CHAPTER IV

P68 RNA helicase in human transcriptional and post transcriptional gene silencing
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Abstract
Proteins involved in RNA silencing mechanism, DICER1 and Argonaute family members are predominantly found in the cytoplasm. Here we found that p68, a RNA helicase that is implicated in miRNA silencing and myriad of cellular functions, localizes to both nucleus and cytoplasm in human cells. Moreover it is co-localized transiently on inactive X chromosome in cell lines of female origin. Depletion of p68 protein from human female cell lines reduces the levels of sXiRNA. Reduction of sXiRNA and p68 localization on the inactive X chromosome does not play any significant role in Xist coating and enrichment of H3 me3K27 for the maintenance of X silencing. But loss of p68 level decreases its ability on the siRNA mediated transcriptional and post transcriptional silencing. Together we describe p68 localization on the inactive X and its multifactorial contributions in miRNA mediated silencing, siRNA mediated transcriptional and post transcriptional silencing processes.

Introduction
Post transcriptional silencing mediated by microRNA known to occur in specialized structures known as P bodies (Liu et al. 2005a; Liu et al. 2005b). RNAi effector proteins, such as DICER1 and Argonautes specifically localize in the cytoplasm and also in P bodies (Billy et al. 2001; Sen and Blau 2005). In mammals Small RNA also mediates nuclear gene silencing processes at the level of chromatin as conserved as expected. Argonaute family proteins, Ago-1 and Ago-2 are involved in this process (Janowski et al. 2006; Kim et al. 2006). In S. pombe small RNA and Argonaute proteins localize to peri centromeric DNA and mediate HP1 binding and modification of Histone H3 on lysine 9 there by rendering higher order packaging of targeted DNA (Volpe et al. 2002; Noma et al. 2004; Verdel et al. 2004). Similarly, involvement of RNAi machinery including argonaute family proteins on heterochromatin like structure formation was demonstrated in Drosophila by Pal-Bhadra et al. 2004. Later binding of piwi protein to DNA has been reported in Drosophila, where Piwi is known to localize in specific bands of the polytene chromosomes depending on the presence and absence of RNA molecules (Brower-Toland et al. 2001).
P68 RNA helicase commonly referred as Lip is reported to be involved in miRNA mediated gene silencing in both Drosophila and mammals (Ishizuka et al. 2002; Fukuda et al. 2007; Salzman et al. 2007). In fly, dmp68 performs multiple functions in the nucleus specially RNA transport from the active transcription sites, rapid RNA turn over and transcriptional repression, heterochromatin silencing etc. As a member of nuclear RNAi machinery, it also intervenes in chromatin organization and chromatin insulator activity. (Buszczak and Spradling 2006; Lei and Corces 2006).

**Results and Discussion**

To investigate whether p68 localizes in the nucleus of mammalian cells, we have performed immunofluorescence assay in human WI-38 cells using p68 antibodies. In this cell line, P68 protein was localized predominantly in nucleus but a considerable amount appeared in the cytoplasm as dot like structure/s (Fig. 4.1). We have reported earlier that small RNA were produced from the XIST locus of the human X chromosome (chapter 1) and p68 is reported to be involved in small RNA maturation (Fukuda et al. 2007) and heterochromatin silencing in fly. These findings tend us to test whether nuclear fraction of P68 protein plays any significant role in X silencing. We double immunostained WI-38 cells, a female 44, XX cell line containing inactive X chromosome by using both H3K27 trimethyl antibodies (a marker for inactive X chromosome) and p68 specific antibodies. P68 protein was colocalized with enriched H3K27 trimethyl signals in nearly 20% of the nuclei (Fig. 4.2).

