ZFP36L1 negatively regulates erythroid differentiation of CD34+ hematopoietic stem cells by interfering with Stat5b pathway


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Abstract.
ZFP36L1 is a member of a family of CCCH tandem zinc finger proteins (TTP family) able to bind to AU-rich elements in the 3’-untranslated region of mRNAs, thereby triggering their degradation. The present study suggests that such mechanism is used during hematopoiesis to regulate differentiation by post-transcriptionally modulating the expression of specific target genes. In particular, it demonstrates that ZFP36L1 negatively regulates erythroid differentiation by directly binding the 3’ untranslated region of Stat5b encoding mRNA. Stat5b down regulation obtained by ZFP36L1 over-expression results, in human hematopoietic progenitors, in a drastic decrease of erythroid colonies formation. These observations have been confirmed by silencing experiments targeting Stat5b and by treating hematopoietic stem/progenitor cells with drugs able to induce ZFP36L1 expression. Moreover, this study shows that different members of ZFP36L1 family act redundantly, since co-overexpression of ZFP36L1 and family member ZFP36 determines a cumulative effect on Stat5b downregulation. This work describes a mechanism underlying ZFP36L1 capability to regulate hematopoietic differentiation and suggests a new target for the therapy of hematopoietic diseases involving Stat5b/JAK2 pathway, such as chronic myeloproliferative disorders.
Introduction.
The TTP family of tandem zinc finger proteins includes TTP/ZFP36, TIS11b/ZFP36L1, and TIS11d/ZFP36L2, all of which have been shown to directly bind AU-rich elements (ARE) and promote degradation of the host transcript (Carballo et al., 2000; Ogilvie et al., 2005). Their central RNA-binding domain interacts with AU-rich elements (UUAUUUAUU), whereas the N- and C-terminal domains recruit enzymes involved in the mRNA degradation pathway. Expression of all TTP family genes can be induced by phorbol esters (TPA) and various other mitogenic stimuli, such as growth factors, in a broad variety of cell types (Gomperts et al., 1992; Corps and Brown, 1995). For ZFP36 and in part also for other members, binding to and destabilization of TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and Vegf mRNAs has been demonstrated (Carballo et al., 1998; Lai et al., 1999; Carballo et al., 2000; Ciais et al., 2004). Furthermore, ZFP36 is a transcriptional regulator (Murata et al., 2000; Murata et al., 2002) and mitogens induce its rapid nuclear to cytosolic translocation (Taylor et al., 1995), might be targets of the p38 MAPK pathway (Carballo et al., 2001; Mahtani et al., 2001) and might be involved in the regulation of apoptosis (Johnson et al., 2000; Johnson and Blackwell, 2002). Finally, TTP family members have recently been involved in cell differentiation and cancer. In particular, ZFP36 has been indicated as a critical regulator of dendritic cells maturation (Emmons et al., 2008), it has been demonstrated that targeted disruption of ZFP36L2 results in defective hematopoiesis (Stumpo et al., 2009) and it has been shown that ZFP36 is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis (Brennan et al., 2009).

The Stat5/JAK2 pathway is widely studied. Its aberrant activation is involved in several diseases (Benekli et al., 2003; Choudhary et al., 2007; Kotecha et al., 2008; Wagner and Rui, 2008; Funakoshi-Tago et al., 2009) and particularly its constitutive activation dependent on JAK2 V617F mutation underlies chronic myeloproliferative disorders (Kralovics et al., 2005; Heller et al., 2006; Guglielmelli et al., 2007; Levine and Gilliland, 2008; Grimwade et al., 2009). Conversely, the same pathway is also involved in the control of normal hematopoiesis, since it plays a fundamental role during erythroid maturation (Grebi et al., 2008; Olthof et al., 2008). The Stat5a and Stat5b genes belong to the signal transducer and activator of transcription (Stat) family of transcription factors. The family of Stat genes is believed to originate from successive genome duplication and functional divergence of a single ancestral Stat gene early in vertebrate evolution. A recent duplication of Stat5 is believed to have given rise to the closely related Stat5a and Stat5b homologous genes that encode proteins that in humans are approximately 91% identical, the main difference between the two proteins residing in a five amino acid insertion at the COOH-terminus of Stat5b (Crispi et al., 2004). Stat5a and Stat5b exert redundant yet distinct functions (Basham et al., 2008) both in physiological and pathological conditions; for instance Stat5b and not Stat5a is responsible for inducing an increase of cell motility and invasiveness in hepatocellular carcinoma (Lee et al., 2006). The common aspects and the differences between Stat5a and Stat5b’s behavior depend upon multiple factors such as differences in mRNA levels (Ambrosio et al., 2002), activation by phosphorylation (Moucadel and Constantinescu, 2005) or, as we point out in the present work, a different pattern of tissue specific expression.
The present study has its starting point in the analysis of a gene expression profile of human hematopoietic cell populations (data not shown) that consistently shows that ZFP36L1 expression is substantially low in erythroid progenitors compared to the other hematopoietic cell types. On these premises, the role of ZFP36L1 was evaluated by ectopic expression and gene silencing experiments in human hematopoietic cord blood derived CD34+ stem/progenitor cells and on the erythroleukemia cell line carrying the JAK2 V617F mutation, Hel. Such experiments confirmed that ZFP36L1 expression negatively regulates erythroid maturation and the results were further validated by monitoring the effect of cinnamon derived polyphenols, a drug able to induce the expression of TTP gene family (Schoene et al., 2005; Cao et al., 2007), on the same cell populations. To investigate the mechanisms underlying such biological behavior, we hypothesized that ZFP36L1 might interfere with Stat5/JAK2 pathway, whose activity is pivotal for erythroid differentiation and survival. We demonstrated that ZFP36L1 and also ZFP36 are able to directly bind the 3’-untranslated region of Stat5b mRNA, thereby triggering its degradation. As confirmed by gene silencing experiments targeting Stat5b, its down-regulation is sufficient to inhibit the formation of erythroid precursors from CD34+ human stem/progenitor cells. Moreover, the current study demonstrates that at least two members of the TTP family, ZFP36L1 and ZFP36, are capable of acting redundantly, since their common over-expression results in a cumulative effect on Stat5b down-regulation.
Materials and Methods

