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Hls5 REGULATES ERYTHROID DIFFERENTIATION BY MODULATING GATA-1 ACTIVITY

Short title: Hls5 modulates GATA-1 activity

Raelene Endersby^{1,2}, Ian J. Majewski^{1,3}, Louise Winteringham¹, Jennifer G Beaumont¹, Amy Samuels¹, Robin Scaife¹, Esther Lim¹, Merlin Crossley⁴, S Peter Klinken¹ and Jean-Philippe Lalonde¹

¹Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Centre for Medical Research, The University of Western Australia Crawley, Australia

²Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN, 38105

³Division of Molecular Medicine, Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville VIC 3052, Australia.

⁴School of Molecular and Microbial Biosciences, G08, University of Sydney, Sydney, NSW 2006, Australia

Corresponding Author:

S Peter Klinken

Western Australian Institute for Medical Research

Level 6, Medical Research Foundation Building

Rear, 50 Murray Street

PERTH WA 6000

AUSTRALIA

Telephone: +61-8-9224.0334

Facsimile: +61-8-9224.0322

Email: pklinken@waimr.uwa.edu.au

Abstract

Hemopoietic Lineage Switch (Hls) 5 and 7 were originally isolated as genes up-regulated during an erythroid to myeloid lineage switch. We have shown previously that Hls7/Mlf1 imposes a monoblastoid phenotype upon erythroleukemic cells. Here we show that Hls5 impedes erythroid maturation by restricting proliferation and inhibiting hemoglobin synthesis; however, Hls5 does not influence the morphology of erythroid cells. Under the influence of GATA-1, Hls5 relocates from cytoplasmic granules to the nucleus where it associates with both FOG-1 and GATA-1. In the nucleus, Hls5 is able to suppress GATA-1 mediated transactivation and reduce GATA-1 binding to DNA. We conclude that Hls5 and Hls7/Mlf1 act co-operatively to induce biochemical and phenotypic changes associated with erythroid/myeloid lineage switching.

Introduction

The erythroblastoid J2E cell line responds to erythropoietin (Epo) by morphological maturation and hemoglobin synthesis.¹ However, on rare occasions, these cells have undergone a spontaneous lineage switch, and display features of monoblastoid cells which do not respond to Epo.² Thus, regulated expression of structural/functional genes involved in commitment to the erythroid lineage can be breached, as previously demonstrated with B cell to macrophage lineage switching.³

Hls5 and Hls7 were isolated as genes markedly up-regulated in the J2E monoblastoid variants.^{2,4} Hls7 is the murine orthologue of Myeloid Leukemia Factor 1 (Mlf1), a gene involved in the t(3;5), associated with acute myeloid leukemia.⁵ Importantly, ectopic expression of Hls7/Mlf1 in J2E cells imposes a dramatic phenotypic change upon the cells, rendering them monoblastoid.² Hls5 is a recently identified member of the RING finger, B Box, Coiled Coil (RBCC)⁴ or Tripartite motif (TRIM) family,⁶ which includes PML, a gene involved in acute promyelocytic leukemia. Hls5 is expressed in a wide variety of hemopoietic cell types, including fetal liver progenitors.⁴ The role of Hls5 in erythroid maturation was investigated in this study.

Materials and Methods

Cell lines were cultured +/- Epo, as described previously.^{1,2} Assays for differentiation, cell cycle progression, clonogenicity, confocal microscopy, cytocentrifugation and staining have been detailed elsewhere^{1,2,4}, as have methylcellulose colony assays.^{1,2,7} Immunoblotting, immunoprecipitation and *in vitro* pull down assays were used as reported previously.^{1,2} Yeast 2 hybrid analyses were described by Ingley et al⁷, and electrophoretic mobility shift assays by Spadaccini et al.⁸ The M1 α construct was used to determine GATA-1 activity⁹, while chromatin immunoprecipitation experiments utilised primers for the β^{maj} globin promoter and HS2 regions of the β -globin locus¹⁰.