P68 in fly is required for heterochromatin silencing. So, next to test whether P68 has any role/s in heterochromatic marker localization on the inactive X chromosomes and whether it is involved in sXiRNA production, we have depleted endogenous p68 RNA levels in the HEK-293 cells using specific short interfering RNA (siRNA). Estimation of RNA by Semi-quantitative RT-PCR from the p68 siRNA transfected cells showed that p68 RNA level was reduced more than 60% of normal level by the siRNA mediated inhibition (Fig. 4.3). It indicates that similar to other effector proteins, p68 RNA helicase can also be effectively inhibited by using siRNA.

Earlier we have described that both sense and anti sense small Xist RNA that is complementary to Exon1 of human XIST locus were produced. To investigate the requirement of p68 RNA helicase in the formation of sXiRNA, we depleted p68 by transfecting siRNA
complementary to p68 mRNA and a quantitative Northern blot analysis of RNA extracted from control siRNA and p68 siRNA transfected cells was performed using XIST Exon-1 specific probes. The results showed that sXistRNA level was significantly reduced (~65%) in the p68 depleted cells (Fig. 4.3). These results revealed that p68 is required for sXistRNA processing from the human XIST locus. To verify further the efficiency of P68 on maturation of miRNA in human cells, p68 was functionally depleted in HeLa cells. Genome wide profile of miRNA expression was analyzed using mature miRNA microarray analysis in both control siRNA and p68 siRNA transfected cells. The relative expression profile of microRNA pools revealed that p68 alters the microRNA expression grossly. Most of microRNA was proportionately reduced by the reduction of P68. But a reasonable number of miRNA showed an enhanced expression. These results demonstrated that P68 plays a crucial role in majority of the microRNAs expression and maturation in HeLa cells (Fig. 4.4).

To evaluate further the role of p68 RNA helicase in the heterochromatic formation, we have selected heterochromatin marker bound to inactive X chromosomes in HEK 293 cell lines in the p68 depleted background. We processed HEK-293 cells after transfecting with control or p68 siRNA and cells were probed by Xist RNA on the inactive X chromosomes. XIST RNA found to be localized normally on the inactive X chromosome in the p68 depleted cells as exemplified by the two distinct spots in majority of nuclei (Fig. 4.5). It is reasonable to predict that p68 might act downstream of the XIST recruitment. Xist binding on the silent X creates an environment for coating of histone repressive marks for down regulating the gene repression. So, to investigate whether p68 has any role on X specific histone repressive modifiers including H3K27, we have immunostained control and p68 depleted cells using H3K27 tri methyl histones antibodies on to inactive X chromosomes. Similar to XIST, H3K27 trimethylation did not alter the binding pattern on the 2 Xi of HEK293 cells in majority of their nuclei. Together these results suggested that localization p68 is not important for binding of Xist and H3 trimethyl K27 on the silent X chromosome for down regulation the X-linked gene expression, but we could not rule out their specific role and subtle contribution after Xist and H3me3K27 establishment.

Next to establish the contribution of P68 on siRNA mediated silencing in human. We designed and synthesized fluorescent tagged siRNAs that specifically target human GAPDH mRNA and a control siRNA not complementary to human genome. The efficiency of GAPDH siRNA for effective GAPDH mRNA silencing was monitored in the MCF-7 cells (Fig. 4.6).
p68 deficient cells, transfection of siRNA did not alter normal GAPDH expression level. Therefore a marked reversion of siRNA mediated silencing of GAPDH occurred in the presence of reduced p68 expression. These results demonstrated that P68 is necessary for siRNA mediated post transcriptional silencing.

Apart from repressing genes at the level of mRNA, promoter associated siRNA appears to induce transcriptional repression by DNA methylation in mammals and plant, while in flies in the absence of major DNA methlyase activity, promoter specific RNA promotes transcriptional repression via a group of histone tail modifiers. Therefore promoter associated transcriptional repression is well conserved cross species, but processes employed are distinct in each species. To evaluate if p68, a member of nuclear RNAi machinery has any specific role in promoter specific transcriptional silencing, in particular by inducing repressive histone modifications directly on the DNA sequence, we have used siRNA specifically designed to silence X linked androgen receptor gene promoter as a model gene, along with similarly designed control siRNA sequence non homologous to none of the sequences in human genome. We transfected AR specific siRNA or control siRNA into cells in which endogenous p68 protein was already silenced. The silencing of AR gene expression triggered by the introduction of AR promoter specific siRNA was disrupted in the p68 depleted cells, while control siRNA used in the place of p68 siRNA did not alter the AR siRNA induced AR gene expression levels, as detected by relative real time PCR analysis (Fig. 4.7). Therefore promoter associated siRNA induced silencing requires p68.