Cell cultures and treatments.
U937 cell line was obtained from ATCC (Rockville, MD) and cultured in RPMI1640 medium (Euroclone, Devon, UK), supplemented with 10% heat inactivated fetal bovine serum (Biowhittaker, Walkersville, MD, USA) and 1mM L-Glutamine (Euroclone). Hel cell line was obtained from ATCC and was cultured in IMDM medium (Euroclone) supplemented with 10% heat inactivated fetal bovine serum and 1mM L-Glutamine. GP+envAm12 cells and Human Embryonic Kidney (HEK) 293 cells were cultured in DMEM (Euroclone), supplemented with 10% heat inactivated fetal bovine serum and 1mM L-Glutamine. Ecotropic Phoenix cells were cultured in IMDM medium supplemented with 10% heat inactivated fetal bovine serum and 1mM L-Glutamine. Human CD34+ hematopoietic stem/progenitor cells (HSCs) were purified from umbilical cord blood (CB) samples as already described (Piacibello et al., 1998; Montanari et al., 2005), and seeded (5-10x 10^4/ml) in IMDM (Euroclone) containing 20% heat inactivated fetal bovine serum and early acting human hematopoietic cytokines: 50ng/ml stem cell factor (SCF) and Flt3-ligand (Flt3-l), 10ng/ml interleukin-6 (IL-6) and interleukin-3 (IL-3) (R&D Systems, Minneapolis, MN, USA). These experimental conditions normally promote a mixed granulo-monocyte differentiation of CD34+ cells that is generally achieved within 14 days from plating (Piacibello et al., 1998; Montanari et al., 2005).

For CFC assay CD34+ cells were cultured for 48h prior to transfection with non targeting control siRNA / anti-Stat5b siRNAs or prior to infection with viral supernatants; cells were then plated as already described in a complete methylcellulose medium (MethoCult™ H4434, Stem Cell Technologies, Vancouver, BC, Canada) between 24h and 48h post transfection or 24h after purification of transduced cells. Colonies were scored 14 days after plating as erythroid [burst forming unit-erythroid (BFU-E) + colony forming unit-erythroid (CFU-E)], CFU-GEMM, CFU-GM, CFU-G or GFU-M. Error bars represent SEM calculated on a set of three to four independent experiments (* p value < 0.05).

Human primary myeloblasts, monoblasts, erythroblasts and megakaryoblasts were generated by in vitro culture of cord blood CD34+ HSCs performed as already described (Gemelli et al., 2008).

Cinnamon extracts were kindly provided by Dr. R. A. Anderson; they were resuspended in water and added once to IMDM culture medium / methylcellulose medium at the concentrations indicated in figure legends and medium wasn’t changed until cells were lysed or colonies were scored.

Plasmids, expression constructs and retroviral vectors.
Full length ZFP36 and ZFP36L1 cDNAs were generated by RT-PCR performed on total RNA extracted from U937 cells using ZFP36-DP (5’-ATGGATCTGACTGCCATCTACG-3’) and ZFP36-RP (5’-CGGGCAGTCACCTTTGTCACT-3’), or ZFP36L1-DP (5’-GAACGCACAGGATGCCACCCA-3’) and ZFP36L1-RP (5’-CCTACCTGGCTTAGTCATCGA-3’). Amplification was carried out using FastStart High Fidelity PCR System (Roche Applied Science, Penzberg, Germany) and the
amplified fragments were inserted into the pCR2.1-TOPO T/A cloning vector (Invitrogen, Carlsbad, CA, USA) where they were fully sequenced to exclude polymerase-induced mutations.

ZF3P36 / ZFP36L1 cDNAs were then EcoRI excised and cloned into an EcoRI digested pcDNA3.1 expression vector (Invitrogen) or into an EcoRI digested LxIΔN retroviral vector, resulting in the construction of LZFP36IΔN and LZFP36L1IΔN retroviral vectors.

FlagZFP36 / FlagZFP36L1 fragments were RT-PCR amplified using primers FlagZFP36-DP (5’-ATGGACTACAAGATGACGACGACAAAGGATCTGACTGACTGACCATCTAC-3’) and ZFP36-RP or FlagZFP36L1-DP (5’-ATGGACTACAAGATGACGACGACAAAGACCACCACCACCCTCGTGTCT-3’) and ZFP36L1-RP and the amplified fragments were inserted into the pCR2.1-TOPO T/A cloning vector, from which they were EcoRI excised and cloned into an EcoRI digested pcDNA3.1 vector.

In order to obtain vectors for in vitro transcription and translation, FlagZFP36 / ZFP36L1 RT-PCR amplified fragments were inserted into the pEXP5-CT-TOPO T/A cloning vector (Invitrogen) where they were fully sequenced to exclude polymerase-induced mutations.

Vegfa and Stat5b ARE-containing 3’UTR were amplified by RT-PCR on total RNA extracted from U937 cells using Vegfa-3’UTR-DP (5’-ACAGAGAGACAGGGCAGGAT-3’) and Vegfa-3’UTR-RP (5’-GGAATATCTCGAAAAACTGCAC-3’) or Stat5b-3’UTR-DP (5’-GATGGCCCCAATAACCTTAT-3’) and Stat5b-3’UTR-RP (5’-TCCCTTCAGAGGAGCTTTA-3’). The amplified fragments were then inserted into the pCR2.1-TOPO T/A cloning vector and they were fully sequenced. The 3’UTR fragments were then EcoRI excised, blunted and cloned into a SmaI digested pGL3-Promoter Vector (Promega, Madison, WI, USA) that had previously been modified in order to transfer the multiple cloning region downstream the luciferase reporter gene.

**DNA transfection and retroviral infection.**

Transient transfection of pcDNA3.1-based constructs in Hel cells was obtained using the Amaxa Nucleofector Technology (Lonza Cologne AG, Cologne, Germany). Briefly: 1x10^6 Hel cells were electroporated in 100μl Ingenio Electroporation Solution (Mirus Bio LLC, Madison, WI, USA) containing 5μg of the desired DNA and using T016 electroporation program. Cells were lysed for RNA or protein extraction 72h after electroporation.