Results and Discussion

To determine the effects of elevated Hls5 on normal erythroid progenitors, fetal liver cells were infected with retroviruses expressing wild-type Hls5, myc-tagged Hls5 or empty vector control. Methylcellulose assays revealed that both Burst

Forming Units-Erythroid (BFU-E) and Colony Forming Units-Erythroid (CFU-E) were suppressed between 40 and 70% by Hls5; in contrast, Hls5 increased myeloid colony formation (Fig 1A). Thus, elevated Hls5 levels inhibit the development and maturation of erythroid progenitors, which could be due to cell death, as over-expression of Hls5 can induce apoptosis⁴.

To examine the effects of Hls5 on immature erythroid cells in greater detail, J2E cells¹ were infected with vector alone, or Hls5-expressing retroviruses and termed J-Hls5 or J-Hls5-myc (Fig 1B). Confocal microscopy showed that Hls5 was present primarily in granular structures throughout the cytoplasm, a feature characteristic of RBCC/TRIM family members;⁶ however, the protein was also detected in nuclear structures (Fig 1C).

Enforced expression of Hls5 did not alter the erythroblastoid morphology phenotype of the cells (Fig 1D). This observation contrasts markedly with J2E cells expressing Hls7/Mlf1, which appeared much larger and monoblastoid.² It was concluded that, unlike Hls7/Mlf1, Hls5 does not primarily affect structural genes which control cellular morphology. It is noteworthy that Hls5 and Hls7/Mlf1 do not induce expression of each other (data not shown). Increasing Hls5 levels in J2E cells induced a slight reduction in clonogenicity (Fig 1E) and cell growth (Fig 1F), which could be attributed to reduced transit through G₁/S of the cell cycle (Fig 1G). This is consistent with our previous observations that elevated Hls5 perturbs cell cycle progression in transiently-transfected COS and HeLa cells.⁴

Strikingly, Epo-induced hemoglobin synthesis was almost completely abolished in Hls5-expressing cells (Fig 1H). Globin and GATA-1 transcripts were reduced in these cells (Fig 1I), as were protein levels (Fig 1J). Hls5, therefore, had a significant effect on globin synthesis and hemoglobin production, which could be caused, in part, by reduced GATA-1 levels. However, the elimination of globin proteins indicates that post-transcriptional factors, also influence globin content in J-Hls5 cells (Fig 1J).

Although the Hls5-expressing cells were unable to manufacture hemoglobin, they were still able to mature morphologically, when induced to differentiate⁷ (Fig

1K). The cells progressed beyond the proerythroblast/basophilic erythroblast boundary displaying decreased cell volume, nuclear condensation and even nuclear extrusion. Elevated Hls5, therefore, inhibited hemoglobin synthesis, but not morphological maturation. Similarly, suppression of the transcription factor EKLF has been shown to reduce hemoglobin production, but not interfere with morphological differentiation.⁸ From these experiments it was concluded that the effects of Hls5 and Hls7/Mlf1 on erythroid differentiation are quite distinct – while Hls7/Mlf1 mainly affects cellular morphology, Hls5 influences biochemical aspects of erythroid differentiation such as globin production.

A yeast two-hybrid screen was conducted to identify partner proteins which may elucidate the function of Hls5 in erythroid cells. FOG-1 was isolated in this screen as an Hls5-interacting protein. The FOG-1 clones which associated with Hls5 comprised amino acids 817 to 980, a region spanning FOG-1's seventh and eighth zinc fingers (Fig 2A). Figure 2B demonstrates that FOG-1 bound to full length Hls5, but not to truncation mutants lacking the B Box; thus the B Box of Hls5 is crucial for the interaction with FOG-1. Hls5 also associated with FOG-1 in mammalian cells, as demonstrated by co-immunoprecipitation (Fig 2C). The specific interaction of Hls5 with zinc fingers 7 and 8 of FOG-1 is interesting, as other fingers (1, 5, 6 and 9), but not 7 and 8, associate with GATA factors.⁹

It has recently been shown that zinc finger 3 of FOG-1 binds TACC3, a protein that also contains a Coiled Coil domain.¹² As FOG-1 is a transcriptional co-factor for GATA-1,¹³ and TACC3 inhibits the function of FOG-1,¹² the impact of Hls5 on GATA-1 activity was investigated. Data presented in Fig 2D show that Hls5 modestly enhanced the FOG-1 repression of the GATA-1 activity. Together, these data indicate that Hls5 associates with FOG-1 and affects GATA-1 transactivation.

