cells, than wild type ES cells at 3, 5 and 7 days after the start of differentiation, with cells expressing the ES cell marker SSEA-1 still persisting at day 7 (fig 2b). To evaluate *in vivo* multi-lineage differentiation of factor-independent JAK2V617F ES cells, injections into mouse kidney capsule were performed which resulted in formation of teratocarcinomas composed of all three germ layers (fig 2c). Unlike parental teratocarcinomas however, teratocarcinomas from factor-independent JAK2V617F ES cells were composed predominantly of undifferentiated or poorly differentiated cells (fig 2c), indicating that while differentiation was possible; it was dramatically reduced by the presence of JAK2V617F. Factor-independent JAK2V617F ES cells were also injected into 8

cell stage mouse embryos and transplanted into recipient females; no chimaeras were observed either embryonically or postnatally following three independent rounds of injections (Supplementary figure 1). Correct timing of differentiation is essential for integration of ES cells into the developing blastocyct. Delayed or inefficient differentiation is likely to have excluded factor-independent ES cells from contributing to chimaeras thus making them fail one of the classical criteria of pluripotency.

Our demonstration that mutant JAK2 influenced ES cell self-renewal raised the possibility that this pathway may be required in wild type ES cells. We therefore investigated the clonogenicity of wild type and JAK2V617F ES cells in the presence of pan-JAK (AG490[18] and Jaki1[19]) and JAK2 selective (TG101209[20]) inhibitors when grown in 2i conditions which obviate the requirement for STAT3 activity[13]. There was a dose dependent decrease in the number of ES cell clones formed in the presence of all inhibitors. Moreover, JAK2V617F ES cells grown in 2i or N2B27 show a similar sensitivity to JAK inhibitors (fig 3a, Supplementary table 1) and these effects were seen at concentrations considerably lower than routinely used [14],[18-20]. Time-lapse microscopy of parental or JAK2V617F ES cells showed that ES cells cannot be maintained in a self-renewing state in 2i media following the addition of AG490. Instead, they lost characteristic ES cell morphology and began to form cells that appear more differentiated (Supplementary movies). Together with the *in vitro* differentiation and teratocarcinoma assays, these results demonstrate that factor-independent JAK2V617F ES cells are not transformed into a permanently self-renewing ES cell state.

Factor-independent ES cell self-renewal is independent of the JAK/STAT pathway

To understand the molecular basis of factor-independent self-renewal and the role of JAK2 in LIF-independent ES cell self-renewal, the pathways regulated by JAK2 were analysed. Extracellular binding of cytokines induces receptor-bound Janus kinases to recruit and phosphorylate STAT (Signal Transducer and Activator of Transcription) proteins, which then dimerize and translocate to the nucleus where they regulate transcription[21]. STAT3 is the only STAT family member expressed in wild type or JAK2V617F ES cells (Supplementary figure 2) and is essential in LIF dependent ES self-renewal[11],[13]. LIF induces STAT3 phosphorylation [10],[22], and constitutively active STAT3 removes the need for LIF to promote self-renewal [23]. JAK2V617F ES cells, in either N2B27 alone or 2i media do not contain detectable STAT3 phosphorylation (fig 3b). Due to the importance of the JAK-STAT axis in ES cell self-renewal [10],[11],[22],[23] and in the MPNs [24],[25], JAK regulation of STAT3 was investigated further. Firstly, the expression profile for a number of known transcriptional targets of STAT3 signalling were compared; genes including SOCS3 were significantly down-regulated in factor-independent JAK2V617F ES cells compared to wild type or JAK2V617F ES cells growing in LIF (Supplementary figure 2). Secondly, STAT3 null ES cells grown in 2i conditions were treated with the same panel of JAK inhibitors described previously and their clonogenicity was tested. STAT3 null ES cells were similarly sensitive to wild type ES cells when treated with JAK inhibitors, suggesting that the diminished self-renewal caused by inhibiting JAK signalling in 2i conditions occurs independently from STAT3 (fig 3c, Supplementary table 1). Finally, the JAK2V617F construct was introduced by homologous recombination into STAT3 null ES cells (Supplementary figure 3), and JAK2V617F STAT3 null ES cells were tested for their ability to be maintained in factor independent conditions. 11 JAK2V617F STAT3 null ES cell clones were successfully grown for 5 passages or more in N2B27 alone (Supplementary figure 3), and continued to express the key ES cell markers Oct4 and Nanog (fig 3d). Taken together these experiments demonstrate that factor independent self-renewal is not conferred by LIF-independent activation of STAT3.

Phosphoinositide-3-OH kinase (PI3K) is a down-stream target of JAK signalling and promotes ES cell self-renewal by a pathway that entails AKT phosphorylation [22],[26].