U937 cell line and CD34+ HSCs were transduced using LxIΔN empty control vector or LZFP36IΔN / LZFP36L1IΔN retroviral vectors. Packaging lines for the LxIΔN-based constructs were generated by transinfection in the ecotropic Phoenix and amphotropic GP+envAm12 cells, as previously described. Viral titers were assessed by flow cytometry analysis of the expression percentage of the reporter gene ΔLNGFR upon infection of U937 or CD34+ hematopoietic progenitors. Incubation of cells with viral supernatants and purification of transduced cells were performed as previously described (Zanocco-Marani et al., 2006). Transduced U937 / CD34+ HSCs were lysed for RNA extraction between 7 and 10 days after transduction, as indicated in figure legends.
Gene silencing.
Stat5b silencing in HeL cells and in CD34+ cells was obtained by using a mix of two pre-designed siRNA (Cat.#: AM16708; siRNA ID#: 115945 and 3618; Applied Biosystems, Foster City, CA, USA). Electroporation was performed using the Amaxa Nucleofector Technology (Lonza Cologne AG) according to the manufacturer’s instructions. For each cycle, 5-10x10^5 cells were electroporated in 100μl Ingenio Electroporation Solution (Mirus Bio LLC) containing 3μg of each siRNA duplex. Every experiment included a negative control represented by 6μg of a non-targeting siRNA (Dharmacon Inc., Lafayette, CO, USA) in order to exclude non-specific effects of siRNA nucleofection. HeL cells were lysed for protein or RNA extraction 48h after electroporation; CD34+ HSCs were lysed between 4 and 7 days post electroporation, as indicated in figure legends.

Western Blot.
20μg of total protein extracts obtained by cell lysis in RIPA buffer or 2 μl of in vitro translated proteins were loaded onto 10% SDS-polyacrylamide gel and blotted. Membranes were pre-blocked in blocking solution, 5% milk in 0.1% TBST, for 1h at room temperature and then incubated with a 1:200 dilution of mouse monoclonal anti-Stat5b (G-2) Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a 1h incubation at room temperature with goat anti-mouse IgG conjugated to horse-radish peroxidase 1:10000 (sc-2005, Santa Cruz Biotechnology). Expression of actin was analysed with a mouse anti-human pan-actin MoAb (Sigma Aldrich) to normalize protein samples. Detection of in vitro translated proteins was carried out with anti ZFP36 polyclonal antibody (Abcam, ab36558), anti ZFP36L1 polyclonal antibody (Abcam, ab35801) or anti Flag M2 Mouse Monoclonal Ab (Sigma Aldrich, St. Louis, MO, USA). Detection was carried out by using the BM Chemiluminescence Blotting Substrate (Roche Applied Science).

Microarray data analysis
Expression profiles of CD34+ HSCs and CD34-derived erythroblasts, megakaryoblasts, monoblasts and myeloblasts were obtained and analysed as previously described (Gemelli et al., 2008). Stat5a, Stat5b and ZFP36L1 expression levels during in vitro erythroid differentiation of CD34+ hematopoietic progenitor cells were obtained from the GEO-Profiles database (http://www.ncbi.nlm.nih.gov/geo/; GDS2431).

RT-PCR and Real Time PCR.
Total cellular RNA was extracted using “EuroGOLD Total RNA kit” (Euroclone) or “RNeasy micro Kit” (Qiagen GmbH, Hilden, Germany). RNA integrity and concentration was then assessed by the Bio-Analyzer technique (Applied Biosystems). For RT-PCR 3μg total RNA were reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and then expression of specific genes was assessed by polymerase chain reaction amplification performed using specific primers: GAPDH-DP (5’-GAAGGTGAAGGTCGGAGTC-3’), GAPDH-RP (5’-GAAGGCGATGCGGAGCT-3’); ZFP36L1-DP (5’-
GAACGCACAGGATGACCACCA-3'); ZFP36L1-RP (5’-
CCTACCTGGCTTAGCTCATCTGA-3'); Stat5b-DP (5’-
GATGGCCCCAAAAACCTTAT-3'); Stat5b-RP (5’-
TCCCTTCAGAGGAAGGCTTTA-3'); Jak2-DP (5’-GAGCCTATCGGCATGGAATA-
3’); Jak2-RP (5’-TCACCTGAAGGACCACTTCC-3'); IRF8-DP (5’-
ATGTGTGACCCGAATGGTG-3'); IRF8-RP (5’-CCAGACAGAGGGATCCACCAT-
3’). Normalization of the amplified samples was obtained by the glyceraldehyde 3-
phosphate dehydrogenase (GAPDH) housekeeping gene.

For quantitative Real time PCR (QRT-PCR) 100ng total RNA were reverse transcribed
using High Capacity cDNA Archive Kit (Applied Biosystems) according to the
manufacturer’s instructions; QRT-PCR was then performed with an ABI PRISM 7900
sequence detection system (Applied Biosystems) as already described (Zanocco-Marani
et al., 2006). Primers and probes for the different genes’ amplification were provided by
Applied Biosystems; quantitation was performed by amplifying GAPDH mRNA as
endogenous control. Error bars represent SEM calculated on a set of three to four
independent experiments (* p value < 0.05; ** p value < 0.01).

REMSA.
RNA electrophoretic mobility shift assays (REMSAs) were performed on in vitro
transcribed / translated FlagZFP36 and FlagZFP36L1 proteins. Such samples were
produced by using the “Expressway Cell-Free E.Coli Expression System” (Invitrogen)
following the manufacturer's guidelines. Mobility shift reactions were carried out in gel
shift buffer (10mM Hepes pH 7.9, 10mM KCl, 2mM MgCl2, 0.5mM DTT, 10% glycerol,
0.15% NP40) in a total volume of 20μl. RNA probes were synthesized by Eurofins MWG
Operon (Ebersberg, Germany); their sequences are: GMCSF-ARE: 5’-
UAUUUAUUUAUUUAUUUAUUAU-3’ (NM_000758, nt from 695 to 716); Stat5b-
ARE: 5’-AUAGUAAAUUAUUAUUAUGGAAAGAU-3’(NM_012448, nt from 5005
to 5028). Single stranded RNA probes were 5’-end labelled using γ32P-ATP (6000Ci/mmol;
PerkinElmer, Waltham, MA, USA ) and T4 polynucleotide kinase (New England
Biolabs, Ipswich, MA, USA). The binding reactions contained 1.0×105cpm of the
indicated labelled probe and 2μl of in vitro ranscribed / translated protein. Supershifts
were determined by an extra 20' incubation in the presence of 1μg Flag M2 Mouse
Monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA). Reactions were resolved on
a non-denaturing 5% polyacrylamide gel. Electrophoresis was performed at 4°C at 160V
for 3h.