A possible interplay between Hls5 and GATA-1, in the absence of FOG-1, was then explored. Interestingly, GATA-1 imposed a marked relocation of Hls5 from cytoplasmic granules⁴ into the nucleus, where it co-localized with GATA-1 (Fig 2E); furthermore, GATA-1 co-immunoprecipitated with Hls5 (Fig 2F). Yeast two-hybrid experiments showed that this interaction occurred in the absence of FOG-1, and deletion mutant analysis revealed that the B Box, Coiled Coil domain of

Hls5 associated primarily with the N-terminal finger of GATA-1 (Fig 2G, H). Significantly, Hls5 was able to repress GATA-1 transcriptional activity (Fig 2I), and interfere with GATA-1 binding to DNA (Fig 2J), in a dose-dependent manner. Deletion of the B Box, Coiled Coil domain abrogated the Hls5 inhibition of GATA-1 transactivation (data not shown). While these data indicate Hls5 can modulate GATA-1 transactivation independently, it is likely that Hls5 forms a complex with FOG-1 and GATA-1. The B Box, Coiled Coil domain of Hls5 appears crucial for these interactions which influence GATA-1 binding to DNA. As numerous GATA-1 partners have been identified,¹⁴ it is possible that different pools of GATA-1 exist which interact with specific proteins to influence expression of discrete sub-sets of genes.

Chromatin immunoprecipitation assays revealed a significant reduction in GATA-1 binding to the β globin locus of Hls5-expressing cells (Fig 2K); consequently, J-Hls5 cells had decreased transcripts for GATA-1 target genes, α and β globin (Fig 1I). Thus, Hls5 modulates GATA-1 by reducing mRNA and protein levels (Fig 1I, 1J) and by influencing DNA binding / transactivation (Fig 2I, 2J, 2K). However, some GATA-1 responsive genes (FOG-1 and Epo-receptor) were not affected by the increased Hls5 levels. These data suggest either concentration – dependent effects of GATA-1 on transcription of different genes, or site-specific effects of Hls5 on distinct GATA-1 elements.

The biological consequences of elevated Hls5 in erythroid cells, viz severely impaired globin synthesis and restricted cell growth (Fig 1F-1I), are consistent with Hls5 inhibiting the activity of GATA-1, since GATA-1 regulates globin gene transcription and cell cycle progression.^{15,16} Hls5 may, therefore, play an important role in the maturation of red blood cells by modulating GATA-1 activity and expression of lineage-restricted functional genes^{9,12-14,17}. The presence of numerous GATA binding sites in the Hls5 promoter,⁴ adds another potential layer of cross-regulation between these molecules. Interestingly, other RBCC/TRIM family members (Herf1 and TIF1 γ) have been implicated in transcriptional regulation in erythroid cells.^{18,19} Moreover, another family member PML has been shown to associate with, and influence the activity of, GATA-2.²⁰ These observations suggest that RBCC/TRIM proteins represent a class of molecules which can modulate the activity of GATA factors.

Hls5 was originally isolated as a gene up-regulated during an erythroid to myeloid lineage switch,⁴ and independently as a pro-apoptotic gene activated during macrophage maturation.²¹ The suppression of GATA-1 activity by Hls5 could potentially promote maturation along the myeloid pathway, as inhibition of GATA-1 favours myelopoiesis over erythropoiesis.²² Collectively, these data suggest that during the J2E erythroid / myeloid lineage switch spontaneous activation of Hls5 suppresses globin synthesis, while Hls7/Mlf1 contributes to the altered morphology of the cells. We postulate that Hls5 and Hls7/Mlf1 act cooperatively to promote this lineage transition. These data add to the body of knowledge building on the genes involved in lineage switching.^{2,3,23-25}

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Contribution: RE, IJM and J-PL designed and performed experiments, analyzed data and contributed to writing the manuscript. LW, JGB, AS, RS and EL designed and undertook experiments, while MC and SPK supported the research, designed experiments, analyzed data and contributed to writing the manuscript.