However, the level of phosphorylated AKT in JAK2V617F ES cells was unaffected by the addition of the JAK inhibitor AG490 (fig 3b), and was only elevated following the addition of LIF (fig 3a). Moreover, GSEA analysis of the microarray data shows that there was no significant change in PI3K pathways in factor-independent JAK2V17F ES cells grown in N2B27 compared to wild type ES cells grown in LIF and BMP4 (Supplementary figure 4). JAK2V617F regulation of PI3K therefore was unlikely to be important for factor-independent self-renewal. Activation of ERK 1/2 and Ras, two other downstream targets of JAK signalling promote ES cell differentiation[27-30]. However, ERK 1/2 was still phosphorylated in factor-independent JAK2V617F ES cells (Supplementary fig 4), and JAK2V617F ES cell self-renewal was enhanced by inhibiting ERK signalling (fig 1c), suggesting that factor-independent self-renewal is not conferred by loss of ERK activation.

JAK2 signals directly to chromatin in ES cells by phosphorylating H3Y41

Studies in *Drosophila* have shown that JAK signalling globally counteracts heterochromatic gene silencing by antagonising the function of heterochromatin protein 1 (HP1)[31,32]. Moreover, we have recently identified a novel role for JAK2 in the nucleus of haematopoietic cells where it can phosphorylate tyrosine 41 of histone H3 (H3Y41) which interferes with HP1α binding[14] and thus provides a molecular explanation for the JAK2 activity uncovered from Drosophila genetics. Phosphorylated JAK2 was present in the nucleus of ES cells (fig 4a) and we therefore investigated whether JAK2V617F alters the distribution of HP1α. Chromatin bound HP1α, but not Oct4, was lower in JAK2 mutant compared to wild type ES cells, under multiple growth conditions (fig 4b).

To confirm that HP1α is dynamically regulated by JAK2, JAK inhibitor TG101209 was added to JAK2V617F ES cells grown in factor-independent conditions. Following 2 hours of treatment there was a significant increase of chromatin-associated HP1α in the nucleus (fig 4c), and a decrease in the global level of H3Y41ph (fig 4d, Supplementary figure 5). The decrease in H3Y41ph levels was observed as early as 15 minutes after JAK inhibitor treatment (Supplementary figure 5) which is not only consistent with the direct link between Jak2 and H3Y41 phosphorylation but also highlights the very dynamic nature of the H3Y41ph histone modification. Importantly, these results indicate that inhibition of JAK2 in ES cells is accompanied by an increase in chromatin bound HP1α.

Nanog is a critical target for LIF independent JAK signalling

The pluripotency transcription factor Nanog is expressed at elevated levels in JAK2V617F ES cells compared to wild type cells in both cytokine containing and cytokine independent conditions (fig 1f). Nanog was also down-regulated at the protein level following a 2 hour treatment with TG101209 (fig 4c and d). Based on the data presented above, these observations are consistent with a model whereby JAK2 may regulate Nanog expression by controlling the level of phosphorylated H3Y41 at the Nanog promoter. We therefore carried out chromatin immunoprecipitation (ChIP) for phosphorylated H3Y41 in factor-independent JAK2V617F ES cells grown in N2B27 and in N2B27 plus the JAK inhibitor TG101209 for 6 hours. We mapped a 8kb window spanning the Nanog transcriptional start site (TSS); phosphorylated H3Y41 was present surrounding the TSS of Nanog, but was reduced following treatment with TG101209 (fig 5a). These changes were coupled with an increase in the binding of HP1α and decrease in H3K4me3 at the Nanog promoter (Supplementary fig 5). These reciprocal changes in H3Y41ph and HP1α binding following JAK inhibition were also seen in wild type ES cells growing in LIF independent conditions (fig 5b); suggesting that loss of H3Y41ph and HP1α recruitment are involved in regulating Nanog expression.

To further characterise this newly discovered link between JAK2 and Nanog, Nanog over-expressing ES cells [33] were analysed in the ES cell clonogenicity assay with the panel of JAK inhibitors described above. Although JAK inhibition caused a reduction in the self-renewal ability of Nanog over-expressing ES cells, this was significantly less than the decline observed with wild type ES cells (fig 5c), indicating that Nanog over-expression can largely overcome the effect of JAK inhibition. Conversely, factor-independent ES cell self-renewal of JAK2V617F ES cells transduced with shRNA vector targeting Nanog [34] was severely compromised when compared with control vector transduced cells (fig 5d), indicating that Nanog is required for factor-independent self-renewal of JAK2V617F ES cells.

