Genome-wide localization of histone 4 arginine 3 methylation in a differentiation primed myeloid leukemia cell line.

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Abbreviations used : H4R3 stands for Histone type 4 arginine in the position 3; kb for kilobases; bp for basepairs; Chip for chromatin immunoprecipitation; TFBS for transcription factor binding site. All other abbreviations are introduced in the text.

Abstract

Methylation of arginine residues in proteins is involved in modulation of a various protein-protein interactions. On the chromatin level H4R3 methylation is providing a signal integration step during myeloid differentiation. In order to globally characterize the role of arginine methylation in signal integration and developmental processes we decided to map genomic loci marked by PRMT1 (Protein Arginine Methyl Transferase 1) via histone H4 arginine 3 methylation. For this, we used the myeloid leukemia cell line, HL60, which is known to differentiate along the monocyte/macrophage or granulocyte lineage. We used chromatin immunoprecipitation and cloning to isolate genomic loci marked by this modification. After sequencing and in silico analysis we found that all of the genomic hits identified were intronic or within 5 kb of 5‘ ends of specific genes. The locations identified were enriched in conserved transcription factor binding sites of POU2F1, MEF-2 and FOXL1 factors. A significant number of the genes in the proximity of the identified genomic loci are involved in signaling pathways and developmental processes including immune response of myeloid cells.

Introduction

Immune responses are mediated through multiple signaling cascades. Proteins involved in these cascades have to transmit signals on the scale of seconds. Biological systems efficiently use posttranslational modifications of proteins in order to change very rapidly the conformation of signaling molecules (Pawson, 2000). These mechanisms are able to increase the number of effector molecules by as much as an order of magnitude. The array of known posttranslational modification is extremely wide; it includes from palmitoylation and ubiquitination to sumoylation, acetylation and methylation various posttranslational changes. One studying signaling networks has to take into consideration all of these modifications (Kabuyama, 2004).

One of the lesser-appreciated protein modifications is methylation. Methylations were described early in the '70s, but the characterization in the '80s of the enzymes involved in the methylations gave a new impact to the study of processes mediated by methylation (Disa, 1986; Gallwitz, 1971). Recently a plethora of substrates were identified (Boisvert, 2003) (Lee, 2002) (Wada, 2002) and the enzymes involved in demethylation were also described (Cuthbert, 2004; Shi, 2004; Wang, 2004). It is easy to see that this will further increase the pace of research on this field.

We have recently shown that methylation on arginine residues of the H4R3 histone tail is modulating cellular transcriptional memory and provides a signal integration mechanism in the process of myeloid cell differentiation (Balint et al. submitted manuscript). HL60 myeloid leukemia cells enter in a transient primed state during their differentiation, characterized by an increased level of H4R3 methylation. HL60 cells become more susceptible for retinoid signals in this primed state. By the removal from the media of differentiating agents, this primed state is lost after several cell divisions, thus showing that increased arginine methylation provides a signal integration step during cell differentiation. This state is characterized by an increased level of PAD4, the pepdydil-deiminase responsible for demethylation of methyl-arginines via citrullination (Wang, 2004) (Balint et al. submitted manuscript).

Arginine methylation, beside a general role in gene expression regulation, seems to modulate proteins involved in immunological processes (Boisvert, 2004) (Mowen, 2001). Chromatin immunoprecipitation studies previously established that PRMT1 is one of the first proteins involved on the chromatin level in the events that in a concerted manner lead towards transcription (Metivier, 2003). To further characterize how PRMT1 modulates gene expression on the chromatin level we decided to isolate chromatin fragments that are methylated on arginine H4R3. For this we used a polyclonal antibody that recognizes specifically methylated H4R3 residues (Wang, 2004). The immunoprecipitated fragments were cloned and sequenced. Here we report that the immunoprecipitated fragments are more abundantly located in introns and a great majority of them contain conserved transcription factor binding sites.

Materials and Methods

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was carried out as described by Kuo and Allis (Kuo, 1999) with modifications. Briefly: cells were fixed with 1% formaldehyde for 10 min at room temperature. Fixation was stopped by adding chilled glycine to a final concentration of 150 mM. Cells were scraped and washed twice with ice-cold PBS that contained proteinase inhibitors (1mM PMSF, 1μg/μl aprotinin and 1μg/μl pepstatin A). Nuclei were prepared by incubation for 10 min on ice in a buffer containing 5mM Pipes pH8, 85mM KCl, NP40 0.5% and proteinase inhibitors. After centrifugation with 3000g for 10 minutes at 4 °C, nuclei were resuspended in sonication buffer (1%SDS, 0.1M $NaHCO_3$ and proteinase inhibitors), lysed on ice for 10 minutes and sonicated on ice to an average fragment size of 500 base pairs. Cell debris was pelleted twice by centrifugation with 10000g for 30min at 4°C in a bench-top centrifuge. Soluble chromatin was aliquoted and frozen in liquid nitrogen and stored on –70°C. For immunoprecipitation chromatin was diluted, 10-fold in an IP buffer (0.01%SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7 mM Tris pH8.1, 16.7 mM NaCl and proteinase inhibitors). 1 ml of diluted chromatin was precleared twice with 40μl, blocked protein A-sepharose beads. Immunoprecipitation was carried out with specific antibodies purchased from Upstate Biotech and Abcam against modified histones as it follows: , #07–213 Anti dimethyl H4 Arg3 6μl/IP. Incubation with the antibodies was carried out overnight, on a rotating plate at 4°C. Complexes were collected with 40 μl blocked protein A Agarose (Upstate #16–157). Beads were pelleted, washed twice with each of the following buffers: buffer A (low salt 0.1% SDS 1% Triton X-100 2mM EDTA 20mM Tris pH8.1 150 mM NaCl + PI), buffer B (high salt 0.1%SDS 1% Triton X-100 2mM EDTA 20mM Tris pH8.1 500 mM NaCl + PI), buffer C (0.25 M LiCl 1% NP40 1% sodium deoxycholate 1mM EDTA 10 mM Tris pH8.1+PI) and TE buffer (10mM Tris 10mM EDTA pH8+PI). Immunoprecipitated nucleosomes were eluted twice from beads with elution buffer (1% SDS, 0.1M $NaHCO_3$) and eluates were combined. Crosslinks were