Conflict of Interest Disclosure: The authors declare no competing financial interests for this project.

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Figure Legends

Figure. 1. Effect of Hls5 on erythroid differentiation. (A) Fetal liver progenitors were exposed to Hls5 or Hls5-myc retroviruses, then plated in methylcellulose in the presence of Epo (5 U/ml); CFU-E and BFU-E were determined after 2 and 7 days, respectively. CFU-M were determined separately after 7 days. Each result is the mean \pm SD ($n = 3$) and displayed as percentage of control cultures. (B) Immunoblot analysis of exogenous Hls5 in J-Hls5 / J-Hls5-myc clones, probed with antibodies against Hls5 and Erk2 (loading control). (C) J-Hls5-myc cells were exposed to Epo (5U/ml) and 48h post-induction Hls5 protein was visualized using anti-myc antibodies and confocal microscopy. DNA was identified by Hoescht staining. (D) Wright-stained cytocentrifuge preparations of J2E and J-Hls5 cells. (Bar represents 20 μ m) (E) The clonogenicity of various J2E and J-Hls5 clones was determined by plating cells in soft agar. Each result is the mean \pm SD ($n = 3$). (F) Growth rate of various J2E and J-Hls5 clones was determined by MTT assays. Each result is the mean \pm SD ($n = 3$). (G) J2E (blue trace) and J-Hls5 (red trace) clones were released from a nocodazole-induced G₂/M block, and at the times indicated cell cycle progression was assayed by propidium iodide staining and visualized by flow cytometry. (H) Hemoglobin production of J2E and J-Hls5 cells following Epo induction was determined by benzidine staining. Each result is the mean \pm SD ($n=3$). (I) Quantitative RT-PCR was performed on J2E cells expressing vector-alone, Hls5 or Hls5-myc. Transcript levels are displayed relative to β -actin (loading control). Each bar represents the mean of 3 independent assays performed in triplicate. (J) Immunoblot (IB) analysis of J2E (vector alone) and J-Hls5 clones during Epo-induced erythroid differentiation, probed with antibodies against globins, GATA-1 and v-raf (loading control). GATA-1 levels (relative to v-raf) were quantitated and represented as a percentage of the GATA-1 (vector-alone) value at 0h. (K) Wright-stained cytocentrifuge preparations of J-Hls5 clones during erythroid terminal differentiation. (Bar represents 20 μ m).

Figure. 2. Effect of Hls5 on FOG-1 and GATA-1. (A) Schematic representation of Hls5 and FOG-1 with various domains displayed. The boxed area of FOG-1 bound Hls5 in the yeast two-hybrid screen. (B) Hls5 bound FOG-1 through its B Box, Coiled Coil domains, as determined by yeast 2 hybrid analysis. (C) Hls5 bound full length FOG-1 in *in vitro* pull down assays. COS cells were transfected

