Epigenetic Transcriptional Regulation of the Growth Arrest-Specific gene 1 (Gas1) in Hepatic Cell Proliferation at Mononucleosomal Resolution

Natalia Sacilotto, Antonio Espert, Josefa Castillo, Luis Franco, Gerardo López-Rodas*

Chromatin Laboratory, Department of Biochemistry and Molecular Biology, University of Valencia, Burjassot, Valencia, Spain

Abstract

Background: Gas1 (growth arrest-specific 1) gene is known to inhibit cell proliferation in a variety of models, but its possible implication in regulating quiescence in adult tissues has not been examined to date. The knowledge of how Gas1 is regulated in quiescence may contribute to understand the deregulation occurring in neoplastic diseases.

Methodology/Principal Findings: Gas1 expression has been studied in quiescent murine liver and during the naturally synchronized cell proliferation after partial hepatectomy. Chromatin immunoprecipitation at nucleosomal resolution (Nuc-ChIP) has been used to carry out the study preserving the in vivo conditions. Transcription has been assessed at real time by quantifying the presence of RNA polymerase II in coding regions (RNAPol-ChIP). It has been found that Gas1 is expressed not only in quiescent liver but also at the cell cycle G1/S transition. The latter expression peak had not been previously reported. Two nucleosomes, flanking a nucleosome-free region, are positioned close to the transcription start site. Both nucleosomes slide in going from the active to the inactive state and vice versa. Nuc-ChIP analysis of the acquisition of histone epigenetic marks show distinctive features in both active states: H3K9ac and H3K4me2 are characteristic of transcription in G0 and H4R3me2 in G1/S transition. Sequential-ChIP analysis revealed that the “repressing” mark H3K9me2 colocalize with several “activating” marks at nucleosome N-1 when Gas1 is actively transcribed suggesting a greater plasticity of epigenetic marks than proposed until now. The recruitment of chromatin-remodeling or modifying complexes also displayed distinct characteristics in quiescence and the G1/S transition.

Conclusions/Significance: The finding that Gas1 is transcribed at the G1/S transition suggests that the gene may exert a novel function during cell proliferation. Transcription of this gene is modulated by specific “activating” and “repressing” epigenetic marks, and by chromatin remodeling and histone modifying complexes recruitment, at specific nucleosomes in Gas1 promoter.

Introduction

The delicate balance between positive signals, that induce cells to enter and progress through the cell cycle, and negative signals, which maintain them in a resting state, controls cell proliferation. Among the models of cell proliferation, the murine partial hepatectomy (PH) offers the advantages of analyzing cell cycle events in a synchronized cell population proliferating in an organism (reviewed in [1,2]). Although the hepatocytes in adult healthy livers rarely divide, surgical resection, chemical or viral injury have the ability to trigger a regenerative response. The hepatocytes leave the G0 state, progress through the cycle and restore the lost hepatic mass. Once this process is completed, cells exit from the cycle to return to reversible growth arrest in an active process that requires growth-inhibitory gene products (reviewed in [2–5]).

Special attention has been paid to the identification of genes expressed in quiescent conditions to discover genes that could arrest proliferation of growing cells, for instance, during the development of cancer. Schneider and co-workers [6] analyzed, by subtraction hybridization techniques in quiescent mouse fibroblasts, genes expressed when cells were arrested by serum starvation or contact inhibition. By these means, six cDNA clones were isolated (Gas1 to Gas6). Among these growth arrest-specific (Gas) genes, only Gas1 demonstrated the ability to inhibit cell proliferation when over-expressed in normal and transformed cell lines [7–9], and to reduce tumor cell growth [10–13]. Apart from these antiproliferative functions, other reported roles for Gas1 include promotion of apoptosis [10,14], involvement in mouse embryonic development [15], and suppression of melanoma metastases [16].

Therefore, it seems evident that Gas1 is a pleiotropic gene, which exerts its functions according to the tissue, the developmental stage or the cellular context. However, no data on the implication of Gas1 in the maintenance of quiescence of adult tissues, such as liver, are available to date.
The control of eukaryotic cell proliferation requires the integration of several signals that specify a precise transcriptional program, which obviously ought to be developed in a chromatin context (reviewed in [17–19]). Epigenetic mechanisms, such as the covalent modifications of DNA and histones, as well as the nucleosomal distribution along the DNA, are strictly regulated, crucial facts that determine the appropriate transcriptional behavior [17,20–25]. Some epigenetic marks have been correlated with transcriptional activation, such as acetylation of histone H3 and H4; or with silencing, such as methylation of cytosines at the CpG islands and of some histone residues (reviewed in [22,24,25,26]). However, it is more likely that a specific combination of marks, acting in a complex network of interactions, drive the transcriptional response in particular cell types or environments. Therefore, understanding how proliferation of normal cells is epigenetically controlled is a pre-requisite to define the mechanisms of deregulation of cellular behavior, such as in cancer development.

To better know how the transcriptional regulation of Gas1 is carried out under controlled proliferation, we analyze in this work the expression of the gene during mouse liver regeneration, a process in which cells proliferate synchronously. We find that Gas1, expressed in quiescent liver, is repressed when the cells enter the cell cycle, to be again expressed at the G1/S transition. The epigenetic marks and the changes in nucleosomal positioning around the transcriptional start site have been studied and some distinctive features of the repressed state and of both the active ones are described. Our results suggest that the mechanisms regulating Gas1 transcription are different at a chromatin level in both active states.