In this study, we described inactive X specific localization of p68 RNA helicase, a protein implicated in microRNA based post transcriptional gene silencing mechanism. P68 RNA helicase homologue, Lip has been implicated in the process of gene deactivation and maintenance of position effect variegation in flies (Csink et al. 1994) though the exact mechanism by which this is achieved has been a puzzle. Not only Lip plays an active role in nuclear gene silencing pathways, it has been reported to be a very important component of Drosha containing microprocessor complex involved in the processing and generation of microRNA both in flies and humans (Fukuda et al. 2007). The involvement of p68 in the siRNA mediated silencing mechanisms at transcriptional and post transcriptional levels is poorly understood. In our study using specific antibodies, we found that p68 RNA helicase localizes to specific domains within the nucleus. During the process of Xi establishment, it selectively
localizes to certain perinucleolar domains (Zhang et al. 2007). This process is specific to S phase of the cell cycle. P68 RNA helicase is known to be a component of nucleolus and interacts with quite a few nucleolar proteins though very little are understood regarding its function in this nuclear compartment (Nicol et al. 2000; Kahlina et al. 2004). Recent evidences relating to its roles in rRNA processing implicates a functional relevance towards its nucleolar localization (Fukuda et al. 2007). In plants, nucleolus plays an important role in generation and establishment of transcriptionally silent chromatin domains through RNAi related processes (Pontes et al. 2006). It is not known whether in animal kingdom similar nucleolar related processes/proteins play any important roles in gene silencing pathway. Our data indicates an association between p68 and inactive X chromosome. It is possible that p68 RNA helicase might regulate production of small RNA involved in nuclear silencing processes via nucleolar proteins that are involved in rRNA biogenesis. Indeed we found an essential role for p68 RNA helicase in sXiRNA generation from the XIST locus of human X chromosome. P68 and its closely related homologue p72 are indeed known to interact with proteins that are involved in both gene activation (Rossow and Janknecht 2003) as well as repression (Wilson et al. 2004). Both p68 and p72 bind to and brings about gene deactivation at the target promoters in mammalian cells (Wilson et al. 2004; Jacobs et al. 2007). Though it is localized to inactive X chromosome and involved in sXiRNA formation, like other proteins involved in RNAi mechanism, depletion of p68 does not have any effect on the localization of inactive X chromosome specific repressive marks, XIST and H3K27 tri methylation. There are two possible explanations for this paradox. X chromosome inactivation in humans is not complete and nearly 30% of X linked genes show differential escape from inactivation (Carrel and Willard 2005). As p68 localization on inactive X chromosome doesn’t spread to the entire Xi domain unlike H3K27 trimethylation, there might be a portion of X linked genes that might be susceptible for p68 mediated silencing. Lack of genome wide binding patterns of p68 precludes further explanation in this regard. Alternatively, the recalcitrant behavior of chromatin in somatic cells unlike that of ES cells, towards small changes in local milieu is responsible for lack of visible alterations in the Xi heterochromatin. It is also possible that large heterochromatic regions such as inactive X chromosome might be regulated simultaneously by several redundant pathways. Apart from its miRNA mediated roles, our study indicates that p68 RNA helicase plays important roles in siRNA mediated post transcriptional and transcriptional silencing processes. This is interesting considering that very few proteins
apart from argonaute family proteins were implicated in both transcriptional and post transcriptional gene silencing pathways. As p68 is present in both nucleus and cytoplasm, it will be interesting to see whether this protein directly binds to target DNA sequences and recruits other gene deactivating proteins including HDAC’s to the respective siRNA target promoters.
Fig 4.1. Localization of p68 RNA helicase predominantly in nucleus