with Flag-tagged FOG-1 and lysates were immunoprecipitated with anti-Flag antibodies in the presence of [³⁵S]-labelled Hls5. **(D)** COS cells were transfected M1 α reporter together with various combinations of Vector, GATA-1, FOG-1 and Hls5 (200 ng each). Constant DNA levels were maintained by varying the amount of pcDNA3; Hls5 did not affect expression of GATA-1 or FOG-1 in these transient transfection experiments. Luciferase activity was determined using the Dual-Luciferase system (Promega) after 36 h, normalized to the activity of a Renilla reporter plasmid (pRL-TK) and are shown relative to GATA-1 activation (100). Error bars represent \pm SD (n=3). **(E)** COS cells were transiently transfected with Hls5 alone (upper panel), or Hls5 and GATA-1 together (lower panel). Hls5 and GATA-1 were visualized using anti-myc and anti-GATA-1 antibodies, and confocal microscopy. DNA was identified by Hoescht staining. **(F)** The GATA-1 and Hls5 interaction was demonstrated by transfecting COS cells with myc-tagged Hls5 and GATA-1, followed by immunoprecipitation (IP) and immunoblotting (IB). Vertical lines have been inserted to indicate a repositioned gel lane. **(G)** Yeast two-hybrid analyses demonstrated that the B Box, Coiled Coil domains of Hls5 were required for GATA-1 binding. **(H)** Yeast 2-hybrid assays showed that the GATA-1 N-terminal finger domain was required for binding to Hls5. **(I)** Hls5 alone (200-500 ng) was able to repress GATA-1 transcriptional activity of the M1 α promoter, as described in panel D. **(J)** Electrophoretic mobility shift assay using a radiolabelled GATA-1 oligonucleotide and J2E cell nuclear extracts revealed a GATA-1/DNA complex, which decreased with increasing amounts of purified Hls5. **(K)** Chromatin immunoprecipitation assay of GATA-1 recruitment to the β globin promoter in J2E (vector-alone) and J-Hls5 cells. GATA-1 binding, relative to input DNA, was expressed as a percentage of J2E (vector-alone) cells. Similar data were obtained for the HS2 site.