Results

The Gas1 gene shows a biphasic pattern of gene expression in adult liver after PH

Although extensive information implicating Gas1 in growth control of different cell types is available, no data on the possible involvement of Gas1 in regulating cell proliferation in adult tissues have been reported to date. The adult liver is mainly a quiescent organ and Gas1 has been described as a quiescence gene marker in vitro [6]. We first analyzed Gas1 expression pattern during liver regeneration after partial hepatectomy (PH) in mice. The semi-quantitative and quantitative RT-PCR (Figure 1A), show that Gas1 is expressed in control livers (0 h, non-operated mice) and that its expression decreases rapidly to reach a minimum at around 7 h after PH. The gene also shows an unexpected maximum of expression at 24 h after PH, a time at which hepatocytes are about to enter S-phase, as estimated by analysis of the induction of cell cycle markers CyaE2 and CyaB1 (Figure 1B), and by BrdU incorporation (Figure 1C).

We also analyzed Gas1 expression pattern in sham-operated mice, since surgical stress, even without liver resection, could induce physiological responses that either activate or silence the expression of many genes. The results show that Gas1 expression is not altered by surgical stress itself (Figure 1A).

These data suggest that GAS1 may have a new potential function during the G1/S transition since, according with its classical function in quiescence, it was not expected an increase of gene expression in liver regeneration until the end of the first round of division when cells start to arrest in a reversible G0 state.

Since not only did we find Gas1 expression during quiescence, but also unpredicted up-regulation of the gene during the G1/S transition in liver, we focused our interest in studying the regulation of Gas1 in these transcriptional active states in a chromatin context. We further confirmed that Gas1 was actively transcribed by RNApol ChIP (Figure 1D) and that the protein was synthesized (Figure 1E), at 0 and 24 h after PH. On the contrary, the RNApol II was not present at the coding region of the gene (Figure 1D), and protein level was reduced significantly, at 7 h after PH (Figure 1E).

Analysis of nucleosome positioning at the Gas1 promoter

As we have pointed out before, eukaryotic gene transcription is regulated in a chromatin context, so we wondered whether the changes in Gas1 transcription during liver regeneration are accompanied by changes in chromatin structure. Taking into account that remodeling of chromatin at the promoters is a feature of most inducible genes (see recent reviews in [27–29]), we first analyzed, by classical ChIP assay, the recruitment of the main chromatin remodeling complexes using antibodies against components of the SWI/SNF (BRM or BRG1), ISWI (SNF2 h) and CHD (MTA1) families of remodelers (Figure S1). The results indicated that none of the analyzed remodeling complexes is bound to Gas1 promoter in the inactive state (7 h after PH), while in the active state during liver quiescence, the SNF2 h-containing ISWI remodeling complex appears bound to the promoter. In the second active state, at 24 h after PH, a BRM-containing remodeler binds the promoter. These results strongly suggest that a remodeling of the promoter chromatin is required for Gas1 to be expressed, and this process is accomplished in different ways in both transcriptionally active states. The other complexes checked, i.e., those containing BRG1 or MTA1, do not participate in these remodeling events under these conditions.

We next determined, in the inactive state and in both the active ones, the position of nucleosomes near the transcriptional start site (TSS). First, the theoretical prediction of nucleosome positioning near the TSS, based on the PHASE program algorithm (available in http://wwwmgs.bionet.nsc.ru/mgs/programs/phase/), showed a high probability of nucleosomal presence between −50 to −200 from TSS, a moderate probability between −300 to −500, and a low probability between +50 to +200 (Figure 2A). In view of this prediction, we designed a series of primers giving tiled amplicons between −500 and +300 to examine the nucleosome positioning. To do this, two methodologies were used, namely the micrococcal nuclease protection (MNP) assay and the mononucleosomal immunoprecipitation (Nuc-ChIP) assay. The first method consisted in an extensive digestion of crosslinked liver nuclei with micrococcal nuclease, followed by DNA purification and isolation of the mononucleosomal DNA band from an agarose gel (Figure 2B). The second method consisted in an immunoprecipitation of mononucleosomal-sized chromatin fragments using an antibody against the histone H3, with the consequent DNA  isolation. In both cases, the DNA was further used as a template in PCR reactions using the designed primers.

The results obtained from both MNP assay (Figure 2C) and Nuc-ChIP assay (Figure 2D) showed a strong signal for the amplicons located at −329, at −211 and at +125 /+187, that should correspond to three positioned nucleosomes, further referred to as N-2, N-1 and N+1 (Figure 2E). The data also suggest that N+1 and N-1 nucleosomes may experience a sliding during the transition between the actively transcribed and the repressed state, since the signals of the amplicons −211 and +187 are significantly lower at 7 h in comparison with 0 and 24 h after PH (labeled with asterisks in Figure 2C, 2D and 2E). On the contrary, the nucleosome N-2 seems to be located at the same position, within the resolution limits of this experiment, in the three transcriptional states of Gas1, since the signal at the amplicon −329 bp is similar at 0 h, 7 h and 24 h after PH.

The above results encouraged us to determine which chromatin remodeling complexes could be implicated in the sliding of
nucleosomes N-1 and N+1 during the transition between the different transcriptional states of Gas1. To address this issue, a Nuc-ChIP experiment was carried out. Our results (Figure 3) showed that BRM-SWI/SNF complex was bound to N-1 when Gas1 is repressed at 7 h after PH, and very weakly at 24 h post-HP. Moreover, this complex was strongly bound to nucleosome N+1 in G1/S transition at 24 h post-HP. On the contrary, SNF2 h-ISWI complex was bound neither to N-1 nor to N+1 at the three analyzed Gas1 transcriptional states. The functional importance of the nucleosomal remodeling of the Gas1 TSS vicinity by BRM-SWI/SNF is not yet clear, but it might be related to the fact that some transcriptional factors, namely CREB, C/EBPβ, SMAD4 or SP1, are bound to their transcriptional binding sites located near these nucleosomes, as it was assessed by standard ChIP assay using antibodies against these factors (Figure S2).