WI-38 human lung fibroblast cell line was cultured on cover slips or in chamber slides. At 80% confluency, cells on the coverslips were fixed with 3.7% paraformaldehyde and immunostaining was performed. Mouse monoclonal antibody that specifically recognizes p68 RNA helicase was used to detect the localization of p68 in the cells. Nuclei was counter stained with DAPI.

Lower panel: p68 RNA helicase(Green), Nuclei(Blue)
Top Panel: Adapted from NCB 2005 showing the localization of RISC complex protein Argonaute-2 in cytoplasmic processing bodies (P bodies). hAGO2(Green), Nuclei(blue)
Fig 4.2. Localization of p68 RNA helicase to inactive X chromosome in human cells
To confirm whether any of nuclear localized p68 RNA helicase specifically localizes to inactive X chromosome, we have fixed the actively growing WI-38 female fibroblast cell line with 3.7% paraformaldehyde and performed double immunostaining on those cells with both H3K27 tri methyl antibody (as a marker for inactive X chromosome) along with p68 RNA helicase antibody.

p68 RNA helicase (Green) H3K27 tri methylation (Red). Xi region intensely stained by H3K27 tri methylation. White arrows indicate co-localization between H3K27 tri methylation and p68 RNA helicase on Xi (Inset)
100nM control or p68 RNA helicase specific siRNA was transfected into HEK 293 cells using Hiperfect transfection reagent twice on day 1 and day 3. Cells were harvested six days after first transfection and total cellular RNA was isolated using Trizol reagent. cDNA was prepared from total RNA using superscript reverse transcriptase.

Upper panel: PCR was performed using p68 RNA helicase gene specific primers and GAPDH primers were used to amplify GAPDH transcripts, that are used as loading controls.

Middle Panel: Enriched small RNA from control or p68 RNA helicase depleted HEK 293 cells was separated on 15% Urea PAGE gels and transferred on to Hybond N+ membrane. Blots were probed with ³²P-UTP labeled XIST Exon-1 derived probes. Blots were exposed to phosphor imager screens for 16-24 hrs.

Lower panel: EtBr stained rRNA bands in gel before transfer to membrane were used as loading controls.
<table>
<thead>
<tr>
<th>microRNA</th>
<th>Fold change in expression in p68 RNAi</th>
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<tr>
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<tr>
<td>hsa-miR-190</td>
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<td>hsa-miR-206</td>
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<td>hsa-miR-150</td>
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Fig 4.4. HeLa cells were transfected with 100 mM of either control or p68 RNA helicase specific siRNA. Total RNA was isolated from the transfected HeLa cells using Trizol reagent. Small RNA (<150 nt) was separated from higher molecular weight RNA and quality analysis was performed using agilent bioanalyzer. After determining the quality of RNA, small RNA was labeled using Agilent’s miRNA complete labeling and hybridization kit and hybridized to human miRNA microarray (V2) slide. After washes slide was scanned with Agilent scan software. Data analysis was performed using GeneSpring GX version 7.3. The decrease in mature levels of microRNA was shown above.
HEK 293 cells were transfected twice with 100nM of either control siRNA or p68 RNA helicase siRNA. After six days of transfection cells were fixed with paraformaldehyde and RNA FISH was performed on fixed and permeabilized cells. For detecting chromosomal localized XIST RNA, Cy3 labelled and random primed probes were prepared using Decaprime II kit. Equal amounts of probes were used to hybridize the fixed cells. DAPI is used to counterstain nuclei.

HEK 293 cells contain 2 inactive X chromosomes (Xi) and one active X chromosome.