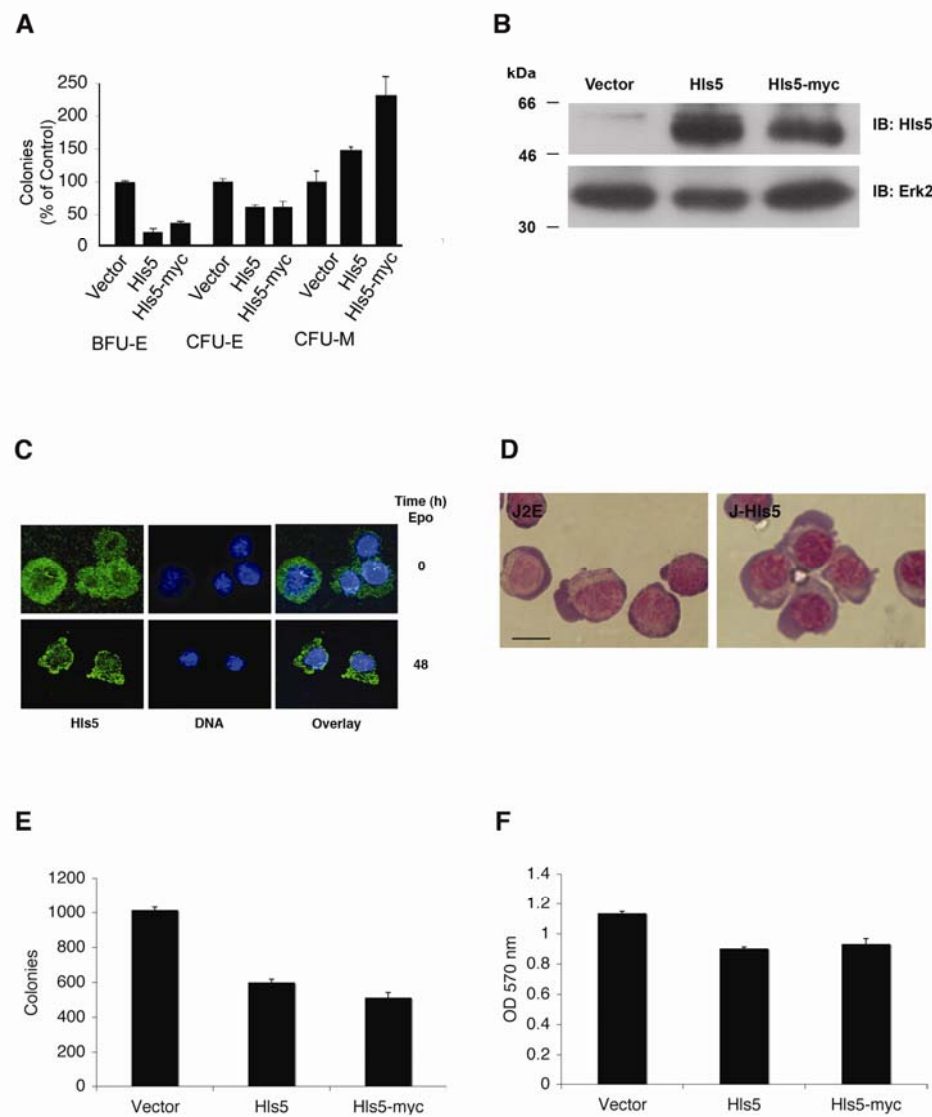


Figure 1