Luciferase Assays.
HEK293 cells were plated at a density of 50.000 cells/well 12h prior to transfection in
24-well plates. Transfections were carried using Lipofectamine 2000 reagent
(Invitrogen). In a typical assay, each well received 200 ng of pGL3-based reporter
construct, 200ng of CMV-βGalactosidase plasmid (Clontech Laboratories Inc., Mountain
View, CA, USA) and 10ng to 20ng of pcDNA3.1 FlagZfp36 or pcDNA3.1 FlagZfp36L1,
as indicated in figure legends; the difference in total DNA transfected was scaled up with
pcDNA3.1 empty vector. After 24-48h cells were harvested and cell lysates were assayed
for luciferase and βGalactosidase activity (Zappavigna et al., 1994). Each transfection
was done in duplicate in the same experiment and the plotted luciferase activities
represent the average of 5 different experiments; error bars represent SEM (\(* \ p \ value < 0.05; \ \ast\ast \ p \ value < 0.01\)).

**Ribonucleoprotein complex immunoprecipitation and analysis by RT–PCR.**
HEK293 cells were co-transfected with pGL3Stat5b-3'UTR and pcDNA3.1-ZFP36L1 plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's guidelines. 24h after transfection ribonucleoprotein complexes were immunoprecipitated following a protocol described in literature (Niranjanakumari et al., 2002). Lysates were immunoprecipitated in the presence of protein A Sepharose (Protein A Sepharose CL-4B, GE Healthcare) previously incubated with anti ZFP36L1 polyclonal antibody (Abcam, ab35801), anti GST polyclonal antibody (Santa Cruz Biotechnology, sc-459), or with no antibody. Immunoprecipitated mRNAs were retro-transcribed using random hexamers and M-MLV reverse transcriptase (Invitrogen). Primers used for subsequent PCR analysis were DP: 5'-TCAAAGAGGCGAAGCTGTGTG-3' and RP: 5'-GCTCCTCAGAGGCAGAGA-3'.
Results.

**ZFP36L1 ectopic expression down regulates Stat5b expression and interferes with erythroid differentiation of human CD34+ cord blood derived stem/progenitor cells.** Based on the main hypothesis that ZFP36L1 could be a negative regulator of erythroid differentiation, we searched the 3'UTR regions of mRNAs encoding proteins involved in such maturation process, in order to find AREII sequences. Among several others not included in the current study, Stat5b appeared to be a suitable candidate, since its 3'UTR carries several canonical and non-canonical AREII sequences. To preliminarily evaluate a possible relation between ZFP36L1 and Stat5b expression, we over-expressed ZFP36L1 in U937 monoblastic and Hel erythroleukemia cell lines and monitored changes in Stat5b expression. The results are shown in figure 1. In particular, panels A and B show the results of over-expression in U937 cells while panels C and D show the results of the same experiment performed on Hel cells. Panel A depicts an RT-PCR showing at the mRNA level that ZFP36L1 over-expression is coupled to a decrease of Stat5b mRNA. Fig.1, panel B depicts the same results analyzed by Real time Quantitative PCR (QRT-PCR), showing that ZFP36L1 over-expression is also coupled to the down regulation of CCL2, a gene previously described as a target of TTP family proteins (Sauer et al., 2006). Fig.1, panel C shows a QRT-PCR showing the extent of ZFP36L1 over-expression obtained in Hel cells. The same panel includes a western blot showing Stat5b protein down regulation following ZFP36L1 over-expression. To gather further information on the effect of ZFP36L1 ectopic expression in Hel cells, we also analyzed by QRT-PCR whether transgene expression is capable of perturbing the expression of a set of erythroid differentiation genes. The results are shown in fig.1D and show that indeed such perturbation occurs. In the same panel TNFα expression, previously described as a TTP family target (Carballo et al., 1997; Johnson and Blackwell, 2002), and β-actin were monitored as positive and negative controls. Consequently to these preliminary experiments, the biological effect of ZFP36L1 over-expression was assessed in CD34+ human cord blood derived stem/progenitor cells. Therefore, CD34+ cells were infected with a recombinant retrovirus allowing ZFP36L1 ectopic expression and then seeded in methylcellulose to evaluate a possible interference with differentiation. The results are shown in fig.1, panel E and show that, compared to the control (empty vector), ZFP36L1 ectopic expression determines a drastic decrease in the formation of erythroid colonies (CFU/BFU-E). In the same primary cell populations we monitored by QRT-PCR the expression of several erythroid differentiation markers (Fig.1, panel F) and observed that expression of several among them (GATA1, LMO2, HbA1 and RhAG) decreases accordingly to the observed suppression of erythroid differentiation.

**ZFP36L1 directly binds Stat5b 3'-UTR and thereby affects its stability.** Fig.2 panel A is a REMSA assay showing that an in vitro translated, Flag-carrying ZFP36L1 protein is able to directly bind to radioactively labelled RNA probes corresponding to the putative AREII regions of Stat5b mRNA (lanes 5-8) and to the AREII regions belonging to GM-CSF mRNA described in literature (lanes 1-4) (Carballo et al., 2000). The REMSA assay also shows Flag antibody dependent super-shift formation (lanes 2 and 6)
and cold probe competition (lanes 3 and 7) for both Stat5b and GM-CSF probes. Fig.2 panel A also includes a western blot performed on the in vitro translated proteins used in the REMSA assay showing that both α-Flag and α-ZFP36L1 antibodies are capable of binding such proteins. The sequences of the probes have been described in the materials and methods section of this paper.

To complete the previous observations, data had to be gathered on the functional meaning of the bond between ZFP36L1 and Stat5b mRNA. Therefore, we performed a luciferase reporter assay, generating luciferase reporter constructs (pGL3 based) allowing transcription of a luciferase mRNA carrying the 3'-UTR of Stat5b or, as a control, the 3'-UTR of Vegfa, a target gene of TTP family (Ciais et al., 2004; Bell et al., 2006; Suswam et al., 2008). The results are shown in fig.2, panel B and show that co-expression of the reporter vectors encoding Stat5b or Vegfa 3'UTRs with a vector expressing ZFP36L1 significantly decreases the basal reporter activity, demonstrating that by directly binding the described mRNAs, ZFP36L1 directly impairs protein production by either promoting mRNA degradation or by inhibiting mRNA translation.

After characterizing the interaction between ZFP36L1 and Stat5b 3'UTR in vitro, next step was to demonstrate that this interaction could also occur in live cells. For this purpose, we transfected HEK293 cells with pcDNA3.1-ZFP36L1 and pGL3-Stat5b-3'UTR plasmids and subsequently subjected them to in vivo crosslinking. ZFP36L1-containing ribonucleoprotein complexes were then immunoprecipitated from cell lysates using a specific anti-ZFP36L1 antibody and the immunoprecipitates were subjected to RT–PCR amplification of the firefly luciferase mRNA. As shown in fig.2 panel C, luciferase mRNA was specifically detected in anti-ZFP36L1 immunoprecipitates and in the input sample, whereas it was not detectable in the control immunoprecipitations (α-GST Ab, no antibody). These results demonstrate that the ZFP36L1–Stat5b 3'UTR interaction occurs not only in reconstituted in vitro systems but also in living cells.