reversed by incubating for 6 hours at 65°C after adding 20µl 5M NaCl. Eluate was combined with 10 µl of 0.5M EDTA, 20µl 1M Tris pH 6.5 and 2µg Proteinase K and incubated for 1 hour at 45°C. DNA was recovered after phenol: chloroform extraction and ethanol precipitation using 20 µg of glycogen as a carrier. DNA was resuspended in 50 µl of 50 ng/µl yeast tRNA (Invitrogen).

Generating of Blunt Ends

Protruding ends of the isolated DNA fragments were filled by treatment with Klenow enzyme as it follows.
The DNA fragments from 10 independent chromatin immunoprecipitations were mixed, divided in 10 alliquotes, supplemented with 0.5µl of 2.5mM dNTP, 3U Klenow enzyme, 3.5µl 10X Klenow buffer in a total of 35 µl reaction mix. Reaction was carried out at room temperature for 10 minutes and stoped by incubation for 10 minutes at 75 C°.

Ligation and Plasmid Preparation

Ligation was performed with 15 U (0.5µl) T4 DNA Ligase (Fermentas) with addition of PEG at 16C° overnight with a vector: insert ratio of 10:1according to the manufacturers recomendations. We used Zero Blunt® PCR cloning vector (Invitrogen). The ligation product was transformed in competent E. coli, shaked in SOC medium at 37 C° for 2 hours and plated in Kanamycin containing plates according to the manufacturers recomandations. On the second day colonies were isolated, plated on 96 well plates in liquid medium and let to grow for 24 hours at 37 °C degrees. Medium was suplemented with 30% of glycerol and frozen at -70C°C. At the analysis of colonies an aliquot of the frozen medium was plated on kanamycin containing plates and individual colonies were grown up. Plasmids were isolated with Miniprep Wizard (Promega) according to the manufacturers recommendations. The size of the insert was verified with XbaI and BamH1 double digestion and PCR with M13 primers. Results were visualized by standard agarose gel electrophoresis in a 2% agarose containing gel.

Sequencing

Sequencing of the insert was performed with standard M13+ sequencing primer according to the manufacturers recommendations of the ABI Big Dye Terminator 2.1 kit.

In Silico Analysis

In silico analysis of the sequences was performed as it follows:
The sequences of plasmid origin were removed from the ends of the raw sequence and the resulting sequence was first analysed with NCBI Blast (Altschul, 1990). Sequences that past the quality control described in the results section were further analysed. In the second step the genomic location of the sequences was analysed with BLAT program (Kent, 2002) on the UCSC Human Genome Browser (Karolchik, 2003). Conserved regions, known genes, conserved transcription factor binding sites and the position of the analysed sequence towards these genomic elements was monitored. For annotation we used PANTHER Analysis. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) (Mi, 2005) (Thomas, 2003) database allows complex annotation of proteins and genes. We selected the data described as “Molecular functions” and “Biological processes” of the Version 5.0 (release date Jan. 1, 2005).

Results

A key arginine methyltransferase of the mammalian cell is PRMT1. One of the methylation reactions mediated by this enzyme is the H4R3 methylation (Tang, 2000a). H4R3 methylation was shown to be linked with gene expression (Wang, 2001). Methylation of histones by PRMT1 makes them a better substrate for histone acetyltransferases and by this provides a mechanistic link between gene expression regulation and arginine methylation (Wang, 2001). We decided to map genomic loci of arginine methylation in a differentiation primed myeloid leukemia cell line using an unbiased technique. The „Chip to clone” strategy was used previously to identify binding sites of transcription factors (Weinmann, 2001). Transcription factors have consensus

binding sites that can be identified by in silico analysis and several biochemical methods can be used to verify the binding. In the case of a transcriptional cofactor that binds to a whole array of sequence specific transcription factors and has a well defined enzymatic activity identification of the genomic loci it binds to can be problematic. The post translational modification produced by this cofactor can be used for such analysis. It was shown previously that posttranslational histone modifications localised to specific loci are produced on much wider genomic areas than the localization of the transcription cofactors responsible for them (Bernstein, 2005) (An, 2004). This phenomenon has not been explained yet, nonetheless we decided to use this observation in order to identify genomic loci marked by PRMT1 via H4R3 methylation. The localization of H4R3 methylated loci was achieved by using chromatin immunoprecipitation with an antibody raised against methylated H4 arginine (Wang, 2004). This antibody should bind specifically to genomic loci marked by PRMT1 (Tang, 2000a; Wang, 2004).