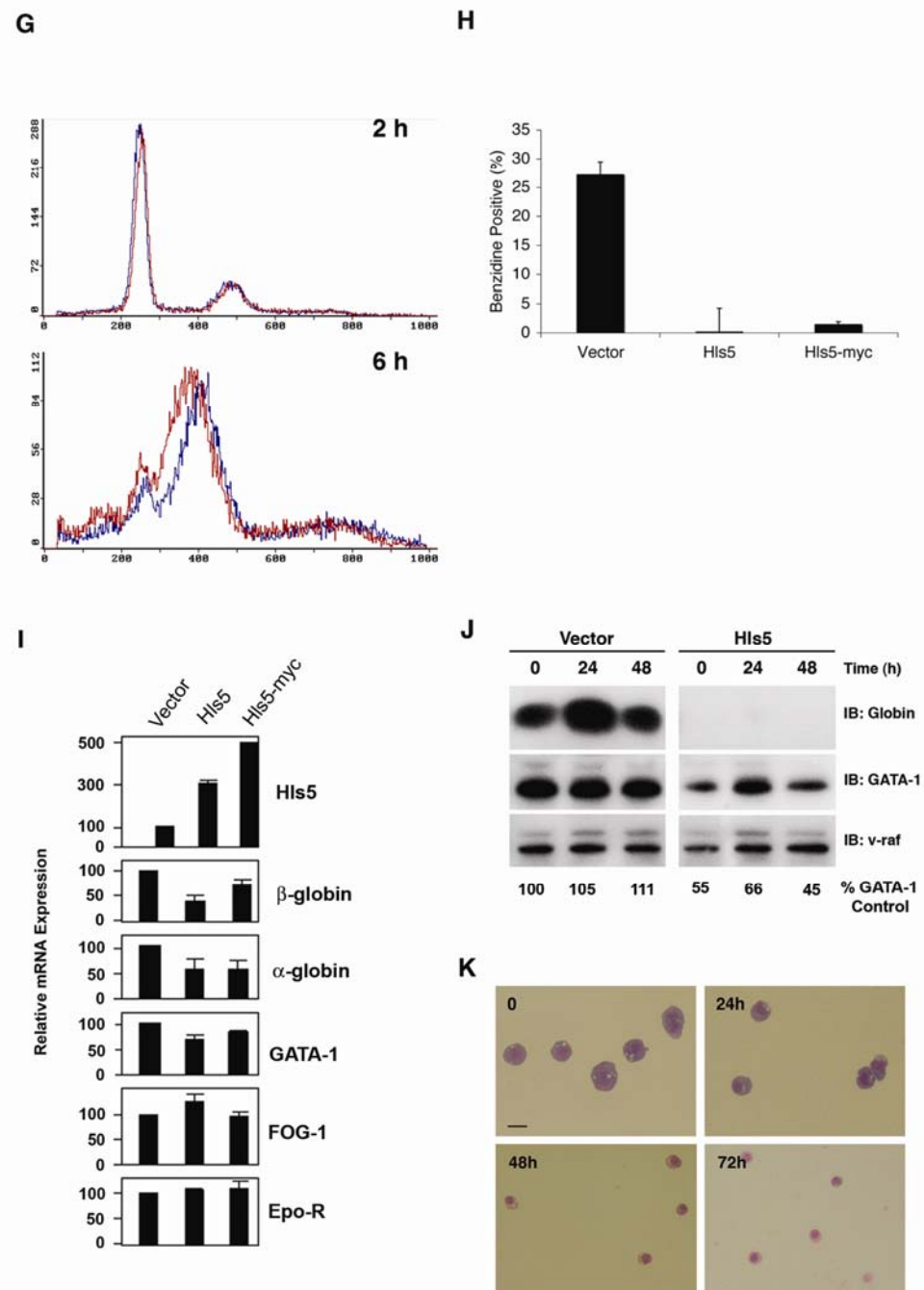


Figure 1

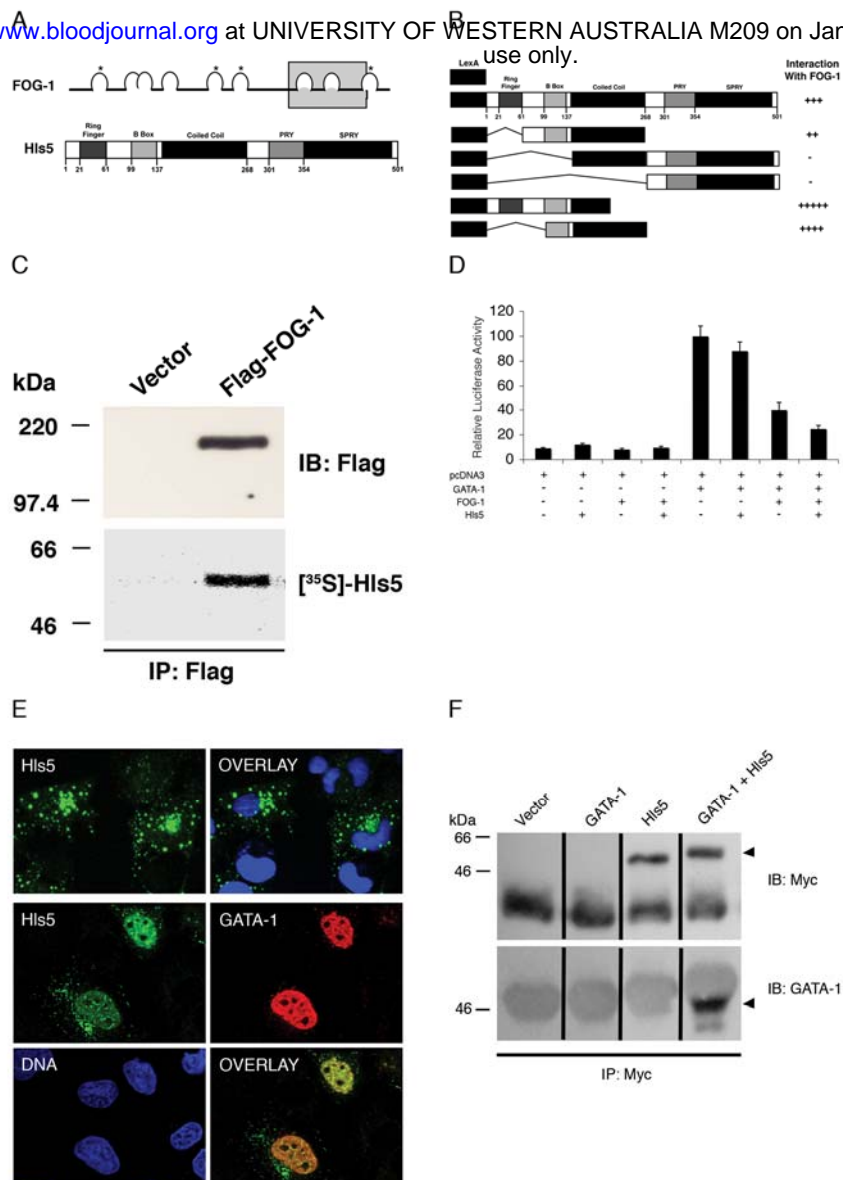