HAT and HDAC recruitment to Gas1 promoter at mononucleosomal resolution

To explore the epigenetic regulation of Gas1, we carried out analyses of the recruitment of histone acetyltransferases (HAT) and histone deacetylases (HDAC) to the gene promoter by chromatin immunoprecipitation (ChIP) assay at mononucleosomal resolution. We again selected the two transcriptional active states of Gas1, quiescence and G1/S transition, and the transcriptional inactive state, at 7 h after PH.

The preliminary studies, using standard ChIP assay, indicated that GCN5 was bound at the Gas1 promoter in quiescent liver. GCN5 binding was less clear at 7 h after PH and it was released from the promoter at the G1/S transition. By contrast, CBP is constitutively bound to Gas1 promoter at the three selected time-points. The other HAT complexes analyzed, p300 and PCAF, do not seem to be implicated in the acetylation pattern of Gas1 promoter in the time-points under study (data not shown). In relation with the HDACs, mSIN3A was bound to Gas1 promoter in quiescence and at 7 h, and the ChIP analysis only gave a faint band 24 h after PH. It is also noteworthy that the NCoR-containing histone deacetylase complex was constitutively bound to Gas1 promoter at the three selected transcriptional states (data not shown).

Consequently, we analyzed the recruitment/release of those histone modifying complexes to the specific nucleosomes located at the Gas1 TSS vicinity. Figure 4A shows that GCN5 is bound to

Figure 1. Expression of growth arrest gene-1 (Gas1) during mouse liver regeneration after partial hepatectomy (PH). (A) Steady state mRNA levels of Gas1 were measured by semiquantitative (top) and quantitative (bottom) RT-PCR in liver at the indicated times after PH. The values were normalized by the loading control rRNA 18S and expressed as relative to control liver at 0 h after PH. Mice that underwent only laparotomy (Sham operation) were used as control. The bars of errors correspond to the standard deviation of 5–6 independent RT-PCR measurements. (B) Expression of cyclines CycE2 and CycB1. Steady state mRNA levels of cyclines were measured by semiquantitative RT-PCR (top panel) and the bands were integrated by ImageJ software analysis (bottom panel). The values were normalized by the loading control rRNA 18S and expressed as relative to control liver. (C) Immunohistochemical detection of BrdU incorporation (bottom panel). The values were normalized by the loading control rRNA 18S and expressed as relative to control liver. The α- and β- Actin genes were used as negative and positive control respectively, and rRNA 18S as an internal control of the RT-PCR analysis.

doi:10.1371/journal.pone.0023318.g001
A: PHASE software

B

C: MNP

D: Nuc-ChIP

E
nucleosome N-1 in quiescent liver and at 7 h after PH. As to nucleosome N+1, it only binds GCN5 in the inactive state (7 h after PH). On the contrary, CBP is bound to both nucleosomes at the three transcriptional states studied.

On the other hand, the mSin3A-containing HDAC complex was actively recruited at 7 h post-HP, when Gas1 is repressed. Afterwards, the SIN3A complex remains bound to nucleosome N-1, although it was released from nucleosome N+1 in the G1/S transition, when Gas1 is again active (Figure 4B). The binding of NCoR-containing HDAC complex to Gas1 promoter displays a pattern roughly similar to that of GCN5 (Figure 4B).

Epigenetic marks in Gas1 promoter at mononucleosomal resolution

We next analyzed if there are distinct epigenetic marks in the positioned nucleosomes located at the Gas1 TSS vicinity in the three transcriptional states. To perform this analysis, the cross-linked fragments of chromatin of mononucleosomal size, obtained from micrococcal nuclease digestion, were immunoprecipitated with antibodies against specific histone modifications (Figure 5A).

We found different patterns of histone modifications in the nucleosomes under the diverse condition modifications. For instance, H3K9ac seemed to be a specific mark of N+1 at 0 h; H4R3me2 appeared at 24 h in the N-1 and N+1 nucleosomes; H3K4me3 appeared on N-1 at 0 h and 24 h. The H3K14ac and H3K27ac were present with higher or lower intensity at both nucleosomes in the three analyzed time-points.

These results suggest that the regulation of the epigenetic marks level along the Gas1 TSS vicinity is rather complex, since there is combination of “inactive characteristic marks” (as H3K9me2) and “active characteristic marks” (as H3K9ac, H3K14ac, H3K27ac, H4K5ac, H3K4me3 or H4R3me2) not only in neighbor nucleosomes, but also within the same nucleosome. Some “activating” marks are present at both analyzed nucleosomes when Gas1 is transcriptionally active in quiescence and in G1/S transition. It is noteworthy that some distinctive, specific marks have been found at the different transcriptional states, as it happens with the presence of H3K9ac in nucleosome N+1 in quiescence or with H4R3me2 in nucleosomes N-1 and N+1 in the G1/S transition.