The „Chip to Clone" method is summarized in figure 1. Briefly, HL60 cells were treated with 1.25% DMSO for 16 hours to achieve a differentiation primed state characterized by increased H4R3 methylation. Cells were fixed with 1% formaldehyde, sonicated to an average fragment size of 500bp. Figure 2 shows the predominant DNA fragment size of the sheared chromatin. We used anti H4R3-metyl antibody and protein A-agarose beads to immunoprecipitate mono and dinucleosomes that presented this modification. Beads were washed extensivelly, and immunoprecipitated DNA purified. After isolation of DNA, protuding ends of the DNA elements were filled to create blunt ends. Generated fragments were cloned then in a low background vector, transformed and the individual colonies isolated. Plasmids from individual colonies were purified. Inserts were identified by restriction enzyme analysis and by standard PCR with vector specific M13 primers and agarose gel electrophoresis (Figure 3). Further analysis was carried out after sequencing the isolated plasmids.

By sequencing 111 of these cloned sequences we found that 54 of the sequenced clones had DNA fragments substantially smaler than those which could originate from the immunprecipitated nucleosomes. These fragments, considered by us junk DNA, were

large enough to provide disruption of the lethal gene included in the plasmid but unlikely to originate from an immunoprecipitated nucleosme that contains a DNA fragment of at least 140 bp. 57 fragments were containing fragments longer than 150 bp, corresponding to mono-, di- or trinucleosomes. We decided to analyse in detail the genomic localization of these. First we performed a BLAST analysis with the identified sequences. 48 of them were of human origin, 7 of bacterial origin and two of them of salmon origin. The bacterial origin was probablly due to contamination with bacterial genomic DNA fragments and the salmon DNA from the salmon sperm DNA used as a blocking agent for the protein A agarose beads.

We considered a match in the human genome a sequence with more than 95% identity along the blasted query sequence. At the further stages of the analyses of the sequences we used the following categories:

1. sequence with unique localization: a sequence with a unique match in the human genome
2. repetitive sequence: a sequence with a match in one of the known repetitive elements in the genome. In these cases the blasted querry gave hits in the number of thousands along various chromosomes.
3. repeted sequences with unique localization: those sequences that were found several times in our library but had a unique genomic localization.

From the 48 human sequences 38 of them provided us with a perfect match, that means the whole length of our query sequence could be alligned with more than 98% identity to a human sequence. In one case we found a duplicate location with identical score but both of these locations were in gene poor regions. The 38 human sequences contained 26 unique sequences, 12 repeted unique sequnces and 10 repetitive sequences. From the 10 repetitive sequences we found 6 unique repetitive sequences and 4 that were repetition of a single clone from the 6 previously mentioned. We decided to further analyse the genomic localisation of the 26 unique sequence hits with the UCSC genome browser. From the 26 analysed sequences we found 14 nearby known genes. We set up a cut off value of 10kb for the analyses of proximity of genes. 9 of the sequences were found in

introns, one at an intron exon overlap and 4 within 5 kb from 5‘ ends of genes. One hit was at 5‘ of a predicted promoter. 12 of them had no genes within 10kb. 6 of these had regions with conserved TF binding sites within 5 kb. Table 1 presents the names of the 14 identifed genes, their locations on chromosomes, the location of immunprecipitated fragment, relative to the gene and the conserved transcription factor binding sites within 1 kb relative to the immunoprecipitated fragment.

PANTHER Analysis

The PANTHER (**P**rotein ANalysis **TH**rough Evolutionary **R**elationships) (Mi, 2005) (Thomas, 2003) database allows complex annotation of proteins and genes. We selected the data described as “Molecular functions” and “Biological processes” of the Version 5.0 (release date Jan. 1, 2005). This annotation allowed us to align the identified genes along biological processes as shown in Table 2. Signaling, cell differentiation, immune response and development are frequently associated with the identified genes.

Analysis of Conserved Transcription Factor Binding Sites

Next we decided to analyse the conserved transcription factor binding sites (TFBS) in the 5kb region of the 26 identified immunoprecipitated fragments regardless wheter there were located in the proximity of genes or not. We found 39 conserved TFBS, with 12 of them beeing present more than one time. In Table 3 we present those 6 TFBS that were present at least three times in this list, with their corresponding transcription factors, according to the Biobase library (Matys, 2003). Two of these were published to have conections with the family of protein arginine methyltransferases, namely MEF2 with PRMT4 (or CARM1) (Chen, 2002) and FOXO as being an inducer of BTG1 the activator of PRMT1 (Bakker, 2004).

Discussion

The level of methylation on arginine residues depends on the level of methyl donor Adomet, the level of methyltransferases (PRMT-s) and the level of demethylases present in the cell at a specific point in time. Methylation of arginine residues is involved in multiple ways in immunological responses (Boisvert, 2005a). Methionine adenosyltransferase (EC 2.5.1.6) is a key enzyme of cellular metabolism and catalyzes the formation of S-adenosyl-methionine (Adomet) from l-methionine and ATP. Adomet is the main methyl donor of the cell, a precursor of polyamine synthesis of molecules such as spermine and spermidine (Moreira, 2004). An arginine group may be methylated to produce methyl-arginine, while the aminoacid L-arginine may be oxidized by NOS to produce citrulline and NO and may be hydrolyzed by arginase to produce ornithine and urea. Importantly, methyl-arginine groups may be de-iminated to form citrullinated proteins. The link between Th1 and Th2 switch regarding arginase or NOS was described previously (Moreira, 2004) (Bronte, 2003) (Hesse, 2001). Th1 cytokines (INFγ and TNFα) induce NOS and produce NO release while Th2 cytokines (IL4 and TGFβ) induce Arginase. NO in the culture medium is an extreamly potent inhibitor of T cell proliferation. On the other hand arginase induction by IL4 depletes arginine from the enviroment and by this also blockes T cell proliferation (Bronte, 2003). LPS treatment of macrophages or the presence of both IL4 and INFγ activates NOS and arginase at the same time. This is producing arginine depletion as a result of arginase activity and is switching NOS to produce O_2^- in addition to NO and by this the formation of peroxynitrite is achieved. Peroxynitrite will favor the nitrosylation of several signaling mollecules and interferes by phosphorylation on thyrosine residues. In activated T lymphocytes the enzymes responsible for synthesis of the main methyl donor molecule, Adomet, namely MAT II, MAT I/III were shown to be markedly upregulated (Halim, 2001; LeGros, 1997; LeGros, 2001). This means that in activated T cells there is an increase in the Adomet level and a depletion of arginine, two modifications that favor methylation of arginine residues of the proteins. One may assume that the inhibition of tyrosine phosphorylation by nitrosylation and activation of arginine methylation will further broaden the translation of specific immunological signals into distinct genetic

