Insulators are crucial cis acting elements which demarcate and maintain independently regulated domains in the eukaryotic genome. Insulators work to restrain the enhancers to their cognate promoters preventing nonspecific activation by them and also several insulators act as barriers to spread of chromatin states across them and are thus able to protect against position effect variegation or inappropriate silencing of genes due to advance of silencing heterochromatin. The insulators are able to manipulate the higher order structural organization of the genome and also modify the local chromatin environment to cause insulation activity. These activities can coexist within the same insulator or may be present independently depending upon the genomic context in which the insulator is operational.

Several proteins bind to insulator elements and cause formation of an active insulator. The best characterized vertebrate insulator protein CTCF has been shown to bind and organize functional insulators at several vertebrate insulator sites and its homologues have been shown to mediate insulation activity in several other organisms also. CTCF has been characterized to bind about 20,000 sites in the human genome. However, the exact functional nature of these sites as enhancer blockers or barrier elements or both in the endogenous context remains unclear. CTCF binding has been reported at chromatin domain boundaries in some studies implicating a chromatin barrier like activity associated with CTCF. However, in other studies, CTCF association was also seen with an absence of a chromatin barrier function. Thus, conflicting evidence exists regarding the chromatin barrier activity of CTCF dependent insulators and this raises doubt regarding the role of endogenous CTCF binding sites as chromatin barrier as well as enhancer blocking elements. Currently in context of insulator mechanisms the interdependence of chromatin barrier and enhancer blocking activities for insulator functioning is also not clear.

In order to address these issues, analysis of a well defined CTCF dependent insulator, the \textit{H19-ICR} insulator which acts as an enhancer blocker \textit{in vivo} in a CTCF dependent manner at its endogenous location, was carried out. The chromatin structure was examined in the \textit{H19-ICR} insulator region using chromatin immunoprecipitation assays. Specifically the distribution pattern of hallmark histone modifications associated with active and repressive chromatin was determined in an allele specific manner upon maternal and paternal inheritance of wildtype or insulator deleted alleles. The analysis was done in two independent tissue samples namely PMEFs and neonatal liver. The study resulted in the following observations:
Summary

- Analysis of expression pattern of H19 and Igf2 genes in PMEFs of various genotypes generated for this study showed an imprinted expression pattern of wildtype alleles and also clearly demonstrated the role H19-ICR in maintaining imprinted expression pattern of H19 and Igf2 genes in the PMEF system. The H19-ICR deleted alleles showed an altered and biallelic expression profile of Igf2 upon maternal inheritance highlighting the importance of H19-ICR in regulating imprinted expression at the locus. Very surprisingly, in case of PMEFs, the DMRdelG allele harboring the H19-ICR deletion, lacked H19 expression upon either maternal or paternal inheritance which is in contrast to expression pattern of DMRdelG allele in skeletal muscles indicating that some aspects of regulation of H19 gene expression is different in case of PMEFs as compared to tissues like neonatal liver and skeletal muscle.

- The maternally and paternally inherited wildtype alleles were associated with different and contrasting chromatin states. The wildtype paternal allele is associated with dimethyl H3 K9 repressive chromatin modification and thus shows a generalized repressed chromatin structure in the H19-ICR and associated regions. On the wildtype maternal allele, the H19-ICR insulator and the region downstream of it shows an active chromatin structure rich in dimethyl H3 K4 and acetyl H3 K9 modifications. These chromatin states are closely correlated with the expression status of the H19 gene on the wild type maternal and paternal alleles.

- The wild type maternal allele shows distinct domains of chromatin structure, the region upstream of the H19-ICR insulator is rich in repressive dimethyl H3 K9 modification, suggesting a repressive chromatin structure in this region. In the region downstream of the H19-ICR insulator, an active chromatin structure characterized by presence of dimethyl H3 K4 and acetyl H3 K9 modifications is observed. These domains are separated by the H19-ICR insulator. The presence of distinct chromatin domains with contrasting chromatin modifications and a region just downstream of the H19-ICR insulator associated with a sharp enrichment of dimethyl H3 K4 modification is suggestive of a chromatin barrier function of the H19-ICR insulator which separates the two chromatin domains on the maternal allele. The active
The chromatin structure observed in the *H19-ICR* insulator downstream region is consistent with the actively expressing state of the maternal *H19* gene.