The presence of “inactivating” and “activating” epigenetic marks within the same nucleosome could be due to a monoallelic gene expression of Gas1 as an additional mechanism to regulate precisely the level of transcription in response to cellular stimuli during quiescence and G1/S transition. By this mechanism, the hepatocyte could maintain the desired level of transcript depending on whether one or two alleles are transcribed and, at the same time, the transcriptional activity of each allele may be epigenetically regulated through independent ways. Gimmelbrant and co-workers [51] performed a genome-wide search for genes subject to monoallelic expression and found that up to 1000 genes in human genome may be subject to such monoallelic expression, including not only X-inactivated genes in development or autosomal imprinted genes but also other autosomal genes. Among the latter class, these authors found Gas2 and Gas6, members of the growth arrest-specific gene family, as genes that are expressed monoallelically.

To check if the “activating” and “repressing” marks are coexisting in Gas1 promoter in the same nucleosome, but being located at different alleles, we perform a sequential-ChIP analysis (also known as re-ChIP) at nucleosome N-1 by combining Nuc-ChIP assay, using an antibody against H3K9me2, followed by reimmunoprecipitation with an antibody against H3K27ac, H3K4me3 or H4R3me2, and vice versa.

The results represented in Figure 5B indicate that indeed both types of epigenetic marks are located not only at the same nucleosome but also in the same allele since different combination of “activating” and “repressing” epigenetic marks in the sequential-ChIP showed significant band signals as evaluated by the ImageJ software (Figure 5B, bottom panel).

Discussion

In spite of the extensive information on the role of the Gas1 in cell growth control in vitro, programmed cell death and in mouse development in vivo, no data on the role of Gas1 in the quiescence of adult tissues is available. On the other hand, over-expression of Gas1 inhibits cell proliferation in different tumor cell lines [10,31,32], as well as in cultured fibroblasts [9] and, recently, the possible benefits of Gas1 in tumor gene therapy have been advanced [12,16].

In the present study, using the natural synchrony of the cell proliferation model after partial hepatectomy, we describe that Gas1 is expressed not only in quiescent liver but also actively

---

**Figure 2. Nucleosome positioning at Gas1 transcriptional start site (TSS) vicinity.** (A) Theoretical prediction of nucleosome positioning based on PHASE software algorithms. (B) Agarose gel showing mononucleosomal-sized chromatin fragments used for nucleosome positioning. PCR analysis from (C) micrococcal nuclease protection (MNP) and (D) Nuc-ChIP assays for nucleosome positioning. (E) Schematic representation of the positions of nucleosomes empirically determined. The positions of the center (bold letters) and lengths (brackets) of the amplicons used in the PCR reactions are showed at the bottom of the panel. The images are representative of at least three independent experiments.

doi:10.1371/journal.pone.0023318.g002

**Figure 3. Nuc-ChIP assay of BRM-containing SWI/SNF and SNH2 h-containing ISWI remodeling complexes after PH.** The PCR bands were integrated by ImageJ software, the background (no antibody samples, NA) subtracted, normalized by dividing by their corresponding Input samples and represented as histograms at the bottom panel. The images are representative of at least three independent experiments.

doi:10.1371/journal.pone.0023318.g003
transcribed just before entering S phase. This is the first time that expression of \(\text{Gas1}\) at that moment of the cell cycle is described, because, to date it has only been showed expression of the gene when cell lines are arrested in G0 [1,2,4,5,19]. Our data suggest that \(\text{Gas1}\) expression could be playing a dual role in liver cell cycle control, acting to keep liver quiescence, and to control the pass of the cells through the G1/S checkpoint. The expression of \(\text{Gas1}\) would facilitate the arrest of the cell cycle before entering S phase if cell damage has occurred. In this context it should be noted that overexpression of \(\text{Gas1}\) in cultured cells causes arrest of the cell cycle at the interface G1/S [7,9,33]. Further research is obviously needed to ascertain the molecular mechanisms involved.

We have described that c-Myc, which is expressed during liver regeneration in a biphasic manner, is distinctly activated in both expression waves [34], so we wondered whether the mechanisms involved in the transcriptional regulation of \(\text{Gas1}\) in quiescence and in the G1/S transition are also different.

It has been described that c-MYC and v-Src-repressed expression of \(\text{Gas1}\) is not linked to the TGF-β signalling pathway [35,36]. c-MYC also regulates several cell cycle regulatory genes as p15, p21, p27, Gadd34, Gadd45, and Gadd153, c-MYC represses transcription of these genes by at least two distinct mechanisms. One mechanism requires DNA binding of the MYC–MAX complex to Inr element in their promoters, and the inhibition of transcriptional activators via the C-terminal domain of c-MYC. The other mechanism is dependent on c-MYC binding to the SP1 transcription factor via the c-MYC central region inhibiting SP1 transcriptional activity (see review in [37]). We have found that SP1 is present at \(\text{Gas1}\) promoter under repression at 7 h after PH (Figure S2), suggesting that this second mechanism may be acting in \(\text{Gas1}\) repression during liver regeneration after PH. Comparative studies carried out by de Martin and co-workers by EMSA technique using growing and resting NIH3T3 cell extracts suggested that most of the growing and repressing state-specific protein-binding sites are located between nucleotides −195 and −550 [38]. This region is highly conserved from chicken to human, as assessed by \(\text{Gas1}\) promoter alignment (http://genome.lbl.gov/vista/index.shtml) (data not shown), and contains several putative binding sequences for SP1 binding (as estimated by TESS software http://www.cbi.nippon. edu/cgi-bin/tess/tess) (data not shown). Nevertheless, it cannot be ruled out that other mechanisms might be also acting in \(\text{Gas1}\) transcriptional regulation since it has been reported that MYC-box 2 (MB2) and leucine repeat C-terminal region (LZ) of c-MYC are required for \(\text{Gas1}\) repression in Myc-transformed Rat-1a fibroblast cell lines [35], regions that do not interact with SP1 transcriptional factor [37].