While the above demonstrate a central role for Nanog in JAK2V617F mediated factor-independent self-renewal, our immunohistochemical analysis showed JAK dependent H3Y41ph throughout the nucleus (fig 4d). Therefore, a number of regulators of ES cell self-renewal were analysed to determine if there was JAK-dependent dynamic localisation of H3Y41ph at their promoters. Some genes such as Sox2 and SMARCA4 were dynamically regulated in a similar fashion to Nanog with JAK inhibition causing a reduction H3Y41ph and an increase in HP1α. Other genes such as Bicd2, Dnmt1 and Tbx3 showed reduced H3Y41ph, but no increase in HP1α, while Dnmt3b had no H3Y41ph (Supplementary fig 5). Multiple genes involved in ES cell self-renewal therefore display JAK dependent dynamic regulation of H3Y1ph and HP1α, but detailed modes of regulation are likely to be gene-specific. Recruitment of HP1α by sequence-specific transcription factors has been observed previously [35],[36], suggesting that loss of H3Y41ph alone is not sufficient for HP1α binding.

Multiple JAKs can phosphorylate H3Y41

Knock-outs of single Janus kinase family genes can survive beyond the epiblast stage of development [37]-[39], which in light of our JAK inhibitor data suggest functional redundancy. We therefore wanted to ascertain if this new regulatory role for JAK2 signalling in ES cell self-renewal can also be conferred by other Janus kinase family members. We derived JAK2 null ES cells (Supplementary figure 3), in order to identify whether H3Y41 was phosphorylated independently from JAK2. H3Y41ph was present in JAK2 null ES cells, and was dynamically regulated by inhibiting JAK signalling. Moreover, there was a significant reduction in the global level of H3Y41ph (fig 6a) following treatment with AG490, which was accompanied by differentiation of these inhibitor-treated JAK2 null ES cells, as seen by loss of Oct4 expression (figure 6a). JAK1 has long been implicated in ES cell self-renewal [40]. We now show that JAK1 was also present in the nucleus of wild type ES cells grown in 2i (fig 6b) and that JAK1 can directly phosphorylate H3Y41 (Supplementary figure 6). Moreover, ES cells express all Janus kinase family members (Supplementary figure 2), and JAKs other than JAK1 and JAK2 may also be involved in phosphorylating H3Y41. Our observation that H3Y41 can be phosphorylated by JAKs other than Jak2 coupled with our analysis of Jak2 null ES-cells suggests that while this pathway was discovered using JAK2, it likely involves multiple members of the Janus kinase family.

Discussion

Chromatin modifying enzymes restrict ES cell differentiation to specific lineages[41],[42], and chromatin modifications are dynamically redistributed during *in vitro* differentiation of ES cells[43],[44]. Here we not only demonstrate JAK2 mediated control of H3Y41 phosphorylation on the Nanog promoter but also show global effects on heterochromatin. We hypothesize therefore that increased H3Y41 phosphorylation on key gene loci stabilizes the overall transcriptional regulatory state of ES cells. This model is not only consistent with our demonstration of cytokine independent self-renewal but would also explain the inability of factor-independent JAK2V617F ES cells to contribute to chimaeras, and comparatively low

propensity to differentiate in teratocarcinoma assays. Given the original identification of the JAK2V617F mutation in an adult stem cell disease, our results raise the intriguing possibility that at least part of the disease phenotype may arise from changes in the plasticity of chromatin. The JAK2V617F mutation occurs in blood stem cells, and mutant blood stem cells are thought to be biased towards producing myeloid progeny because the mutant allele is rarely detected in lymphocytes. Gene expression studies as well as evolutionary comparisons support the notion that myeloid differentiation represents the default pathway for blood stem cells. It is therefore possible that the myeloid differentiation bias of JAK2V617F blood stem cells is mechanistically related to the cell fate stabilisation phenotype uncovered here in JAK2V617F ES cells. Furthermore, many cancers are characterised by an increase in the level of activated JAK signalling[45], which is often achieved by amplification of the amount of cytokine[46], or sensitivity of the receptor to cytokines[47]. Consequently the level and distribution of H3Y41ph is likely to be altered, suggesting that JAK signalling to chromatin may have a wider role in human cancer and that changes in H3Y41ph should therefore also be examined in these diseases.

Methods

ES Cell culture

ES cells were cultured in N2B27 media prepared as described [48],[49] either with no supplements, supplemented with recombinant mouse LIF (1000 IUml^{-1}) and recombinant human BMP4 (Peprotech), or with CHIR99021 (1μM) and PD0325901 (1μM) both University of Dundee, Division of Signal Transduction Therapy, referred to as 2i. ES cells were also cultured in Knockout-DMEM (Invitrogen) plus 15%FCS, 2mM L-Glutamine (Invitrogen) and 50μM β-mercaptoethanol (Invitrogen) and LIF.