programs. Identification of protein substrates that may be methylated on arginine residues was carried out in several studies. Protein arrays, differential cloning and proteomic approaches were used to identify these substrates (Boisvert, 2003; Lee, 2002; Wada, 2002).

The enzymes involved in arginine methylation, members of the PRMT (protein arginine methyltransferase) protein family use as substrates arginine-glycine rich protein stretches (Wada, 2002) (Lee, 2002) and S-adenosine-methionine in order to catalyze the methylation reaction. PRMT1 is the main enzyme in the mammalian cell responsible for methylation of arginine residues (Tang, 2000a).

Involvement of arginine methylation in immunological processes is suggested by several reports. One of the first reports about the involvement of PRMT1 in regulation of immune responses was the publication of direct interaction between IFNAR1 (a component of INFα/β receptor complex) and PRMT1 (Abramovich, 1997).

STAT proteins that bind to INF receptors seem to be also modulated by arginine methylation. In particular methylation on arginine residues was reported as being involved in regulation of STAT1 (Mowen, 2001). Hypomethylated STAT1 presents a stronger interaction between STAT1-PIAS1 that alters STAT1 –DNA binding and this means that methylation of arginine residue in STAT1 is needed for the proper function of this molecule. On the other hand a member of JAK protein complex, JBP1 (JAK binding protein 1) was found to be another arginine methyltransferase, namely PRMT5 (Pollack, 1999). On the level of gene expression regulation, NFAT- one of the main transcription factor involved in cytokine gene expression- is activated by the presence of the arginine-methylated cofactor, NIP45 (Mowen, 2004) (Hodge, 1996).

If one takes a closer look at the main arginine methyltransferase of the cell, PRMT1 could be considered an apparently promiscuous protein. Methylation of STAT1 and NFAT, cofactor of nuclear receptors, DNA damage checkpoint control (Boisvert, 2005b) regulation of splicing was all described as involving PRMT1. One of the known

methylation reactions mediated by this enzyme is the methylation on the tail of histone H4 arginine residue in position 3. This modification is specific to PRMT1, while another methyltransferase, PRMT4 or CARM1, is involved in the arginine methylation in position 17 of the H3 histone tail. During embryonic development the expression of the PRMT1-reporter fusion gene was greatest along the midline of the neural plate and in the forming head fold from embryonic day 7.5 (E7.5) to E8.5 and in the developing central nervous system from E8.5 to E13.5.

PRMT1 -/- animals die between the embryonic day E4.5 and E6.5 between implantation and gastrulation suggesting that the modification is crucial for organ development in the body (Pawlak, 2000). Interestingly PRMT1 -/- ES cells are viable and are dividing, suggesting that this enzyme is not indispensable for life per se (Pawlak, 2000).

We decided to further characterize the links between gene expression regulation and arginine methylation by PRMT1. HL60, myeloid leukemia cell line if primed with DMSO shows an increased level of H4R3 methylation. This state is characterized by a transient, increased sensitivity towards retinoid signals and by this provides a signal integration step in cell differentiation processes. We used this cellular state to identify genomic loci marked by PRMT1. We used chromatin immunoprecipitation to identify genomic loci marked by PRMT1 with H4R3 methylation. The isolated fragments were cloned into a low background vector and isolated. After sequencing of 111 isolated colonies we identified 26 fragments that had a fragment size corresponding of mono, di- and tri-nucleosomes. 14 of these sequences were intronic or within the 5kb to 5‘ ends of known genes. Regulatory regions show a higher degree of conservation during evolution (Liu, 2004). This is why identification of conserved transcription binding sites in the proximity of H4R3 arginine methylated chromatin streches is a sign of the relevance of the specific loci. We found 39 conserved TFBS, with 12 of them being present more than one time. In table 3 we present those 6 TFBS that were present at least three times in this list, with their corresponding transcription factors, according to the Biobase library (Matys, 2003). Two of these were published to have conections with the family of protein arginine methyltransferases, namely MEF2 with PRMT4 (or CARM1) (Chen, 2002) and FOXO as being an inducer of BTG1 the activator of PRMT1 (Bakker, 2004).

To annotate the identified genes we used the PANTHER (Protein ANalysis THrough Evolutionary Relationships) (Mi, 2005) (Thomas, 2003) database. From the 14 genes 3 remained unclassified. The 9 classified genes contained 1 structural protein (Collagen 9A1), 1 amino acid transporter (SLC36A1), all the other genes are involved in signaling, cell differentiation, immune response and development.