In view of the importance of chromatin structure in eukaryotic gene regulation, we focused our interest in the changes in chromatin when going from one transcriptional state to another. First, we have determined that three nucleosomes, N-2, N-1 and N+1, are positioned in the neighborhood of the TSS. A nucleosome-free region (NFR) is flanked by nucleosomes N-1 and N+1. These results are in agreement with the findings obtained by genome-wide mapping which have revealed that N-1 and N+1 nucleosomes delimiting a NFR is an evolutionarily conserved, common feature of promoters. In our case, both nucleosomes reside in canonical locations at specific distances from the TSS [39–44].

As “gate-keepers” of the NFR at the promoters, the N-1 and N+1 nucleosomes are well positioned to have significant regulatory potential in transcription. Published data show that, when genes are transcribed, nucleosome N+1 is shifted downstream of TSS [40,43], as it happens to \(\text{Gas1}\) nucleosome N+1 in quiescence and G1/S transition (Figure 2). The observed sliding probably leaves the TSS fully accessible for the transcriptional machinery (reviewed in [43]). In our case, the data are also compatible with an upstream sliding of nucleosome N-1 upon transcription of the gene (Figure 2). This sliding may be related to...
changes in the accessibility of transcriptional factors, as CREB, C/EBPβ, SMAD4 or SP1, to their sequence-specific binding sites located near N-1 nucleosome, as we estimated by TESS software and analyzed by ChIP assay (Figure S2).

It is noteworthy that a differential binding of the BRM catalytic subunit to N-1 and N+1 nucleosomes occurred at the analyzed transcriptional states. The data of Figure 3 indicate that the remodeling complex is mainly bound to nucleosome N-1 at 7 h and to nucleosome N+1 at 24 h after PH. That complex could be responsible of the N-1 sliding when going from the active state in quiescent liver to the repressed state at 7 h after PH and for the transition to the second active state at 24 h after PH. On the contrary, the complex does not seem to participate in the sliding of nucleosome N+1 when going from the inactive state to the active one at 24 h after PH (Figure 2). Although the ISWI complex is bound to the active promoter, as revealed by the binding of its component SNF2h detected by low-resolution standard ChIP assay, we have not found evidence for the association of ISWI complex to N-1 or N+1 nucleosomes. It is possible that this complex acts on some neighbor nucleosomes not studied in the present work. Of course, other non-studied remodeling complexes may participate in the remodeling events of Gas1 promoter and proximal transcribed region.

There is a plethora of data showing that covalent histone modifications play a crucial role in transcriptional regulation (see reviews in [17,23,26,45–51]). However, information about histone modifications at specific nucleosomes in different transcriptional states is limited in the literature and this circumstance adds some interest to the data presented in Figures 4 and 5. As Figure 4 shows, GCN5-containing HAT complex is bound to nucleosome N-1 in quiescence, and to N-1 and N+1 nucleosomes at 7 h post-H. On the contrary, CBP is constitutively bound to these nucleosomes flanking Gas1 TSS at the three transcriptional states. On the other hand, the mSIN3A-containing HDAC complex is mainly bound to the analyzed nucleosomes when Gas1 is repressed at 7 h post-HP, while the low signal obtained for the binding of NCoR-containing HDAC complexes make it difficult the drawing of definite conclusions. At any rate, the present data suggest that the regulated acquisition of histone epigenetic marks depends on the balanced and controlled activities of the opposite histone modifying enzymes activities acting over the same specific nucleosome.

The pattern of histone modifications in N-1 and N+1 nucleosomes is rather complex (Figure 5). We were able to distinguish different “categories” of modifications. For instance, modifications such as H3K9ac and H3K27ac are constitutively present in both analyzed nucleosomes at the three transcriptional conditions and they might be related to the generation of a relaxed chromatin structure at Gas1 promoter as expected for a gene poised for transcription. On the other hand, the modifications H3K9ac and H3K4me2 are mainly related with quiescence, whereas H4R3me2 is mainly related with G1/S transition. Those modifications could be participating in the acquisition of a platform to which regulatory proteins may bind chromatin to activate or repress transcription of Gas1 in response to a specific cellular signaling.

We found that nucleosome N-1 at Gas1 promoter at 0 h and 24 h after PH contains not only “activating” (H3K27ac, H3K4me3 and H4R3me2) epigenetic marks, as expected since the gene is transcriptionally active, but also the known “inactivating” mark H3K4me2. The widespread monoallelic expression analysis on human autosomes, carried out by Gimelbrant and co-workers [30], showed that both Gas2 and Gas6, two members of the growth arrest-specific gene family, belong to the group of genes expressed monoallelically. This finding opens the hypothesis that Gas1 could be differentially regulated in both alleles by differential epigenetic marks leading to a monoallelic gene expression. However, the results obtained by sequential-ChIP (Figure 5) demonstrate that this does not occur in Gas1 since both types of epigenetic marks colocalize within the same nucleosome at Gas1 promoter.

There is a growing body of data in the literature showing that “repressing” H3K9 methylation is distributed at both silent heterochromatin and at active genes [52–56]. Wiencke and co-workers [54], using ChIP-on-chip experiments and conventional ChIP assay, confirmed the positive associations of H3K9me3, H3K4me2 and H3K9ac modifications with gene expression. The data also revealed that H3K9 methylation overlaps with those histone “activating” marks at TSS surrounding of genes. The information obtained in our studies indicate that the “inactivating” H3K9 methylation may colocalize, at least at nucleosome N-1, with several activating marks. It can not be ruled out the possibility that H3K9me2 is located in one of the H3 molecule and the “activating” marks on the other H3 molecule of the nucleosome, but the lack of specific antibodies for H3 against dual H3K9me2 and “activating” H3 marks leave open the question as to whether both modifications occur on the same histone H3 tail.