Generation of JAK2^{V617F} ES cells

A cassette containing a floxed PGKNeoPoly(A) minigene followed by a mutant human JAK2V617F cDNA and a SV40 Poly(A) sequence was then introduced into AB2.2 ES cells into the ATG translation start site in the exon 2 using recombineering[50]. Targeted ES clones with JAK2 $^{F/+}$ allele were identified by Southern blot analysis [15]. Correctly targeted ES cells were subjected to an electroporation of PGKCre. Single colonies were picked and analysed by PCR [15].

Colony forming assays

ES cells were trypsinized to obtain a single cell suspension and 500 cells plated per 10cm^2 well in triplicate. After 3 days the media was changed, and after 6 days the cells were stained for alkaline phosphatase (Sigma) and colonies counted.

Gene expression analysis

Total RNA was collected in triplicate from wild type ES cells grown in N2B27 plus LIF and BMP4, JAK2V617F ES cells grown in N2B27 plus LIF and BMP4 and JAK2V617F ES cells in N2B27 only using Tri reagent (Sigma). cDNA was synthesised and hybridized to Illumina Mouse WG-6 v1.0 Expression BeadChips. BeadChip probe-sets that did not pass the Illumina signal detection statistic at a threshold of $p < 0.01$ in all sample replicates of at least one sample group were removed from further analysis, leaving 18,697 expression values per profile. For all samples, the remaining probe-sets were background corrected and quantile normalized using the lumi package of the Bioconductor suite of software for R. Upon comparison of sample group profiles, inter-group profile correlations were calculated using average linkage and the Spearman correlation measure, accompanied by standard

error. Gene categories enriched upon inter-group comparison were identified using the Gene Set Enrichment Analysis Software [51] with default settings.

In vitro differentiation

Neural differentiation was performed as described previously[52] except 10μg/ml FGF4 (Peprotech) was supplemented into media. Haematopoietic differentiation was performed by seeding ES cells in suspension in IMDM (Invitrogen) plus 15% FBS (Hyclone), 10% PFHM (Invitrogen), 1% L-glutamine (PAA Laboratories), 1% human transferrin (Roche), 0.3mM MTG (Sigma) and 50ng/ml AA (Sigma).

Teratocarcinoma and chimaera contribution assays

For teratocarcinoma induction, 1×10^6 cells of each ES cell line were injected subcutaneously into the kidney capsule isoflurane-anesthetized 129SV mice. Teratocarcinomas were recovered 3–4 weeks post-injection, fixed overnight in formalin, paraffin embedded and sectioned. Sections were stained with haematoxylin and eosin and imaged using an Improvision Openlab deconvolution camera. For chimaeras, factor-independent JAK2V617F ES cells were injected into the 8 cell stage embryos of Agouti 129SV/BL6 mice. For two rounds of injections, mice were born and examined for chimaeric coat colour, the third round JAK2V617F ES cells had eGFP inserted into the ROSA26 locus and embryos were examined for eGFP positive cells at E12.5.

JAK2 null ES cell derivation

Heterozygous non-recombined JAK2V617F mice were crossed and blastocysts harvested at E3.5, ES cell derivation was performed as described in [53].

Immunohistochemistry, microscopy and flow cytometry

Images were captured with a Zeiss LSM510 meta confocal microscope. Image processing was performed with Photoshop (Adobe Systems). For fluorescent intensity analysis all images were captured using the same settings and unprocessed images were measured using ImageJ (NIH). Live cell imaging was performed using the IncuCyte platform (Essen instruments). Flow cytometry was performed on FACScalibur (BD).

JAK inhibitors

AG490 (Millipore), Jaki1 (Calbiochem) and TG101209 (TargeGen Inc) were all used at 1μM unless indicated otherwise.

Antibodies

Oct4 (Santa Cruz sc-5279) 1:125, Nanog (R&D systems AF2729) 1:250, Tuj1 (Abcam AB14545) 1:1000, JAK2 (Cell signalling technology, no. 3230) 1:100, HP1α (Cell signalling technology, no. 2616) 1:1000, H3Y41ph (Abcam) 1:1000, H3K9me3 (Millipore 07-523) 1:1000, H3 (Abcam Ab1791), pAKT Ser473(Cell signalling technology, no. 9271) 1:1000, β-Tubulin (Sigma T-5293) 1:1000, STAT3 (Cell signalling technology, no. 9132) 1:1000, pSTAT3 Y705 (Abcam AB30646) 1:1000, Alexa Fluor 647 Donkey anti goat (A21447) 1:500, Alexa Fluor 555 Donkey anti rabbit (A31572) 1:500, Alexa Fluor 488 Donkey anti mouse (A21202) 1:500, PE α-mouse Flk-1 (VEGF-R2, Ly-73) (Avas 12α1) (BD Pharmingen) 1:100 and α-h/m SSEA-1 APC conjugated IgM (R&D, Cat: FAB2155A) 1:50.