To illustrate the possible connection between the H4R3 modification and its role in regulation of immune responses by PRMTs we summarize the known data regarding one of the identified sequence, the one located in the promoter of IL1α. IL1α was shown to be expressed in both blood poly-morphonuclear (PMN) and mononuclear (MNL) cells (Lord, 1991). Moreover IL1α in the presence of histamine or PGE2 controls the expression of IL1β (Vannier, 1993).
One of the genomic localizations identified by this screen is at 3kb upstream from the transcription start site of the gene IL1α. This location is on chromosomal band 2q13. Two conserved transcription binding sites are found within 2kb towards the 5 prime end of the gene V$FOXP1_01 V$BRN2_01 responsible for binding of FOX P1a and POU3F2. More interestingly in the first intron, at 5 kb from our located sequence two conserved transcription factor binding sites V$YY1_Q6 and V$COUP_01 are located that bind YY1 and COUP TF respectivelly.

PRMT 1 was shown to interact with YY1 and this interaction results in histone arginine methylation (Rezai-Zadeh, 2003). YY1 forms a complex with PRMT1 mediated by DRBP76 called also ILF3 (Interleukin enhancer factor 3) (Rezai-Zadeh, 2003). This complex favors H4R3 methylation and subsequent acetylation. The activated gene is transcribed and RNA formed. Arginine methylation controls elongation also, via SPT5. SPT5 a regulator of elongation is also methylated on arginne residues by both PRMT1 and PRMT5 on partially overlaping methylation sites (Kwak, 2003).

At the RNA methyltransferase complex level, PRMT1 and PRMT5 (JKB1-janus kinase binding protein1) produce asymmetric and symmetric methyl arginines (Yanagida, 2004).

After splicing and formation of mRNA, ILF3 through its RNA binding domain binds the formed mRNA and interacts with exportin5 (Brownawell, 2002). This interaction between ILF3 and cytokine mRNA was reported to stabilize the formed mRNA molecule (Shim, 2002). This stabilisation is concomittant with the export from the nucleus via XPO5 (expontin 5) which is a Ran Gtp mediated process (Brownawell, 2002; Gwizdek, 2004). This stabilization of cytokine mRNA during nuclear export was documented for IL2 mRNA molecule (Shim, 2002).

Quite interesting members of ILF family (ILF1 and ILF2) were published as being methylated on arginine residues in a proteomic approach (Boisvert, 2003), while ILF3 on a protein array (Lee, 2002). ILF3 was shown to be an activator of the methyltransferase activity of PRMT1 (Tang, 2000b). If taking together these data one may formulate an attractive hypothesis that arginine methylation seems to control the expression of IL1 citokines at various levels from chromatin level through splicing and nuclear export till mRNA stabilization. Although these levels are separated in time and space according to the classical gene expression regulation point of view, the presence of PRMT1 at various levels fits well in the unified theory of gene expression (Orphanides, 2002) and arginine methylation might be a comon regulator of these processes.

Summary

In order to further characterize the role of arginine methylation in signal integration and developmental processes we carried out mapping of the genomic loci marked by PRMT1 via histone H4 arginine 3 methylation. For this we used HL60 cell line previouslly shown to enter in a transient, primed state characterized by increased H4R3 methylation. We used chromatin immunoprecipitation and cloning to isolate genomic loci marked by H4R3 methylation. After sequencing and in silico analysis we found that all of the genomic hits identified were intronic or at 5‘ end of specific genes. The locations identified were enriched in conserved transcription factor binding sites of POU2F1, MEF-2 and FOXL1 factors. A great number of the genes in the proximity of the identified genomic loci were involving signaling pathways and developmental processes.

Furhter analysis of the identified loci needs to be done in order to decipher the role of arginine methylation in regulation signaling pathways and gene expression.

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Figure legends

Figure 1.

Overview of the Chip to clone method. (1) Protein - DNA interactions in a cell result in specific post-translational modifications on the histone tails. (2) Protein - DNA interactions are fixed with 1% formaldehyde. (3) Chromatin is sheared by sonication to mono-, di- and tri-nucleosomes. (4) Specific antibodies against modified histone tails will bind the H4R3 methylated nucleosomes. (5) Antibodies bind to Protein A beads. (6) Nucleosomes with methylated arginine residues are isolated by extensive washing and centrifugation of Protein A beads. (7) DNA is isolated (8) Isolated DNA fragments are cloned in a low background vector.

Figure 2

Average fragment size of the sonicated DNA. Lanes 1-3 show an increasing amount of purified sonicated DNA, while M refers to the marker.

Figure 3

Distribution of different fragments after BLAST and BLAT analysis as described in the Results section. (A) 54 fragments from 111 analysed were of expected size (larger than 150bp). (B) 48 of these 54 fragments were of human origin. (C) 26 human unique sequences were considered for BLAT analysis. (D) 14 hits are within 5kb of known genes.

Figure 4

Cloned fragments are identified with vector specific PCR using M13 primers. The product was run on a 2 % standard agarose gel by electrophoresis. Different lanes show different isolated clones and M refers to the marker.

Figure5

Genomic context of the identified H4R3 methylated site in the promoter of IL1α. Conserved transcription binding sites, and the cloned fragment are shown with arrays.

Table 1

Genes identified by cloning H4R3 methylated nucleosomes, their chromosomal locations, their position towards the genes and the conserved transcription factor binding sites within 1kb.

Table 2

PANTHER (Protein ANalysis THrough Evolutionary Relationships) analysis of the identified genes. Data described as “Molecular functions” and “Biological processes” are shown. One relevant reference is shown independently of PANTHER analysis, if any available.

Table 3

Conserved transcription factor binding sites overrepresented in the regions identified by chromatin immunoprecipitation. The genomic context of 5 kb of all 26 identified immunoprecipitated fragments was analysed. 39 conserved TFBS were found, 12 of them beeing present more than one time. Here we present the 6 overrepresented TFBS with their corresponding transcription factors, according to the Biobase library and the number of their occurencies in the analyzed genomic locations.