The global analysis of the data presented in this paper, together with those showed by other authors [52–56], suggests the need of revising aspects of the histone code involving plasticity of H3 lysine methylation and its “exclusive” association with repressed genes.

Materials and Methods

Biological material and experimental design

All procedures were conducted in accordance with the European regulations (Council Directive 86/609/EEC) and were authorized by the Ethics Committee for Animal Experimentation of the University of Valencia (Approval of procedure for project BFU2007-63120, date 12/03/2007). Eight to ten week-old CD1 male mice were used in the experiments. The partial hepatectomy (PH) technique was based on the classical method described by Higgins and Anderson [57]. Briefly, mice were anaesthetized with isoflurane and the median and left lateral liver lobes (2/3 of liver mass) were excised. Mice were sacrificed under anaesthesia at the indicated time-points after PH, and the remnant livers were harvested for RNA, protein and chromatin extraction. Mice that underwent only laparotomy (sham operation) were used as control.

RNA extraction, RT-PCR and qRT-PCR

The livers were harvested from anaesthetized mice and snap-frozen in liquid nitrogen. After disrupting 30 mg of tissue in an
Ultra-Turrax homogenizer (IKA Werke, Staufen, Germany), RNA was extracted and purified with the Illustra RNA Spin Mini RNA Isolation Kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's instructions. The RNA concentration was estimated by measuring the absorbance at 260 nm, and its purity double-checked by calculating the ratio A260/A280, and by visualizing RNA on agarose formaldehyde gel electrophoresis. 1 μg of total RNA was retrotranscribed to cDNA using SuperScript II RNase H+ (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, with random hexamers (Sigma, St Louis, MO, USA) to prime the elongation reaction. cDNA was analyzed by PCR, the products were size-fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide, photographed with Gel Doc XR+ image analyzer (Bio-Rad, Hercules, CA, USA) and quantified by ImageJ software (http://rsbweb.nih.gov/ij/). The quantitative real time RT-PCR (qRT-PCR) were conducted in an ABI GeneAmp 7000 System Detection (Applied Biosystems, Carlsbad, CA, USA) and analyzed with the ABI Prism Software (Applied Biosystems), and the relative expression values were calculated according to the 2ΔΔCt method.

The primer pairs used for RT-PCR were: Gas1, forward 5′-CGAACACTGAGTGTTCAACGG-3′, reverse 5′-TCGCAAGCAGCTGAGG-3′, amplicon position +937/+1209 and size 272 bp; 18s rRM, forward 5′-TGGTGTATGCTCAGATG-3′, reverse 5′-CTTCTCGGAATTCGACCCTGG-3′, amplicon position +1327/+1580 and size 254 bp. The primer pairs used for qRT-PCR: Gas1, forward 5′-TGGATGAGGAGCCCATG-3′, reverse 5′-GGAAGCTCCGAAGACCC-3′, amplicon position +426/+477 and size 52 bp; 18s rRA, forward 5′-CAGCCGCGGATACGTAGAAA-3′, reverse 5′-AGAGGAGCCGAGCACC-3′, amplicon position +67/+138 and size 72 bp.

Protein extraction and western blot

Fragments of 100 mg of liver were homogenized in 1 ml of ice-cold PBS, supplemented with 2 μl of protease inhibitor cocktail (Sigma). After centrifuging and removing PBS, total protein extracts were run in 12% SDS-PAGE gel [58x303] for PCR analysis of the target gene. Analysis of constitutively expressed β-Actin and α-Actin genes were used, respectively, as positive and negative controls of the experiment. The entire experiment was repeated at least three times.

To study nucleosome positioning as well as to detect histone modifications or modifier binding at the level of single nucleosomes, we employed the Nuc-ChIP technique. To do this, the sonication step of the standard ChIP protocol was replaced by extensive micrococcal nuclease digestion (see below) of isolated cross-linked nuclei. The resulting chromatin fragments were thus enriched in mononucleosomes, which were immunoprecipitated with the desired antibody. The detection of the immunoprecipitated DNA was carried out by PCR by using 1:500 dilutions of the Input and 1:30 dilutions of the IP and NA fractions. The integrated density of the electrophoretic bands (obtained by ImageJ software) of IP fraction was subtracted from that of the NA fraction, and the result normalized by that of the Input fraction.
For the sequential ChIP analysis of histone epigenetic marks colocalization, aliquots from the mononucleosome-enriched chromatin fragments (equivalent to 100 μg DNA) were incubated with the first antibody and, after washings steps, the immunocomplexes were eluted by adding 75 μl TE buffer supplemented with 10 mM DTT and incubating for 30 min at 37°C. The resulting fractions were diluted 20 times with sequential ChIP dilution buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0), supplemented with protease inhibitor cocktail. Afterwards, the diluted samples were reimmunoprecipitated with a second antibody, and the standard procedure was followed. The sequential ChIP results were always confirmed by changing the order in which the antibodies were used. To subtract the background signal to the IP samples, the integrated density of the electrophoretic bands of a parallel sample in which first antibody is replaced by IgG was used.