Chromatin immunoprecipitation and RT-PCR

ES cells were treated for 16 hours with either AG490 or DMSO. Chromatin was prepared and chromatin immunoprecipitation was performed as described previously[35], with the following exceptions. Cells were crosslinked with 1% (v/v) formaldehyde for 15 min at room temperature and DNA was purification with the QIAquick PCR purification kit (Qiagen). Immunoprecipitated DNA was analysed on a Stratagene Mx3005P real-time PCR machine, with SYBRgreen PCR mastermix. The experiment was performed on two separate occasions using independent biological material. Primer sequences are available upon request.

Kinase assay

Active JAK1 protein (Invitrogen) was used in an *in vitro* kinase assay. In brief, assays were performed in 50μl of HTScan kinase buffer (60mM HEPES pH7.9, 5mM $MgCl_2$, 5mM $MnCl_2$, 3μM Na_3VO_4, 2.5mM DTT, 100μM ATP). Recombinant histones were used as substrates, and then probed with anti-H3Y41ph antibody (1:1000) overnight at 4°C, followed by anti-rabbit secondary conjugated to HRP and developed using ECL.

Statistical analysis

Differences in efficiency of ES cell colony formation was analysed in R (R Development Core Team, 2009), using a GLM with a poisson error structure and a log link function. The experimental day was included in the model as a factor variable. The concentration was modelled as both a factor and a continuous variable in separate analyses. Differences in IHC intensity were analysed using a paired Student's T-Test in Excel (Microsoft).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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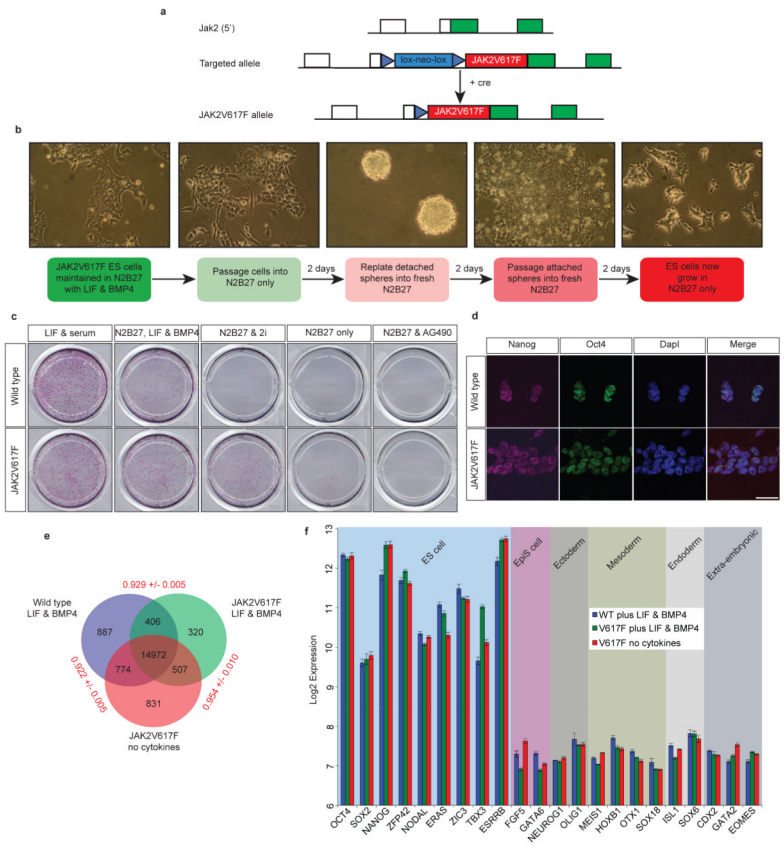

Figure 1.