FISH experiment revealed no differences between XIST localization on to Xi chromosomes in the absence of P68 RNA helicase. XIST (Red), DAPI (Blue).
POST-TRANSCRIPTIONAL GENE SILENCING

Fig 4.6. To test the effect of p68 RNA helicase depletion on post-transcriptional gene silencing, p68 RNA helicase siRNA was transfected along with GAPDH mRNA targeting siRNA into GAPDH expressing cell line, MCF-7. Three days after transfection RNA was isolated from both control and p68 depleted MCF-7 cells transfected with GAPDH siRNA. Real time analysis was performed using SYBR green with AR specific primers using 18S rRNA levels as internal control.
TRANSCRIPTIONAL GENE SILENCING

**Fig 4.7.** To test the effect of p68 RNA helicase depletion on transcriptional gene silencing, we transfected p68 RNA helicase siRNA along with Androgen receptor (AR) promoter targeting siRNA into AR expressing cell line, MCF-7. Three days after transfection RNA was isolated from both control and p68 depleted MCF-7 cells transfected with AR siRNA. Real time analysis was performed using SYBR green with AR specific primers using 18S rRNA levels as internal control.
Materials and Methods

Immunofluorescence

Cells were fixed with 3.7% Paraformaldehyde in PHEM buffer for 10 min and permeabilised with 1% triton X 100. Cells were used immediately for staining or stored in 70% alcohol at 4°C until use at least for a week. Immunofluorescence was carried out using 1:50 or 1:100 dilutions of following primary antibodies: rabbit H3K27 trimethyl (Upstate, Abcam), mouse DDX5 (Upstate), 1:100 or 1:200 dilutions of Secondary anti rabbit or anti mouse antibodies conjugated to FITC, Cy3 or Cy5 (Jackson) were used and finally counterstained with DAPI and viewed under confocal or fluorescent microscope.

XIST Fluorescent In-situ Hybridization

Control siRNA transfected or p68 siRNA transfected HEK293 cells were fixed with 3.7% Paraformaldehyde in PHEM buffer for 10 min and permeabilised with 1% triton X 100. Cells were used immediately used for staining or stored in 70% alcohol at 4°C until further use. RNA FISH was carried out using Cy3 labeled random primed XIST probes that were synthesized using the DECAprime™ II Kit (Ambion) at 37°C overnight as described (Wutz and Jaenisch 2000). Washes were performed at 39°C using 2X SSC and de-ionized formamide and with 2X SSC. Cells were counterstained with DAPI (vectashield) and observed under 20X and 100X objectives in confocal microscope (Carl-Zeiss).

siRNA transfections

Around 60% confluent HEK293 cells or MCF-7 cells were transfected with 100nM control siRNA or p68 siRNA. For analysis of XIST localization and sXiRNA levels, HEK 293 cells were transfected twice on day 1 and day 3 with above mentioned concentrations of siRNA and cells were either fixed with paraformaldehyde or total RNA was isolated from them. For dissecting the roles of p68 in transcriptional and post transcriptional siRNA mediated silencing pathways, MCF-7 cells were transfected with 100nM control p68 siRNA first and subsequently transfected with either 100 nM of GAPDH siRNA or AR specific siRNA or control siRNA. After three days of transfection, total RNA from cells was extracted and real time RT-PCR analysis was performed on the samples.

GAPDH siRNA: AUUCCAUGGCACCCGUCAAG
AR siRNA: CUCUCCACCUCCCCAGGCA (TT)
Control siRNA: GGCACGAUGGCGUACCGG (TT)
P68 siRNA: GCAAGUAGCUGCUAGAAUAU
**Semi-quantitative and real time RT-PCR**

Total RNA was isolated from cells transfected with above mentioned control or gene specific siRNA using Trizol exactly following manufacturer’s protocol. RNA was treated with DNA free (Ambion) to remove DNA contamination and reverse transcribed using superscript II (Invitrogen) following manufacturers protocol. PCR was performed on the DNA using following gene specific primers. Polymerase chain reaction was performed with the following primer pairs.