The antibodies used in our experiments were: α-CBP (sc-369), α-GCN5 (sc-303), α-PCAf (sc-8999), α-P300 (sc-584), α-mSin3A (sc-994), α-NCoR (sc-8994), α-BRM (sc-710), α-BRG1 (sc-0768), α-MTA1 (sc-0813), α-SPI (sc-59), α-CREB (sc-186), α-C/EBPβ (sc-150) and α-rNApol II (sc-899) from Santa Cruz; α-SP1 (sc-1794) and α-CHD4 (ab31542) from Abcam; α-H3K4me3 (ab1012-100), α-H4R3me2 (ab5823) and α-H4K5ac (ab1791-22) from Abcam; α-H3K9ac (ab12189-100), α-H3K36me3 (ab1012-100), α-H1R3me3 (ab3823) and α-H3 (ab1791-22) from Abcam; α-H3K14ac (07-353), α-H1K5ac (07-327) and α-H3K9me2 (05-685) from Upstate-Millipore.

The primer pairs used for RNApol ChIP were: Gas1, forward 5'-CGGAGAGCCGGGCTGAC-3', reverse 5'-TGGCA-CAGGCTGCTGGAG-3', ampiclon position +397/+1209 and size 232 bp; α-Actin, forward 5'-GAGAGTGAGCCCCAGGAC-3', reverse 5'-CCCAATGCCACCCGAC-3', ampiclon position +1325/+1759 and size 434 bp; β-Actin, forward 5'-TGTGCTGTGTGTTGTGCTG-3', reverse 5'-GGGATTCTGGATCTG-3', ampiclon (211, forward 5'-AACAACAGGCTGCTAC-3', reverse 5'-GGTTCTGTTGTGCTG-3', size 97 bp; ampiclon (211, forward 5'-AACACAGGCTGCTAC-3', reverse 5'-TTTCTCGTTCTCTAATG-3', size 82 bp; ampiclon (134, forward 5'-GGAGAGTGGAGAAAGGAGGAGAAG-3', reverse 5'-ATGCTATCGCATAGAAAGGCAGAACACAG-3', size 112 bp; ampiclon (50, forward 5'-TGGTCTTTGTGCTG-3', reverse 5'-GGAGACCCCACTG-3', size 121 bp; ampiclon (125, forward 5'-TTTGGCTGCTGCCTCCTC-3', reverse 5'-CTGTAATGCTGCTG-3', size 127 bp; ampiclon (187, forward 5'-AGGGGACCAAGGCTC-3', reverse 5'-AGGGGACCAAGCAGCTG-3', size 114 bp; ampiclon (242, forward 5'-GCCAAGCGCGCTGAG-3', reverse 5'-GCCAAGCGCGCTGAG-3', size 151 bp.

Nucleosome positioning by micrococcal nucleosome protection assay (MNP)

To estimate the position of nucleosomes, isolated nuclei from cross-linked cells were incubated with micrococcal nuclease to obtain mononucleosomes. After purification of the mononucleosomal DNA, PCR analysis was performed using primers that amplified short tiled fragments covering the region comprised between (500 and +300 bp relative to the transcription start site (TSS) of Gas1. Consequently, amplification products were expected at regions in which a nucleosome was protecting DNA from nucleosome digestion.

Nuclei were obtained from cross-linked liver, as described above, and nuclear pellets were resuspended in RSB buffer for micrococcal nucleosome digestion. 7 × 105 nuclei were centrifuged at 2,000 x g for 3 min at 4°C and washed with 1 ml of ice-cold RSB buffer [10 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 10 mM β-mercaptoethanol, 0.15 μM spermine, 0.5 mM spermidine, 2 μl/ml protease inhibitor cocktail (Sigma), 50 mM Tris-HCl pH 7.5]. After collecting nuclei by centrifugation, the pellets were resuspended in 0.5 ml RSB buffer without protease inhibitors, and incubated with 150 units of micrococcal nuclease (Roche Applied Science, Mannheim, Germany) for 30 min. Reaction was stopped by adding 1/10 volume EDTA 100 mM and putting the samples on ice. Nuclei were incubated at 65°C for 2 h to reverse cross-links in the presence of proteinase K (50 μg/ml). Finally, DNA was extracted and purified by phenol-chloroform extraction and precipitated with ethanol. The DNA pellet was resuspended in 100 μl of TE buffer and incubated 1 h at 37°C with 5 μg/ml RNase A. The DNA from the mononucleosomal band was purified after running in 2% agarose gel by GenClean kit according to the manufacturer's instructions. Afterwards, DNA was used for PCR analysis, after quantifying by absorbance at 260 nm. The Input sample (an aliquot of the samples randomly fragmented by sonication to mononucleosomal size) was included to normalize the results for each primer pair in the PCR reaction. The entire experiments were repeated at least three times.

The primer pairs used for nucleosome positioning were: ampiclon (448, forward 5'-GGGGGAGGAAAGGAAAG-3', reverse 5'-ACACCTTCAGGGCTCAGGATAC-3', size 120 bp; ampiclon (329, forward 5'-TATCCAGCTCCCTGAGAG-3', reverse 5'-CGAGGATCAGGGCTGTTGGA-3', size 82 bp; ampiclon (285, forward 5'-CCTGATAATGGAAGCTGCGCAACA-3', reverse 5'-GTCACAGGTCTGTGTTGAGTA-3', size 97 bp; ampiclon (211, forward 5'-AACAACAGGCTGCTAC-3', reverse 5'-TGCAGCTTCTGAGACTGCAAG-3', size 82 bp; ampiclon (134, forward 5'-GGAGAGTGGAGAAAGGAGGAGAAG-3', reverse 5'-ATGCTATCGCATAGAAAGGCAGAACACAG-3', size 112 bp; ampiclon (50, forward 5'-TGGTCTTTGTGCTG-3', reverse 5'-GGAGACCCCACTG-3', size 121 bp; ampiclon (125, forward 5'-TTTGGCTGCTGCCTCCTC-3', reverse 5'-CTGTAATGCTGCTG-3', size 127 bp; ampiclon (187, forward 5'-AGGGGACCAAGGCTC-3', reverse 5'-AGGGGACCAAGCAGCTG-3', size 114 bp; ampiclon (242, forward 5'-GCCAAGCGCGCTGAG-3', reverse 5'-GCCAAGCGCGCTGAG-3', size 151 bp.