JAK2V617F sustains ES cells in a self-renewing state without any additional factors

a. Targeting strategy to insert patient cDNA containing JAK2V617F mutation into the *jak2* allele by homologous recombination.

b. JAK2V617F ES cells are made factor-independent by transferring ES cells growing in N2B27 plus LIF and BMP4 into N2B27 only. The ES cells then undergo a crisis; detaching and forming spheres, these spheres can then be reattached by transferring into fresh N2B27 on freshly gelatinised flasks.

c. ES cells were plated at 1×10^3 cells per well of a 12 well plate, 6 days later the cells were fixed and stained for alkaline phosphatase to identify ES cell colonies. Only factor-independent JAK2V617F ES cells (JAK2V617F) can form colonies in N2B27 only, this ability is lost following the addition of AG490.

d. Parental ES cells grown in N2B27 plus LIF and BMP4 have a similar pattern of staining for the key ES cell transcription factors Nanog and Oct4 to factor-independent JAK2V617F ES cells in N2B27 alone. Of note, factor-independent JAK2V617F cells have the characteristic variable levels of Nanog seen with wild type ES cells. Scale bar 20μm.

e. Microarray analysis of the three ES cell lines demonstrates that the majority of expressed genes are common to all three ES cell lines. Correlation coefficients were calculated using the mean of each two-way comparisons and show a strong correlation coefficient between all three datasets (red values).

f. Genes known to be critical for ES cell self-renewal are expressed at similar levels in all datasets, and there is no up-regulation of genes expressed in more differentiated cell types. Values are mean of three biological replicates, error bars represent S.E.M.

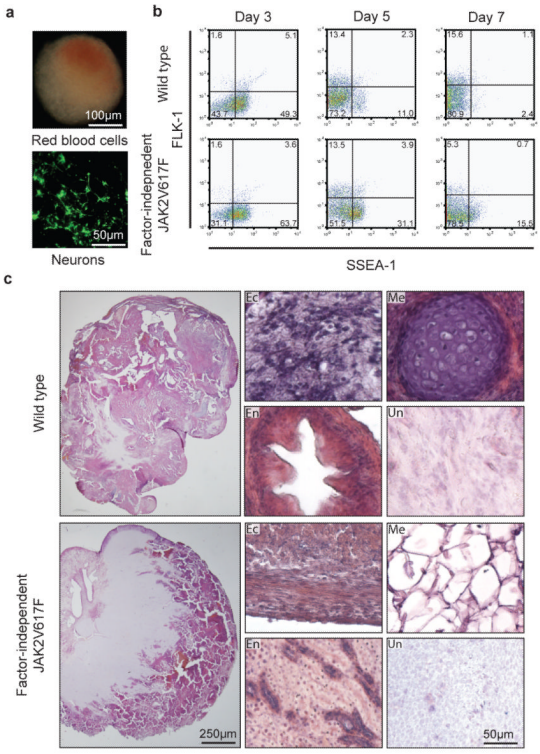

Figure 2.

Factor independent JAK2V617 ES cells are capable of multilineage differentiation *in vitro* and *in vivo*

a. Factor independent JAK2V617F ES cells give rise to embryoid bodies containing red blood cells and differentiate into neurons when transferred into appropriate differentiation conditions.

b. JAK2V617F ES cells generate Flk-1 positive mesodermal cells with delayed differentiation kinetics. Wild type and factor-independent JAK2V617F ES cells were differentiated to haematopoietic lineages, and the proportion of Flk-1 and SSEA-1 positive cells were measured by FACS at days 3, 5 and 7 of differentiation

c. Teratocarcinoma formation from JAK2V617F ES cells. Parental AB2.2 ES cells maintained in N2B27 plus LIF and BMP4 and factor-independent JAK2V617F ES cells were injected into the kidney capsule of 129sv mice and left for four weeks. Teratocarcinomas consisting of cells from all three germ layers formed, but the majority the teratocarcinomas from factor-independent JAK2V617F ES cells remained undifferentiated or were of no immediately discernible cell type. Un -undifferentiated, ec- ectoderm, me – mesoderm, en – endoderm.

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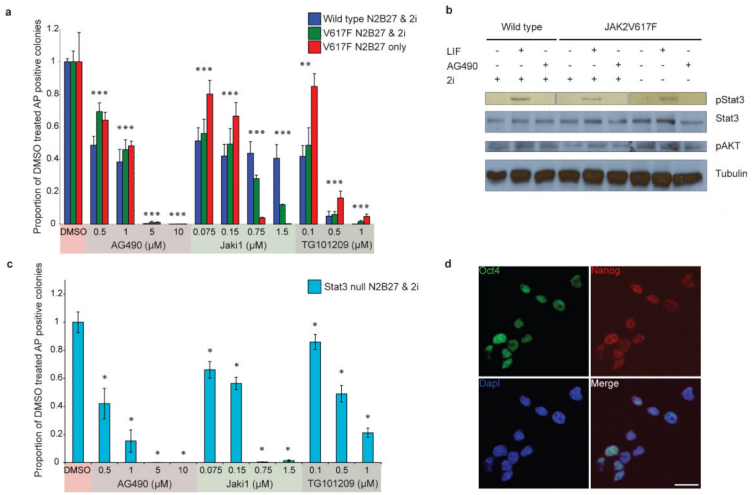

Figure 3.