**GAPDH**

\[ 5'\text{-} TGAAGGTCGGTGTGAACGGATTTG\text{-}3' \]
\[ 5'\text{-} TGATGGCATGGACTGTGGTCATGA\text{-}3' \]

For real time PCR

\[ 5'\text{-} TCAACGACCACCTTTGTCAGCCTCA\text{-}3' \]
\[ 5'\text{-} GCTGGTGGTCCAGGGGTTACT\text{-}3' \]

**p68 RNA helicase**

\[ 5'\text{-} ATGTCGGGTTAATTCCAGTGACC\text{-}3' \]
\[ 5'\text{-} CCATGACATTTGCAAGGAAATTGG\text{-}3' \]

**AR**

\[ 5'\text{-} CCTGCGTTCCGCAAACCTACAC\text{-}3' \]
\[ 5'\text{-} GGACTTGTGATCGCGGTACTCA\text{-}3' \]

p68 RNA levels were observed in ethidium bromide stained gels. AR and GAPDH mRNA levels were measured by SYBR green real time PCR analysis with 18S rRNA as endogenous control in 7900 HT sequence detection system (Applied Biosystems). Transcript levels were calculated according to the $2^{-\Delta\Delta Ct}$ method.

**Small RNA analysis**

Total RNA was isolated from control or p68 siRNA treated samples using Trizol method (Invitrogen). Small RNA was enriched from total RNA using miRNA isolation kit (Ambion). Equal amounts of small RNA was separated on 15% urea-PAGE gels and transferred on to Hybond N* membranes. UV cross linked blots were probed with p$^{32}$-UTP labeled probes derived from XIST Exon-1 region. After extensive washes at 60°C with SDS and SSC, blots were exposed to phosphor imager screens for 16-24 hrs. For miRNA microarray, small RNA was enriched from HeLa cells transfected with control or p68 specific siRNA using miRNA isolation kit (Ambion). This small RNA was used to hybridize Agilent miRNA arrays.
References


Conclusions and Future directions

Gene regulation in eukaryotic cells is achieved by various ways. Recent discoveries implicated a wide range of small RNA molecules play a central and most important regulatory role across phyla. Some classes of small RNA, such as microRNA were predicted to regulate around 30% human protein coding genes (Bartel 2004; Farh et al. 2005). miRNA were also experimentally shown to post transcriptionally regulate several key messenger RNAs involved in several important pathways such as cell death, transformation, differentiation etc (Bartel 2009).

Apart from miRNA, recently several classes of endogenous small RNAs were reported from C.elegans, Drosophila and mouse (Kim et al. 2009). Though the function of majority of these endogenous small RNA are yet to be discovered, most of the these endo-siRNAs derived in yeast were shown to function in guiding heterochromatin complexes to the corresponding target DNA sequences in pericentric DNA (Moazed 2009). In Drosophila, rasiRNA derived mostly from the transposon flamenco locus that has been implicated in heterochromatin formation and regulates the transposon numbers inside fruit flies (Aravin et al. 2007; Brennecke et al. 2007). More over mutations in Drosophila genes that are essential for RNA silencing processes, Piwi, Aubergine and homeless, were also responsible for proper localization of Heterochromatin protein 1 (HP1) and incorporation of H3K9 di methylation on to specific sites in euchromatic regions and constitutive heterochromatin (Pal-Bhadra et al. 2004; Riddle and Elgin 2008).

In mouse ES cells deletion of Dicer gene had pleiotropic effects in differentiation along with failure to incorporate heterochromatin histone marks on to centromeric heterochromatin (Bernstein et al. 2003; Kanellopoulou et al. 2005). Similarly human-chicken somatic cell hybrids containing human chromosome 21 also showed defects in chromosome segregation and incorporation of kinetochoire assembly proteins on to centromeres (Fukagawa et al. 2004). But surprisingly similar studies in mouse Dicer and Argonaute-2 knock out ES cells have shown perfectly normal incorporation of repressive heterochromatin at the centromeres (Cobb et al. 2005; Murchison et al. 2005). These results are surprising considering the fact that same gene knocked out in all of these cell lines produced differential effect in different cellular environment.