Figure 2

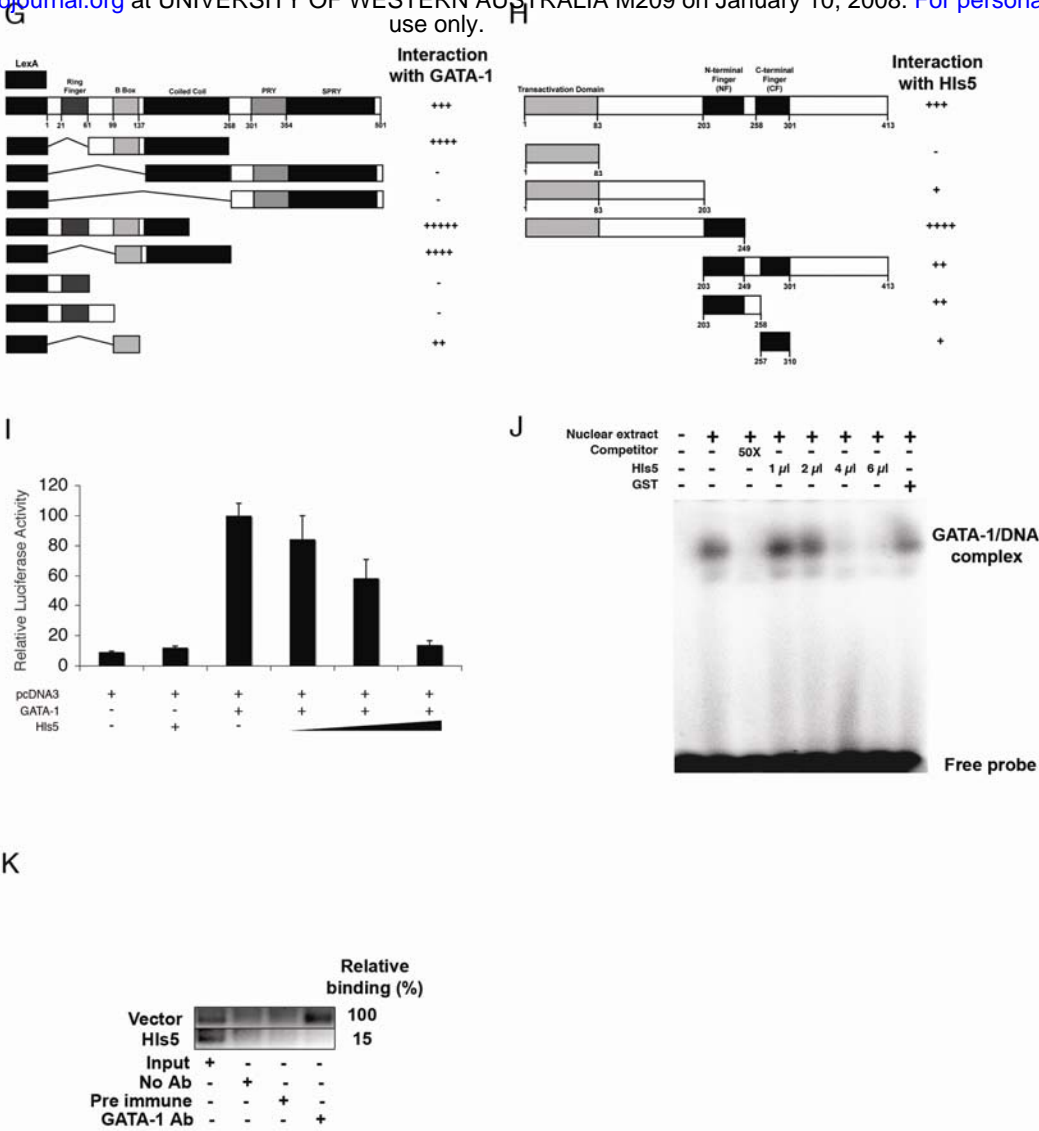


Figure 2