Comparison between the effects of cinnamon derived polyphenols and ZFP36L1 expression on erythroid differentiation. To further validate the data collected so far, the effects of ZFP36L1 ectopic expression were compared to the effects observed on Hel cells and on CD34+ cells following treatment with cinnamon derived polyphenols, that have been described in literature as potent inducers of the genes belonging to the TTP family (Schoene et al., 2005; Cao et al., 2007). The results are shown in fig.3. Panel A shows by QRT-PCR the induction of ZFP36L1 obtained following administration of cinnamon extracts in Hel cells (24h treatment with 0.5 µg/µl cinnamon extracts). Fig.3 panel B shows the subsequent changes in the expression of Stat5b and other erythroid related genes, that are quite comparable to those observed following ZFP36L1 ectopic expression. Panel B also includes, as controls, the expression analysis of the housekeeping gene β-actin, whose expression does not change and of CD44, a marker of monocyte differentiation whose expression increases following cinnamon extracts administration. The upregulation of CD44 is an event we didn’t further investigate on, but could be related to the fact that ZFP family genes are highly expressed in monocytes (ZFP36L1 expression in monoblasts is shown in fig.4A) and are induced by phorbol esters, that are capable of triggering monocyte differentiation. Fig.3, panel C shows cinnamon extracts -driven ZFP36L1 induction in CD34+ cells 24 hours following drug administration (0.25 µg/µl cinnamon extracts). Fig.3 panel D is a western blot of the
same cells showing the decrease in Stat5b expression. In parallel to these experiments, CD34+ cells treated with cinnamon extracts were seeded in methylcellulose. Colony formation was monitored two weeks after plating and, as well as in CD34+ cells ectopically expressing ZFP36L1, a statistically significant decrease in erythroid colony formation was observed (Fig. 3, panel E). Fig.3 panel F depicts the QRT-PCR analysis of the expression of Stat5b and erythroid markers in CD34+ cells treated with cinnamon extracts. Again, the results are in accord with those observed during ZFP36L1 over-expression. Altogether, these experiments show that the biological behavior of cells ectopically expressing ZFP36L1 is essentially superimposable to that of the same cells treated with a drug capable to induce the expression of the genes belonging to the same family.

**Stat5b gene silencing is sufficient to impair erythroid differentiation of human CD34+ cord blood derived stem/progenitor cells.** Since in several contexts Stat5a and Stat5b act redundantly, we evaluated the expression levels of the two genes in the different human hematopoietic cell contexts. Fig.4 panel A is an extract of a microarray analysis previously performed in our laboratory showing the expression levels of Stat5a, Stat5b and ZFP36L1 in human CD34+ stem/progenitor cells and in the various myeloid precursors. Panel A shows that Stat5a and b are expressed at comparable levels in all the contexts analyzed with the exception of erythroblasts, where Stat5a is scarcely represented compared to Stat5b. The same panel shows that ZFP36L1 is scarcely expressed among the different lineages with the exception of monoblasts. To consolidate this observation, we monitored the expression of Stat5a, Stat5b and ZFP36L1 in a gene expression profile deriving from adult CD34+ hematopoietic progenitor cells differentiating toward the erythroid lineage at various time points up to 11 days, published on GEO-Profile database (GDS2431). The results are shown in fig.4B and show that during maturation of human CD34+ progenitors toward the erythroid lineage a downregulation of Stat5a occurs, while high levels of Stat5b are maintained throughout the process. Again, ZFP36L1 remains absent along the erythroid differentiation process. On these grounds, we hypothesized that erythroid maturation of human CD34+ stem/progenitor cells is sustained mainly by Stat5b, rather than Stat5a. Consequently, in order to evaluate whether the decrease of erythroid differentiation that follows ectopic expression of ZFP36L1 relies specifically on the down regulation of Stat5b, we performed gene silencing experiments targeting Stat5b in Hela and CD34+ cells. The results of such experiment are described in fig. 5. Panels A and C are western blots showing that Stat5b expression decreases following siRNA treatment both in Hela and CD34+ cells. Panel B and D show by QRT-PCR the decrease of the expression of erythroid markers and the expression of β-actin as a control in Hela and CD34+ cells respectively following Stat5b gene silencing. Panel E shows the results of a colony assay performed on CD34+ cells treated with siRNA oligonucleotides targeting Stat5b. The assay shows a strong decrease in the formation of erythroid colonies (CFU/BFU-E) in the cell population where Stat5b has been down regulated. This result in particular, mimics the effect of ZFP36L1 over-expression in the same cell population.

**Transcription factor IRF8 is up regulated after ZFP36L1 ectopic expression.** Since published data suggest that Stat5 is able to inhibit dendritic development by directly
suppressing transcription factor IRF8 (Esashi et al., 2008), we evaluated, to further validate our data, whether ZFP36L1-mediated Stat5b down regulation indirectly determines a change in the expression of transcription factor IRF8. The results of such analysis are shown in fig.6. Panel A depicts an RT-PCR showing that indeed IRF8 mRNA increases in U937 cells following ZFP36L1 ectopic expression. Panel B shows the same data analyzed by QRT-PCR, while panel C exhibits the changes in IRF8 expression in Hel cells following treatment with cinnamon extracts. As in the experiments described in fig. 3, cinnamon extracts induce ZFP36L1 expression; fig. 6 panel C shows that an increase in IRF8 expression is associated with Stat5b down regulation. Since the increase of IRF8 expression is observed both after ZFP36L1 ectopic expression and cinnamon extracts administration, it is plausible that, in both cases, this event depends on ZFP36L1-mediated Stat5b down regulation.