In this study we determined the existence of a novel species of endogenous small RNA which might be essential for formation of heterochromatin on inactive X chromosome. XIST (X inactive specific transcript) non-coding RNA plays an important role in mediating
heterochromatin formation on human inactive X chromosome. Using several oligo riboprobes from the XIST Exon-1, we found that internal transcription in specific regions within the XIST locus transcribes small XIST RNA (sXiRNA). These small RNA are analogous to centromeric repeat derived small RNA as both these classes of RNA are derived from longer non coding RNA that are essential for heterochromatinization of loci from which they are derived. The derivation of small RNA from within the XIST locus is surprising in humans, because of two obvious reasons, one, unlike mouse XIST locus, where in a larger antisense RNA, TSIX overlaps majority of XIST transcript, there are no known overlapping transcripts in the human XIST Exon-1. Recently it has been reported that in mouse XIST and TSIX RNA forms duplexes during ES cell differentiation and these duplexes further processed into unconventional small RNA. In humans, TSIX transcript is non functional, with its RNA encompassing only parts of XIST transcript but not promoter and Exon-1 regions. This virtually rules out formation of XIST/TSIX duplex and its small RNA derivatives from the human cells. So, the sXiRNA described in our work has unique in origin. The existence of novel species of small RNA from XIST locus of human cells proclaim a wide array of regulatory Xist RNA that differs significantly in their origin between mammalian species. This was substantiated by existence of sXiRNA in both male cell lines, that does not produce from normal XIST RNA and female cell lines that express XIST RNA. Moreover, treatment with alpha amanitin, a known inhibitor of RNA Pol II did not inhibit the endogenous levels of sXiRNA further indicating the absence of RNA polymerase II role in the process of small RNA derived from XIST/TSIX transcripts and also other longer polymerase II derived transcripts. This is interesting to consider that no other transcripts were till now reported from any mammalian XIST loci that are independent of RNA polymerase II transcription. Considerable reduction in the levels of sXiRNA upon treatment of N-[1-(3-(5-Chloro-3-methylbenzo[b]thiophen-2-yl-1-methyl-1H-pyrazol-5-yl))-2-chlorobenzenesulfonamide, RNA polymerase III inhibitor indicated a possible role of this enzyme in transcribing sXiRNA precursors. Therefore it is reasonable to believe that small Xist RNA is RNA POL III dependent.

It will be interesting to see whether similar kind of internal transcription by polymerase III enzymes to generate smaller transcripts within the loci transcribed by RNA polymerase II. DICER1 is the enzyme that has been shown to generate 21-23nt small RNA in cytoplasm of somatic cells. Our study showed that sXiRNA depends in DICER1 for their processing.
Mammalian DICER1 is a cytoplasmic RNase III enzyme. Our analysis of localization of sXiRNA indicated that most of the mature sXiRNA are nuclear. How a cytoplasmic DICER1 cleaves nuclear sXirRNA. It can be explained by two alternate possibilities, one of is that like certain endogenous miRNA, that are processed by DICER-1 but localize to nucleus through specific export import pathways. Similarly, sXiRNA precursors also can be regulated in the same way with mature sXiRNA ultimately finding their way back into nucleus. It is also possible that DICER1 may function to some extent as an alternative of nuclear RNase III enzyme, as found in Drosha. Recently several endogenous siRNAs have been described in mammals with characteristic features of DICER1 cleavage that localize to both cytoplasm and nucleus (Tam et al. 2008; Watanabe et al. 2008). These findings together implicate that DICER-1 has a novel function in nuclear localized small RNA processing pathways. No significant difference in XIST and H3K27 localization between control and DICER-1 deficient cells is surprising, considering the dramatic effects of DICER-1 loss on the mouse X chromosome inactivation during initiation and establishment phases (Ogawa et al. 2008). In contrast, recent findings both in vivo in mouse embryos and mouse female ES cells show that DICER-1 does not compromise for both establishment and subsequent maintenance of X chromosome silencing which eventually support our findings in human cells (Nesterova et al. 2008; Kanellopoulou et al. 2009). These contradictory findings are difficult to explain, though there are differences in the techniques used to create DICER-1 knock out animals and ES cells used for this purpose. Our work in humans supports the majority view of redundant role for small RNA and DICER-1 in the processes of Xi maintenance. Recent findings and availability of human female ES cells (Silva et al. 2008) warrants the extension of our analysis to this model system. Analysis of existence and function of small RNA from XIST locus in ES cells before and after differentiation will satisfactorily address the roles of small RNA and DICER-1 at very early initiation and establishment phases in lieu with in vivo human embryonic development. Delineating DICER-1 function with regard to the domains and proteins that are involved in the processing of different small RNA will greatly help in deciphering involvement of RNA in the mammalian heterochromatin formation.