JAK2V617F does not activate canonical signalling pathways independently of cytokines, but JAK is required for ES cell self-renewal.

a. Colony forming assay for wild type and factor-independent ES cells in 2i or N2B27 alone following clonal growth for 6 days in dilution series of JAK inhibitors. ES colonies confirmed by positive staining for alkaline phosphatase. There was a significant reduction ($p<1\times10^{-7}$) for all inhibitors on all cell types except JAK2V617F ES cells in N2B27 alone at the lowest concentrations of TG101209, for details of GLM see Supplementary table 1. Error bars represent S.E.M.

b. Immunoblot analysis of steady state levels, or following 8 hours of treatment of cells with LIF or AG490 for P-STAT3 (tyr705), STAT3, P-AKT (ser 473) and tubulin of wild type ES cells grown in N2B27 plus 2i, JAK2V617F ES cells in N2B27 plus 2i or JAK2V71F ES cells in N2B27 only.

c. Colony forming assay for STAT3 null ES cells in 2i performed as in 3c. There was a significant reduction ($p<1 \times 10^{-16}$) using all inhibitors. For details of GLM see Supplementary table 1.

d. Immunocytochemistry of STAT3 null JAK2V617F factor-independent ES cells confirms continued expression of key ES cell genes Oct4 and Nanog. Scale bar 20μm.

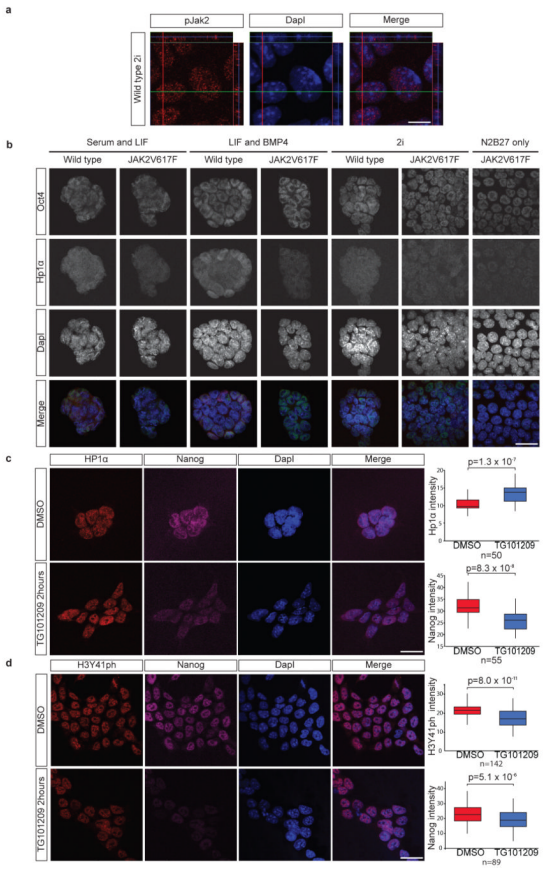

Figure 4.

JAK2 is present in the nucleus of ES cells and JAK2 dynamically regulates HP1α access to chromatin by phosphorylating H3Y41.

a. Immunohistochemistry for phosphorylated JAK2 in wild type ES cells growing in 2i. Orthogonal view confirms the presence of phosphorylated JAK2 in the nucleus. Scale bar 20μm.

b. Immunohistochemistry confirms that HP1α was present at lower levels in JAK2V617F ES cells compared to parental cells when they are maintained in multiple ES cell conditions, but Oct4 remains unchanged. Scale bar 20μm.

c. Immunohistochemistry for HP1α and Nanog in steady state factor-independent JAK2V617F ES cells and following treatment with TG101209 for 2 hours. There was a significant increase in the level of HP1α and decrease in Nanog following inhibitor treatment, two independent experiments combined in box and whisker plot, difference determined by Students T-Test. N is cell number. Scale bar 20μm.

d. Immunohistochemistry for H3Y41ph and Nanog in steady state factor-independent JAK2V617F ES cells and following treatment with TG101209 for 2 hours. There was a significant decrease in the levels of H3Y41ph and Nanog following inhibitor treatment, two independent experiments combined in box and whisker plot, difference determined by Students T-Test. N is cell number. Scale bar 20μm.