**ZFP36 (TTP) behaves comparably to ZFP36L1 and their effect on Stat5b expression is cumulative.** Since ZFP36 and ZFP36L1 share several targets, we evaluated if ZFP36 too is capable of down regulating Stat5b and interfering with hematopoietic differentiation. Fig. 7 panel A is a REMSA assay showing that ZFP36 is able to bind the same AREII regions of GM-CSF and Stat5b mRNA described in fig.2A as targets of ZFP36L1. Panel A also includes a western blot showing that both α-Flag and α-ZFP36 antibodies are capable of binding in vitro translated ZFP36, suggesting that such protein is folded correctly. Fig.7B is a luciferase reporter assay performed by co-transfecting a plasmid expressing ZFP36 and a reporter vector encoding a luciferase mRNA carrying Stat5b 3’-UTR. The results show that ZFP36, as well as ZFP36L1, determines a decrease of luciferase activity by binding Stat5b 3’-UTR. Next we assessed whether ZFP36 is also able to interfere with erythroid differentiation. Therefore, ZFP36 was ectopically expressed in CD34+ human stem/progenitor cells that successively underwent a methylcellulose colony assay. The results are described in fig.7C and show that, compared to the control population, after two weeks a decrease of erythroid colonies (CFU/BFU-E) is visible, and it is super imposable to that observed in CD34+ cells over-expressing ZFP36L1. On these grounds, the hypothesis that the two proteins could act redundantly was tested. To do so, Hel cells were co-transfected with expression plasmids encoding ZFP36 and ZFP36L1 and the effect on Stat5b expression was monitored by western blot. The results are shown in fig.7D and demonstrate that although Stat5b expression decreases after transfection of one or the other mRNA binding protein (lanes 2 and 3), their co-transfection (lane 4) is capable of completely abrogating it. To assess whether the effect on Stat5b depends on a synergistic or redundant behavior, we performed another REMSA that is included in the supplementary material, showing that ZFP36 and ZFP36L1 bind to Stat5b 3’UTR with equal affinity. Such observation, together with published data demonstrating that ZFP36 and ZFP36L1 share the domains that recruit deadenylases and therefore trigger mRNA decay (Lykke-Andersen and Wagner, 2005), suggests that the two proteins are redundant when co-expressed, rather than synergic.
Discussion.

Human hematopoietic differentiation is a strictly controlled process in which regulatory events take place at all the levels of gene expression. Starting from chromatin conformation moving downstream toward the role of transcription factors and microRNAs, a wide amount of data has been gathered so far. With the current study we suggest a novel mechanism to control hematopoietic differentiation based on the activity of mRNA binding proteins whose role, to date, was mostly related to the control of the inflammatory response (Sandler and Stoecklin, 2008; Schaljo et al., 2009). In our hypothesis, such proteins act by repressing the development of a specific blood lineage, namely the erythroid, rather than by promoting the differentiation of specific progenitors and precursors as classically described in many studies that focus on the role of transcription factors.

Starting from the analysis of a gene expression database showing that ZFP36L1 is differentially expressed in the different human blood cell contexts and, particularly, that its expression is consistently low in the erythroid compared to the other lineages (data not shown), we hypothesized that this protein and other members of the same family could play a role during hematopoiesis. By this study we demonstrated that ZFP36L1 and ZFP36 inhibit erythroid development of CD34+ human cord blood derived stem/progenitor cells by inhibiting proliferation of erythroid precursors and suggest that this behavior depends on ZFP36L1-mediated Stat5b mRNA degradation. Moreover, we demonstrated that ZFP36L1 and ZFP36 share Stat5b as a common target and are able to directly bind its mRNA on a canonical AREII sequence located in the 3'-UTR. The inhibition of erythroid colonies formation observed following ZFP36L1 and ZFP36 ectopic expression is statistically significant and it’s coupled to a decrease of precursors’ proliferation rather than to apoptosis or differentiation arrest (see supplementary material). To associate such observation with the sole down regulation of Stat5b was not an obvious step, since this gene is highly homologous to Stat5a and in murine erythropoiesis the proteins Stat5a and Stat5b are capable to some degree of substituting each other (Socolovsky et al., 2001). Therefore, we monitored the expression of Stat5a and Stat5b in human myeloid cell contexts, at different time points during human erythroid differentiation and then performed gene-silencing experiments targeting Stat5b. The expression analysis revealed that, unlike in murine erythropoiesis, during erythroid differentiation of human CD34+ stem/progenitor cells a down-regulation of Stat5a expression occurs, while Stat5b expression persists at high levels (Fig.4). Accordingly, the gene silencing approach showed that Stat5b down-regulation is sufficient to impair erythroid maturation. It remains to be elucidated whether the different expression pattern of Stat5a and Stat5b in human erythropoiesis depends upon transcriptional or post-transcriptional events. The presented results show that the idea underneath is correct, although a more complete hypothesis that rises from the present study suggests that ZFP36L1, ZFP36 and possibly other members of the same family could have among their targets other members of the Stat5b/JAK2 signal transduction pathway that have not been described in the experiments presented here.

The relation between ZFP36L1 and Stat5b is also indirectly described in the experiment (Fig. 6) showing that ZFP36L1 ectopic expression results in the increase of transcription factor IRF8. Such gene is involved at different levels in hematopoietic differentiation as a promoter of specific myeloid and lymphoid lineages (Qi et al., 2009; Saberwal et al.,
but not the erythroid one, it has been described for being negatively regulated by Stat5 (Esashi et al., 2008) and is capable of acting as a pro-apoptotic factor (Yang et al., 2007; Yang et al., 2009). The ability of ZFP36L1 to down-regulate Stat5b thereby promoting the expression of the pro-apoptotic factor IRF8 suggests that this gene family encodes proteins that in specific contexts are able to function as tumor suppressors. Such observation is strengthened by a recently published report that shows that TTP family members are down-regulated in different tumors (Brennan et al., 2009).

Another relevant aspect of the present work resides in the fact that drugs capable of inducing the expression of TTP family genes, such as cinnamon derived polyphenols, do in fact down regulate Stat5b expression and exhibit a very strong influence on erythroid maturation of human CD34+ stem/progenitor cells. The effect of cinnamon polyphenols is observed even at low concentrations of drug (0.25 µg/µl) and this efficiency could depend on the fact that they induce expression of both ZFP36 and ZFP36L1 that, as demonstrated by the experiment shown in fig.7D, share the ability to bind Stat5b mRNA and therefore cooperate in promoting its degradation.