- To test the chromatin barrier activity of the *H19-ICR* insulator definitively, the chromatin structure in the *H19-ICR* insulator associated upstream and downstream regions was studied after deletion of the insulator. Upon deletion of *H19-ICR* insulator from the maternal allele, no significant change in chromatin structure or spreading of upstream silent chromatin into the *H19* gene region was observed. On paternal inheritance of the *H19-ICR* insulator deleted allele, the *H19* gene region gains activating chromatin modifications and the upstream repressed chromatin structure did not spread into the downstream *H19* gene region. Thus, no major alteration in chromatin structure or spreading of chromatin states is seen on either the maternal or paternal alleles upon deletion of the *H19-ICR* insulator which suggests against a possible chromatin barrier activity associated with the *H19-ICR* insulator.

- It is interesting that the active chromatin structure observed in the maternal and paternal *H19* gene region is not correlated with the absence of transcription from the *H19* gene on both the maternal and paternal DMRdelG alleles in PMEFs.

- A large part of the intervening region between the *H19-ICR* and *Igf2* gene was found to be rich in silent chromatin modifications and devoid of activating chromatin modifications irrespective of the transcriptional status of *Igf2* suggesting that these hallmark chromatin modifications do not comprise a signal important for the activation of *Igf2* gene by a possible tracking mechanism of enhancer action.

- The role of enhancer elements in organizing the observed chromatin structure in the *H19* gene region and *H19-ICR* insulator downstream region was investigated. Well defined set of enhancers activate both the *H19* and *Igf2* genes. Recently the enhancers have been shown to physically interact with the maternal *H19-ICR* and the *H19* gene promoter. The chromatin structure in the region downstream to the *H19-ICR* on the maternal allele was studied in the absence of endodermal enhancers in neonatal liver samples. Surprisingly, no major change in chromatin structure was observed in the *H19-ICR*
downstream region upon enhancer deletion. Even though the enhancer deletion leads to a drastic reduction of H19 transcription, no significant alteration of chromatin structure was observed upon enhancer deletion. This suggests that the observed chromatin structure in the H19-ICR downstream region and the H19 promoter region is possibly organized by the sequences in these regions independently. It is likely that other key components crucial for transcription are provided by the enhancer.

In conclusion, the H19-ICR insulator, which is a well characterized CTCF dependent enhancer blocker at the Igf2/H19 locus regulating imprinted expression of Igf2 and H19 genes, in its active form on the maternal allele separates chromatin domains with distinct chromatin structure. However, no chromatin barrier activity is associated with it since upon its deletion from the maternal allele it was observed that no spreading of chromatin states occurs. However, the normally silent maternal Igf2 gene was activated indicating loss of enhancer blocking activity. This suggests that the chromatin barrier and enhancer blocking activities can act independently to organise functional insulators and are thus not interdependent for insulator activity. The in vivo dissection of these activities at the H19-ICR insulator presented here is the first such study to our knowledge and has implications for insulator mechanisms and CTCF dependent insulator elements. This study suggests that CTCF dependent insulators may not necessarily act as chromatin barrier elements and demands thorough investigation of the results obtained from genome wide studies proposing a chromatin barrier activity of CTCF bound insulators, on the pattern of the study presented here. However, this study presents analysis of a single insulator element and thus to more conclusively comment upon the nature of CTCF dependent insulators it is necessary to analyse more insulator elements. Considering current evidence from recent studies it can be inferred that insulator elements binding to CTCF protein may act differently, probably depending upon the context in which they are present and the additional proteins binding to them, as either enhancer blockers or chromatin barrier elements or probably as both. The absence of spreading of chromatin states even in the absence of the H19-ICR insulator on the maternal allele provides another example of maintenance of independent chromatin domains without a fixed chromatin barrier which may be possible due to counteracting the spread of opposing chromatin states. These results provide example of diverse
mechanisms that are operational within the genome to organise independent chromatin domains. Enhancers downstream of the H19 gene which also activate the expression of Igf2 gene do not modify the chromatin domain in the intervening region between the H19 and Igf2 genes in terms of the activating chromatin modifications studied and thus if the enhancer operates by tracking, the activating signal does not involve dimethyl H3 K4 and acetyl H3 K9 modifications. The enhancers also do not contribute significantly to chromatin organization in the H19 gene region. The H19-ICR insulator downstream region on the maternal allele independently organises the active chromatin domain probably due to H19 promoter activity. Both the H19-ICR and the enhancers, however, have important role to play in transcription of the H19 gene since deletion of either of them is associated with severe reduction or loss of H19 expression even though the region downstream of H19-ICR and the 5' portion of the H19 gene are associated with an active chromatin structure. These observations provide interesting leads to further investigate the mechanisms concerning the CTCF dependent insulator activity, mode of enhancer action and chromatin organization at the Igf2/H19 locus. Each of these trails is expected to enhance and clear our understanding of the Igf2/H19 locus and about gene regulatory mechanisms in general.