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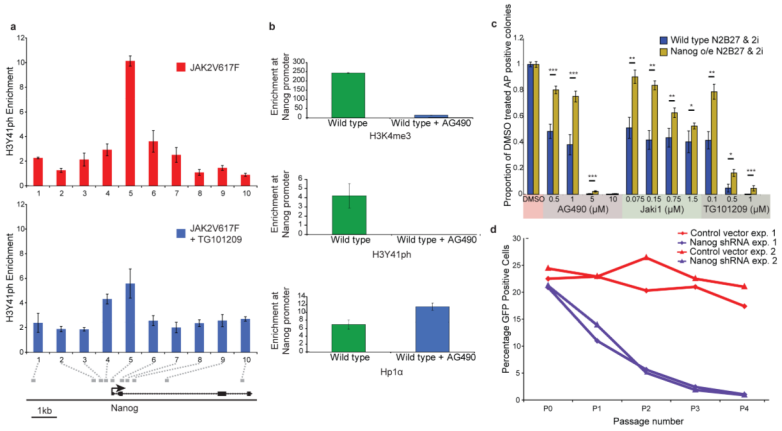

Figure 5.

JAK2 regulates H3Y41ph at the Nanog promoter and Nanog is critical for factor-independent self-renewal.

a. A 8kb window surrounding the Nanog TSS was interrogated using chromatin immunoprecipitation for H3Y41ph in steady state factor-independent JAK2V617F ES cells or following treatment with TG101209 for 6 hours. Data were normalised to H3 occupancy. Representative plot of two independent experiments, error bars represent S.E.M.

b. The Nanog promoter was interrogated using chromatin immunoprecipitation for H3K4me3, H3Y41ph and HP1α in wild type ES cells growing in N2B27 plus 2i or following treatment with AG490 for 16 hours. Data were normalised to H3 occupancy. Representative plot of two independent experiments, error bars represent S.E.M

c. Colony forming assay for Nanog over-expressing ES cells, performed as described in 3b. While JAK inhibition caused a decrease in ES cell self-renewal of Nanog over-expressing ES cells, this was significantly less than wild type ES cells, * $p<0.05$, ** $p<0.01$, ***$p<0.001$, see Supplementary table 1 for details of T-Test. Error bars represent S.E.M.

d. Growth of JAK2V617F ES cells in N2B27 alone is compromised following transduction with a shRNA Nanog knockdown lentivirus co-expressing eGFP.

e. Immunohistochemistry of JAK2 Null ES cells growing in 2i media demonstrated that H3Y41 is phosphorylated in the absence of JAK2, which in turn was dynamically regulated by JAK inhibition treatment with AG490 for 16 hours. There was a significant decrease in the levels of H3Y41ph and Oct4 following inhibitor treatment, two independent experiments combined in box and whisker plot, difference determined by Students T-Test. N is cell number.

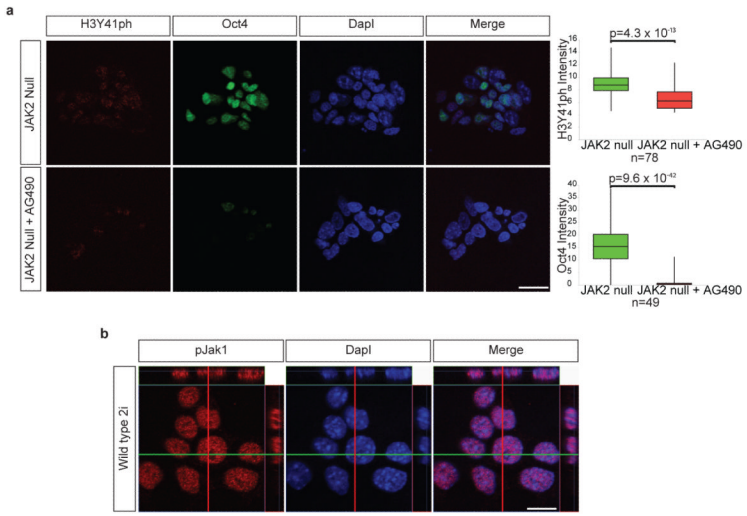

Figure 6.

JAK2 is not the only JAK which can phosphorylate H3Y41

a. Immunohistochemistry of JAK2 Null ES cells growing in 2i media demonstrated that H3Y41 is phosphorylated in the absence of JAK2, which in turn was dynamically regulated by JAK inhibition treatment with AG490 for 16 hours. There was a significant decrease in the levels of H3Y41ph and Oct4 following inhibitor treatment, two independent experiments combined in box and whisker plot, difference determined by Students T-Test. N is cell number.

b. Orthogonal view of immunohistochemistry for phosphorylated JAK1 in wild type ES cells maintained in N2B27 plus 2i.

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