The present study establishes a relation between the TTP family of genes and the Stat5b/JAK2 pathway. Alterations in the genes belonging to such pathway, and precisely the JAK2 V617F mutation that leads to constitutive signal transduction, underlie chronic myeloproliferative disorders (Baxter et al., 2005; Kralovics et al., 2005; Levine and Gilliland, 2008) like polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Today there is considerable optimism that these diseases can be successfully treated with tyrosine kinase inhibitors. In fact specific inhibitors of JAK2 kinase activity have been designed (Gozgit et al., 2008; Hexner et al., 2008; Wernig et al., 2008) and have entered the clinic in Phase I/II trials in PMF and post-PV/ET myelofibrosis. Given the central role of JAK2 signalling to a myriad of cellular processes, there may be significant toxicities associated with JAK2 inhibition, and "off-target" inhibition of JAK1, JAK3, or TYK2 might lead to hematologic, immunologic, and endocrine side effects. Such toxicities could likely preclude their prolonged use; consequently, it is possible that alternative or synergic therapies will have to be developed. On these premises and on the ground of the results presented in this work, we hypothesize an approach for PV, characterized by an increase in absolute quantity of erythroid cells, based on the utilization of drugs that induce TTP gene family expression and that do not present the toxicity problems suggested above, determined by JAK2 inhibitors. TTP-inducing drugs would determine Stat5b down regulation and would thereby allow decreasing the activity of JAK2-Stat5 pathway without interfering with the activity of other necessary, un-mutated tyrosine kinases such as JAK1, JAK3 or TYK2 and others. Such an approach would not likely replace JAK2 inhibitors, but a combined strategy could possibly be useful to lower the dosage of tyrosine kinase inhibitors and therefore limit their toxicity.

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References.


Figure Legends

Figure 1. Overexpression of ZFP36L1 negatively regulates Stat5b and impairs erythroid differentiation of CD34+ HSCs. (A) RT-PCR showing the expression levels of ZFP36L1, Stat5b and Jak2 in U937 cells transduced with empty vector (lane 1) or with ZFP36L1-overexpressing vector (lane 2); RNAs were extracted 7 days post transduction. (B) Real time PCR performed on U937 cells transduced with ZFP36L1-overexpressing vector showing that overexpression of ZFP36L1 downregulates the expression levels of Stat5b; CCL2 was used as a positive control. Data are provided in terms of Log2 of relative quantity compared to the expression levels of the same genes in U937 cells transduced with empty vector; error bars represent SEM calculated on a set of 3 independent experiments (*p<0.05). (C) Left: Real time PCR demonstrating the expression levels obtained in Hel cells via transduction with ZFP36L1-overexpressing vector as compared to cells transduced with empty vector; error bar represents SEM calculated on 3 independent experiments (**p<0.01); RNAs were extracted 72h post transduction. Right: Western blot analysis of Stat5b expression in Hel cells transduced with empty vector (lane 1) or with ZFP36L1-expressing vector (lane 2); cells were lysed 72h post transduction. (D) Real time PCR showing that Hel cells overexpressing ZFP36L1 (grey bars) have reduced levels of Stat5b and of the erythroid markers GATA1, LMO2, RhAG, HbA1 as compared to cells transduced with empty vector (white bars); TNFα was used as a positive control and ACTB was used as control housekeeping gene; error bars represent SEM calculated on 3 independent experiments (*p<0.05; **p<0.01). RNAs were extracted 72h post transduction. (E) Clonogenic assay performed on CD34+ HSCs transduced with empty vector (left) or overexpressing ZFP36L1 (right); error bars represent SEM calculated on 4 independent experiments (*p<0.05). (F) Real time PCR showing that CD34+ HSCs overexpressing ZFP36L1 (black bars) have reduced levels of Stat5b and of the erythroid markers GATA1, LMO2, RhAG, HbA1 as compared to cells transduced with empty vector (white bars); TNFα was used as a positive control and ACTB as control housekeeping gene (SEM calculated on 3 experiments; *p<0.05); RNAs were extracted 10 days after transduction.
Figure 2. ZFP36L1 binding to AU-rich-elements (ARE) in the 3’UTR of Stat5b mRNA confers instability to mRNAs containing such elements. (A) Left: Western blot demonstrating identity and integrity of in vitro translated FlagZFP36L1 protein: immunoblotting was performed with anti Flag or anti ZFP36L1 antibody as indicated. Right: RNA mobility shift assay performed by incubating in vitro translated FlagZFP36L1 protein with labelled RNA probes corresponding to ARE in the 3’UTR of GM-CSF mRNA (used as a positive control, lanes 1-4) or to ARE in the 3’UTR of Stat5b mRNA (lanes 5-8). Probe sequences are (ARE in bold): GM-CSF: 5’-UAUUUAUUUAUUUAUUUAUUUA-3’; Stat5b: 5’-AUAGUAUAUUUAUUUAUGGAAGAU-3’. Supershifts were obtained by an additional incubation with anti Flag Ab (lanes 2 and 6). Competition experiments were performed with cold Stat5b probe (lane 3) or with cold GM-CSF probe (lane 7). Lanes 4 and 8 represent labelled probes incubated with an in vitro translation reaction mix performed on empty vector. (B) Luciferase activity assay performed in HEK293 cells transfected with pcDNA3.1 empty expression vector or with pcDNA3.1 overexpressing FlagZFP36L1 (20ng) together with pGL3 reporter construct encoding for a luciferase gene fused to the 3’UTR of Vegfa (positive control) or to the 3’UTR of Stat5b. Luciferase activity is represented in terms of fold change; error bars represent SEM calculated on a set of 5 independent experiments. (C) Ribonucleoprotein complexes immunoprecipitation assay demonstrating that binding of ZFP36L1 protein to the ARE in the 3’UTR of Stat5b mRNA occurs in vivo: ribonucleoprotein complexes were immunoprecipitated from lysates of HEK293 cells transfected with pcDNA3.1 ZFP36L1 and pGL3 3’UTR Stat5b vectors, RNA was extracted, reverse transcribed and amplified by PCR using primers specific for Stat5b 3’UTR fused to the luciferase gene. Lanes 1 and 2 represent negative and positive control respectively, i.e. PCR amplification performed on no template or on RNA extracted from cell lysates prior to immunoprecipitation; lane 3 shows specific immunoprecipitation obtained with anti ZFP36L1 antibody; lanes 4 and 5 are control immunoprecipitations performed with non-specific antibody or with no antibody.
**Figure 3.** Treatment with cinnamon extracts upregulates endogenous expression of ZFP36L1, downregulates Stat5b levels and inhibits erythroid differentiation of CD34+ HSCs.