In conclusion, our work (See Figure 5.1) describes the formation of small RNA from human XIST locus, exclusively from the XIST locus of active X chromosome without the action of RNA polymerase II. These small RNA named sXiRNA, are processed into 21-23nt mature small RNA by the action of DICER-1. sXiRNA along with DICER-1 might mediate the
exclusion of positive histone modifications, mainly H3K4 di and tri methylation and H3K9 acetylation from inactive X chromosome. Like in yeast it is also possible that sXiRNA containing complexes might regulate incorporation of repressive histone modifications on to inactive X chromosome during early embryonic development where the chromatin might be more amenable to changes in RNA factors. Likewise the roles of sXiRNA on the maintenance of chromatin on active X chromosome also should be investigated in greater detail. DICER-1 knock down results in up regulation of certain tested X linked gene mRNA levels indicating that DICER-1 might regulate X linked gene expression via exclusion of positive histone marks from Xi. Though it is not clear from this study as to what are the effects of DICER-1 repression on gene expression from active X chromosome, some of the effects of DICER-1 on X linked gene expression might be because of its action on active X chromosome. It is also possible that miRNA mediated silencing pathway is inhibited in DICER-1 repressed cells, which in turn might have led to the global increase in H3K4 methylation levels and subsequent changes of the X linked gene expression. p68 RNA helicase, a miRNA pathway protein is found to be localized to inactive X chromosome and it is essential for the formation of sXiRNA from the XIST locus, but show no conspicuous role on Xist binding and enrichment of H3K27 methylation.
5.1 Cartoon depicting the formation and role/s of small RNA and RNAi machinery nuclease, DICER1 in facultative heterochromatin formation in human cells

1. Small RNA precursors are generated predominantly from the XIST locus of active X chromosome. This generation of small RNA does not involve RNA polymerase II but involves RNA polymerase III.

2. These small RNA precursors are processed into 21-24nt small RNA (small XIST RNA, sXistRNA) by RNAse III enzyme DICER1. Majority of mature sXistRNA accumulate in the nucleus.

3. Depletion of cellular levels of DICER1 protein does not affect the localization of XIST RNA and also Xi characteristic heterochromatin marks, H3K27 trimethylation and macroH2A incorporation onto Xi. DICER1 depletion results in decrease in levels of mature sXistRNA. DICER1 RNAi also increases global levels of positive histone mark, histone H3K4 di-tri methylation and its accumulation on Xi.

4. DICER1 depletion results in increased levels of X linked mRNA.

p68 RNA helicase, an gene implicated in miRNA biogenesis in Drosophila was found to co-localise with H3K27 trimethylation and at specific loci on inactive X chromosome. Depletion of p68 RNA helicase also results in decreased levels of sXistRNA but no alteration in XIST localization on Xi.