GENE NAME	CHROMOSOMAL LOCATION	POSITION TOWARDS THE GENE	CONSERVED TFBS WITHIN 1KB
AMPH 1	7p14.1	intronic	V$ICSBP_Q6
Cadherin 23	10q22.1	intronic	V$S8_01, V$PAX2_01
Collagen 9 alpha 1	6q13	5‘	VFOX_Q2, VMEF2_01
DCAMKL1	13q13.3	intronic	-
DSU	2q35	intronic	V$RORA2_01
EYA3	1p35.3	intronic	-
IL1A	2q13	5‘	V$FOXP1_01, V$BRN2_01
IL1RAPL2	Xq22.3	intronic	-
KIAA0476	1q21.3	5‘ from predicted promoter	-
MGC27434	8q24.21	intronic	V$OCT1_B
PAPD1	10p11.23	intronic	V$HP1SITEFACTOR_Q6
ROBO1	3p12.3	intronic	V$SOX5_01
SLC36A1	5q33.1	5‘	-
SPAG16	2q34	intronic	-

Table 1. Balint et al

GENE NAME	PANTHER MOLECULAR FUNCTION	PANTHER BIOLOGICAL PROCESS	REFERENCE
AMPH 1	Membrane traffic regulatory protein	Endocytosis Transport Neurotransmitter release	(Shang, 2004)
Cadherin 23	Cadherin	Cell adhesion-mediated signaling Cell adhesion Oogenesis Embryogenesis Anterior/posterior patterning Heart development Cytokinesis Cell proliferation and differentiation	(Bolz, 2001)
Collagen 9 alpha 1	Extra cellular matrix structural protein	Cell structure	(Mayne, 1985)
DCAMKL1	Non-receptor serine/threonine protein kinase	Protein phosphorylation Calcium mediated signaling Neurogenesis Cell motility	(Sossey-Alaoui, 1999)
DSU	Unclassified	Unclassified	(O'Sullivan, 2004)

Table 2. Balint et al

GENE NAME	PANTHER MOLECULAR FUNCTION	PANTHER BIOLOGICAL PROCESS	REFERENCE
EYA3	Other hydrolase	Vision Developmental processes	(Li, 2003)
IL1A	Interleukin	Cytokine and chemokine mediated signaling pathway Ligand-mediated signaling Macrophage-mediated immunity Cytokine/chemokine mediated immunity Cell cycle control Cell proliferation and differentiation	(Lord, 1991)
IL1RAPL2	Interleukin receptor	Cytokine and chemokine mediated signaling pathway	(Khan, 2004)
KIAA0476	Guanyl-nucleotide exchange factor	Other signal transduction	-
MGC27434	Unclassified	Unclassified	(Storlazzi, 2004)
PAPD1	Unclassified	Unclassified	-
ROBO1	Immunoglobulin receptor family member Defense/immunity protein	Cell adhesion	(Rhee, 2002)
SLC36A1	Other transporter Amino acid transporter	Amino acid transport Transport	(Sagne, 2001)
SPAG16	Microtubule family cytoskeletal protein	Cell motility	-

Table 2. Continued, Balint et al

TF BINDING MATRIX	TRANSCRIPTION FACTOR	NUMBER OF OCCURENCES
V$OCT1_B	POU2F1	6
V$MEF2_Q6_01	MEF-2	5
V$FREAC7_01	FOXL1	4
V$PAX4_04	Pax-4a	3
V$FOXO4_02	FOXO4	3
V$EVI1_05	Evi-1	3