For Nuc-ChIP and sequential-ChIP assays to detect histone modification marks, and binding of chromatin modifier and remodeller complexes to single nucleosomes, ampiclon −211 (for nucleosome N-1) and ampiclon +125 (for nucleosome N+1) were used.

Supporting Information

Figure S1 Gas1 promoter occupation by chromatin remodelling complexes after PH. The samples immunoprecipitated with indicated antibodies were analyzed by PCR using primers of the Gas1 promoter region (Top panel). The integrated density of electrophoretic bands, obtained by ImageJ software analysis of the PCR signals, from the NA fraction was subtracted from that of IP fraction and normalized by that of input fraction. These images are representative of at least three different experiments. (TIF)

Figure S2 Gas1 promoter occupation by transcription factors after PH. The samples were processed as described in Figure S1. (TIF)

Author Contributions

Conceived and designed the experiments: LF GL-R. Performed the experiments: NS AE JG. Analyzed the data: NS LF GL-R. Wrote the paper: LF GL-R.
References

1. Fausto N (2000) Liver regeneration. J Hepatol 32: 19–31.
2. Fausto N, Campbell JS, Richle KJ (2006) Liver regeneration. Hepatology 43: S4–S32.
3. Fausto N, Laird AD, Webber EM (1995) Liver regeneration. 2. Role of growth factors, cytokines in hepatic regeneration. FASEB J 9: 1527–36.
4. Michalopoulos GK (2007) Liver regeneration. J Cell Physiol 213: 286–300.
5. Michalopoulos GK (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 176: 2–13.
6. Schneider C, King RM, Philipson L (1982) Genes specifically expressed at growth arrest of mammalian cells. Cell 54: 787–93.
7. Del Sol G, Collavin L, Ruaño ME, Edmonson P, Saconne S, et al. (1994) Structure, function, and chromosome mapping of the growth-suppressing human homologue of the murine gas1 gene. Proc Natl Acad Sci USA 91: 1848–52.
8. Evdokiou A, Cowled PA (1998) Tumor-suppressive activity of the growth arrest-specific gene GAS1 in human tumor cell lines. Int J Cancer 73: 568–77.
9. Evdokiou A, Cowled PA (1998) Growth-regulatory activity of the growth arrest-specific gene, GAS1, in NIH3T3 fibroblasts. Exp Cell Res 249: 359–67.
10. Zamorano A, Lamas M, Vergara P, Narango JR, Segovia J (2003) Transcriptionally mediated gene targeting of gas1 to glioma cells elicits growth arrest and apoptosis. J Neurosci Res 71: 256–63.
11. Zamorano A, Mellstrom B, Vergara P, Narango JR, Segovia J (2004) Glial-specific retrovirally mediated gas1 gene expression induces gloma cell apoptosis and inhibits tumor growth in vivo. Neurobiol Dis 15: 603–91.
12. Benitez JA, Benitez JP, Segovia J (2007) Targeted-simulated expression of Gas1 and p53 using a histreonic adenoviral vector in gliomas. Cancer Gene Ther 14: 836–46.
13. Dominguez-Monzon G, Benitez JA, Vergara P, Lorenzana R, Segovia J (2009) Gas1 inhibits cell proliferation and induces apoptosis of primary human gliomas, in the absence of Shh. Int J Dev Neurosci 27: 305–13.
14. Mellstrom B, Cena V, Lamas M, Perales C, Gonzalez C, et al. (2002) Gas1 is induced during and participates in excoticotic neuronal death. Mol Cell Neurosci 19: 417–29.
15. Lee KK, Leung AK, Barton MC (2010) Cascades of transcription regulation during liver regeneration. J Hepatol 52: 345–53.
16. Gobeil S, Zhu X, Doillon CJ, Green MR (2008) A genome-wide shRNA screen partially randomized small interfering RNA library. J Biol Chem 284: 26273–85.
17. Lee KK, Leung AK, Tang MK, Cai DQ, Schneider C, et al. (2001) Functions of the liver regeneration gene Id2 in rat. J Biol Chem 276: 22789–93.
18. Mellstrom B, Cena V, Lamas M, Perales C, Gonzalez C, et al. (2002) Gas1 is induced during and participates in excoticotic neuronal death. Mol Cell Neurosci 19: 417–29.
19. Lee KK, Barton MC (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 176: 2–13.
20. Schones DE, Zhao K (2008) Genome-wide approaches for chromatin state determination. Nat Rev Genet 9: 457–69.
21. Riddle, NC, Minoda A, Kharchenko PV, Alekseyenko AA, Schwartz YB, et al. (2009) Chromatin modeling with Histone chIP-seq. Bioinformatics 25: 2360–66.
22. Lu J, Klose RJ, Hnisz J, Gao Y, Montano M, et al. (2012) Distinct properties of nucleosome positioning and flanking chromatin organization in the Drosophila genome. Genome Res 22: 2067–79.