(A) Real time PCR showing upregulation of ZFP36L1 following treatment of Hel cells with 0.5 µg/µl cinnamon extracts for 24h (SEM calculated on 3 experiments). (B) Real time PCR performed on Hel cells treated with cinnamon extracts showing decreased expression of Stat5b and of the erythroid markers GATA1, LMO2, HbA1 and RhAG; CD44 monocyte differentiation marker expression is increased after treatment with cinnamon extracts; ACTB was used as control housekeeping gene. Results are provided in terms of Log2 of relative quantity compared to the expression levels of the same genes in untreated Hel cells; error bars represent SEM calculated on a set of 3 independent experiments (*p<0.05; **p<0.01). (C) Real time PCR showing upregulation of ZFP36L1 following treatment of CD34+ HSCs in liquid culture with 0.25 µg/µl cinnamon extracts for 24h (SEM calculated on 3 experiments). (D) Western blot analysis of Stat5b levels in untreated CD34+ HSCs (lane 1) and in the same cells treated with 0.25 µg/µl for 48h (lane 2). (E) Clonogenic assay performed on CD34+ HSCs in the absence (left) on in the presence (right) of cinnamon extracts. Cinnamon extracts were added directly to methylcellulose medium at a concentration of 0.25 µg/µl (SEM calculated on 3 independent experiments; *p<0.05). (F) Real time PCR showing downregulation of Stat5b and erythroid markers in CD34+ HSCs treated with 0.25 µg/µl cinnamon extracts in liquid culture medium for 48h; CD44 expression is upregulated after treatment with cinnamon extracts; ACTB was used as control housekeeping gene. Data are provided in terms of Log2 of relative quantity compared to the expression levels of the same genes in untreated CD34+ HSCs; error bars represent SEM calculated on a set of 3 independent experiments (*p<0.05; **p<0.01).

**Figure 4.** Stat5a, Stat5b and ZFP36L1 expression in hemopoietic progenitors / precursors and during erythroid differentiation. (A) Microarray analysis results showing Stat5a, Stat5b and ZFP36L1 expression levels in primary hemopoietic cell contexts: normal cord
blood CD34+ HSCs, CD34-derived erythroblasts, megakaryoblasts, monoblasts and myeloblasts. (B) Microarray analysis showing the expression levels of Stat5a, Stat5b and ZFP36L1 during in vitro erythroid differentiation of adult CD34+ hematopoietic progenitor cells at various time points in serum-free medium containing erythropoietin, interleukin-3 and stem cell factor (data from GEO Profiles database: GDS2431).

**Figure 5.** Stat5b silencing is sufficient to downregulate the expression of erythroid markers and to impair erythroid differentiation of CD34+ HSCs. (A) Western blot analysis showing the downregulation of Stat5b obtained in Hel cells transfected with anti Stat5b siRNAs (lane 2) as compared to cells transfected with a non-targeting control siRNA (lane 1); cells were lysed 48h post transfection. (B) Real time PCR performed on Hel cells transfected with control siRNA (white bars) or with anti Stat5b siRNAs (grey bars) demonstrating the efficacy of Stat5b silencing and the downregulation of erythroid markers; ACTB was used as control housekeeping gene (error bars represent SEM calculated on a set of 3 experiments; *p<0.05; **p<0.01). RNAs were extracted 48h post transfection with siRNAs. (C) Western blot analysis showing the downregulation of Stat5b obtained in CD34+ HSCs transfected with anti Stat5b siRNAs (lane 2) as compared to cells transfected with a non-targeting control siRNA (lane 1); cells were lysed 7 days post transfection. (D) Real time PCR performed on CD34+ HSCs transfected with control siRNA (white bars) or with anti Stat5b siRNAs (black bars) demonstrating the efficacy of Stat5b silencing and the downregulation of erythroid markers; ACTB was used as control housekeeping gene (error bars represent SEM calculated on a set of 3 experiments; *p<0.05; **p<0.01). RNAs were extracted 96h post transfection with siRNAs. (E) Clonogenic assay performed on CD34+ HSCs transfected with a non-targeting control siRNA (left) or with anti Stat5b siRNAs (right); SEM calculated on 3 independent experiments; *p<0.05.

**Figure 6.** Increased levels of ZFP36L1 result in upregulation of IRF8 gene. (A) RTPCR showing increased expression of IRF8 in U937 cells transduced with ZFP36L1-overexpressing vector (lane 2) as compared to cells transduced with empty vector (lane 1); RNAs were extracted 7 days post transfection. (B) Real time PCR showing IRF8
upregulation following transduction of U937 cells with ZFP36L1-expressing vector (right); data are provided in terms of relative quantity as compared to cells transduced with empty vector (left); error bars represent SEM calculated on a set of 4 independent experiments; **p<0.01. (C) Real time PCR showing that treatment of Hel cells with 0.5 µg/µl cinnamon extracts for 48h results in upregulation of ZFP36L1, downregulation of Stat5b and upregulation of IRF8 (SEM calculated on 3 different experiments).

Figure 7. ZFP36 behaves similarly to ZFP36L1 in binding to and destabilizing mRNAs spanning Stat5b 3’UTR and in inhibiting erythroid differentiation of CD34+ HSCs. (A) Left: Western blot demonstrating identity and integrity of in vitro translated FlagZFP36 protein: immunoblotting was performed with anti Flag or anti ZFP36 antibody as indicated. Right: RNA mobility shift assay performed by incubating in vitro translated FlagZFP36 protein with labelled RNA probes spanning the ARE in the 3’UTR of GM-CSF mRNA (used as a positive control, lanes 1-4) or the ARE in the 3’UTR of Stat5b mRNA (lanes 5-8). Supershifts were obtained by an additional incubation with anti Flag Ab (lanes 2 and 6). Competition experiments were performed with cold Stat5b probe (lane 3) or with cold GM-CSF probe (lane 7). Lanes 4 and 8 represent labelled probes incubated with an in vitro translation reaction mix performed on empty vector. (B) Luciferase activity assay performed in HEK293 cells transfected with pcDNA3.1 empty expression vector (left) or with pcDNA3.1 overexpressing FlagZFP36 (10ng; right) together with pGL3 reporter construct encoding for a luciferase gene fused to the 3’UTR of Stat5b. Luciferase activity is represented in terms of fold change; error bars represent SEM calculated on a set of 5 independent experiments. (C) Clonogenic assay performed on CD34+ HSCs transduced with empty vector (left) or overexpressing ZFP36 (right); error bars represent SEM calculated on 4 independent experiments (*p<0.05). (D) Western blot analysis showing Stat5b levels in Hel cells transfected with empty vector (lane 1), with ZFP36-overexpressing vector (lane 2), with ZFP36L1-overexpressing vector (lane 3) or transfected with both overexpressing vectors (lane 4). Cells were lysed 72h post transfection.