Table 3. Balint et al

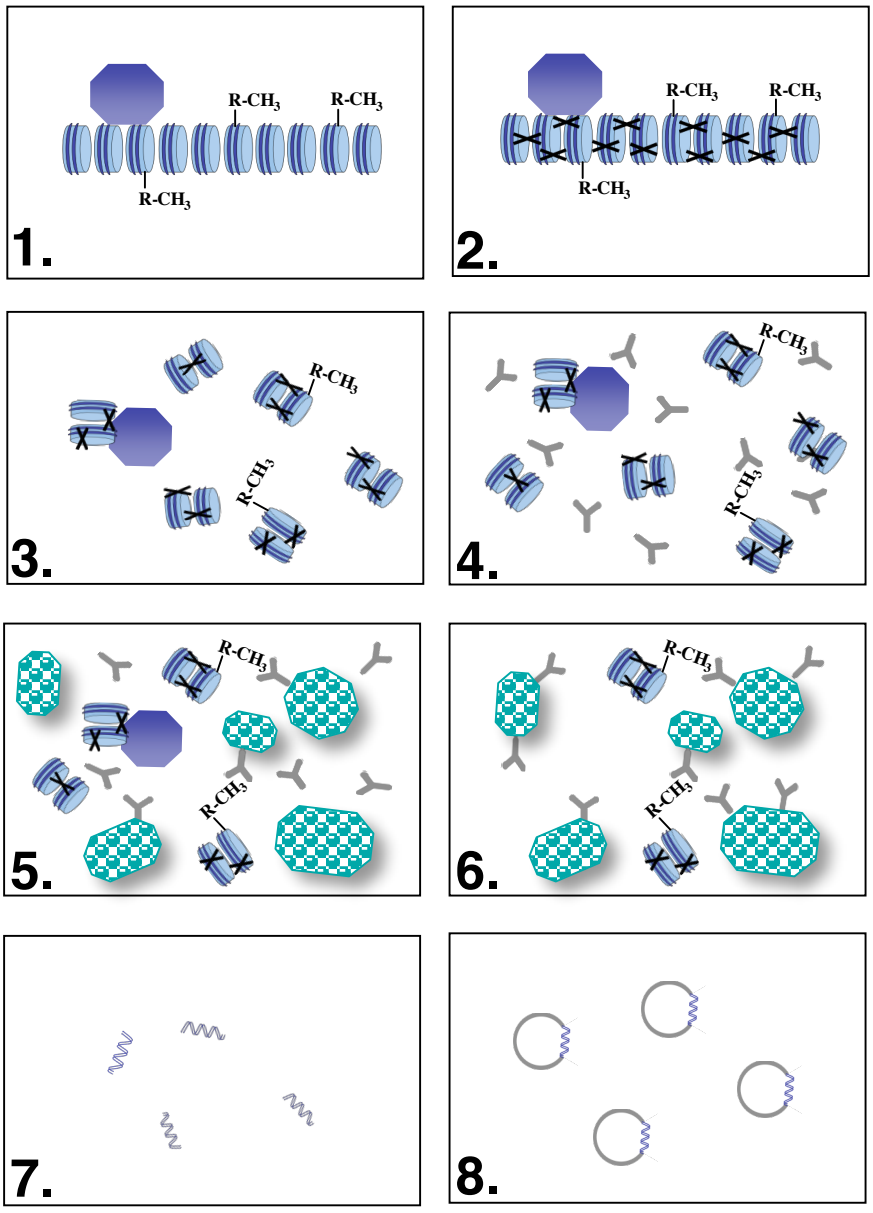

Figure 1. Balint et al

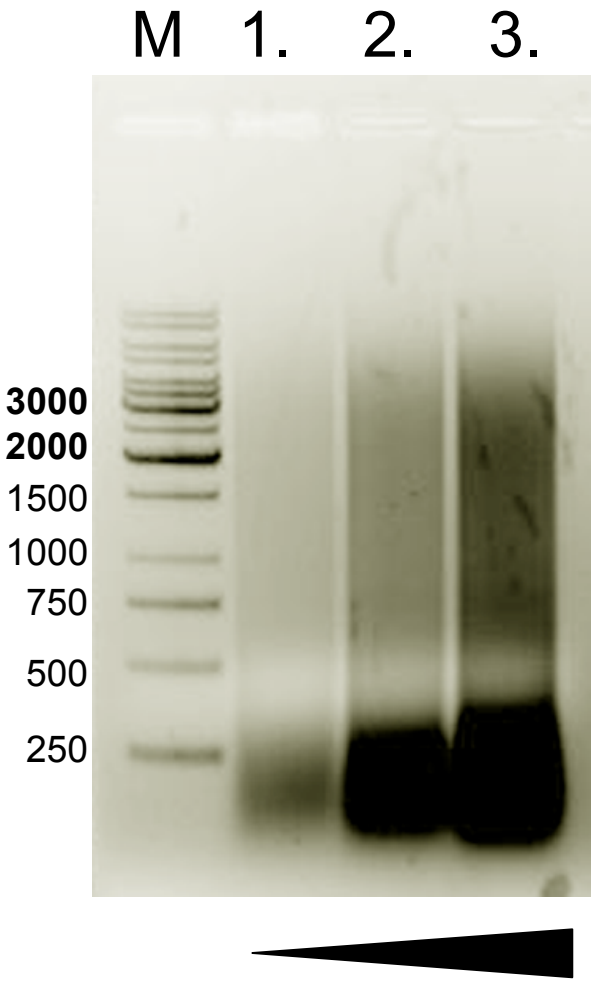

Figure 2. Balint et al

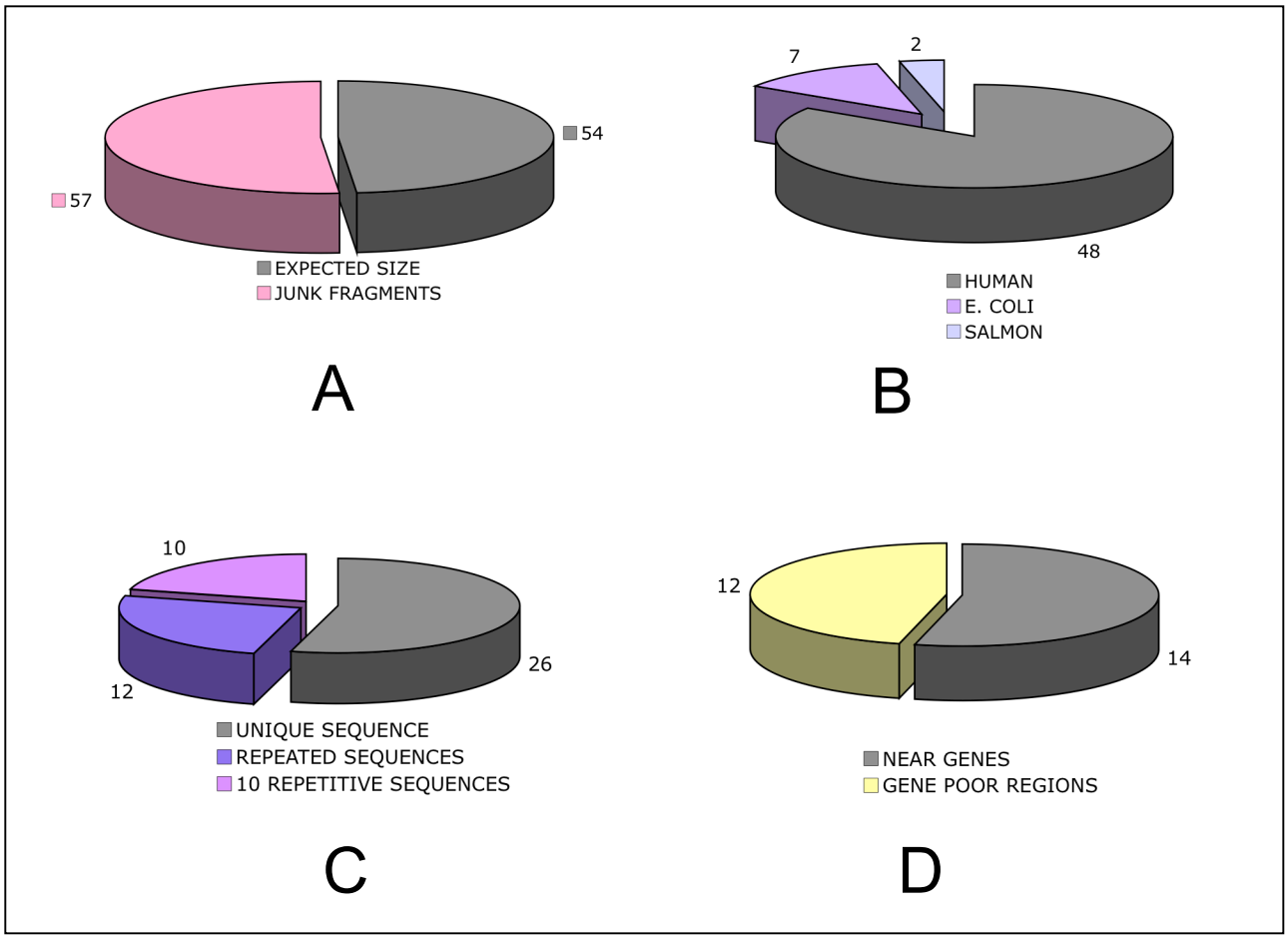

Figure 3. Balint et al

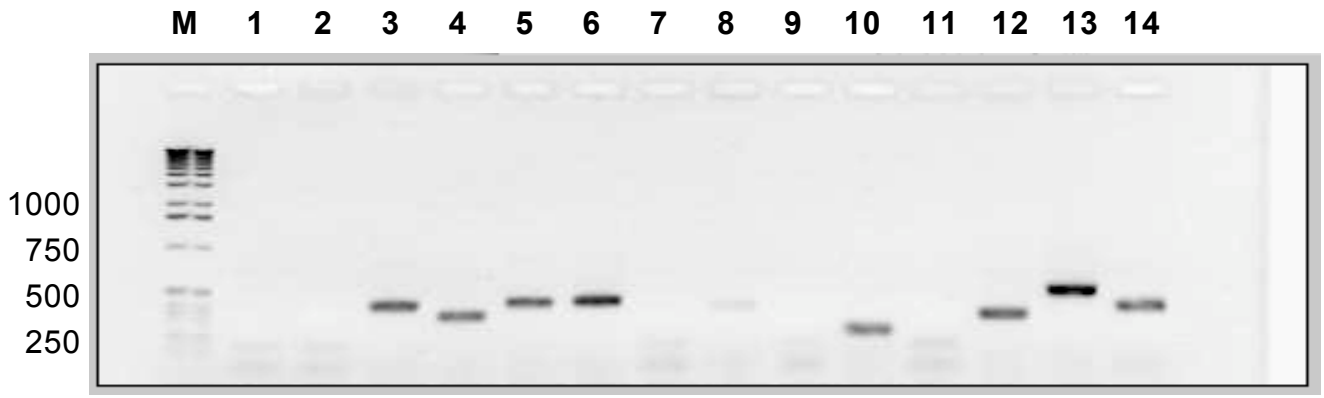

Figure 4. Balint et al

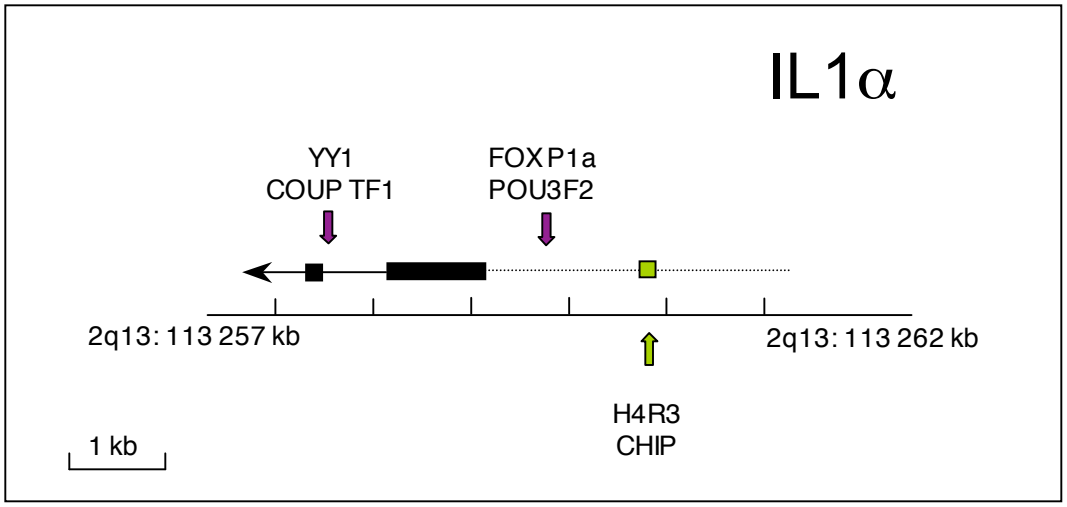

Figure 5. Balint et al