CHAPTER III

Human DICER-1 in sXiRNA processing and X chromosome dosage compensation
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Abstract

In mammals, painting of non-coding XIST RNA on one of the female X chromosomes onsets inactivation (Xi) while its antisense partner Tsix opposes silencing on active X (Xa). But some features of this developmental process vary among human and murines. Here we demonstrate repeat D region of XIST exon1 of human Xa transcribe a novel species of small RNA predominantly of size 21-23nt that are distinct from mouse in their origin and function. They are accumulated mainly in a DICER-1 dependent manner. Inhibition of DICER-1 blocks small RNA production and disrupts dosage compensation by up-regulating selected X-linked genes including XIST. Repression of DICER-1 does not compromise delocalization of XIST binding and H3K27 hypermethylation but promotes global increase in H3K4 di+tri methylation including Xi. Therefore, DICER-1 including small regulatory RNA from repeat D region might function after XIST and H3K27 binding on Xi for maintaining the balance of X-linked gene dosage by modulating Xi chromatin structure in humans.

Introduction

RNA interference is a highly conserved mechanism that is mediated by set of unique RNA binding proteins whose homologues are found in yeast to humans. Small regulatory RNA processing from the non-coding repetitive sequences (rasiRNA) plays crucial roles in transcriptional silencing and heterochromatin like structure formation (Pal-Bhadra et al. 2002; Volpe et al. 2002; Hall et al. 2003; Motamedi et al. 2004; Verdel et al. 2004; Josse et al. 2007). In general double stranded RNA that is generated by sense antisense transcription inside the cells is processed by RNase III class of enzymes into small RNA which are then incorporated into specialized class of protein assemblies whose effector proteins are Argonautes. In yeast, there is only one Dicer homologue which mediates the processing of endogenous double stranded RNA derived from the pericentric repeats into small interfering RNA. These small RNA in turn gets incorporated into an RNA-protein complex, RITS (RNA Induced Transcriptional Silencing) complex and mediates the heterochromatin formation and maintenance at the centromeric DNA.
with the help of Swi6 and Clr4 (Grewal and Rice 2004). Tetrahymena, which contain, germ line specific micronucleus and somatic macronucleus use DCL1p (Dicer like 1) processed micronuclear derived sense-antisense dsRNA into scnRNA (scan RNA) which are ultimately imported into newly formed macronucleus to silence or eliminate the homologous genome regions that are required only in germ line micronucleus but not in macronucleus (Mochizuki and Gorovsky 2004; Mochizuki and Gorovsky 2005). In Drosophila, Dicer-2 mutants show defects in nucleolar organization and heterochromatin formation at certain loci (Peng and Karpen 2007). Similar involvement of Dicer or small RNA in heterochromatin formation in mammals is not clearly established. Indeed small RNAs were reported to control imprinted loci in mammals (Seitz et al. 2003; Seitz et al. 2004; Davis et al. 2005; Cai and Cullen 2007; Royo and Cavaille 2008). Involvement of mouse Dicer in heterochromatin formation has been contradictory. Dicer has been shown to be involved in Histone H3 Lysine 9 methylation of centromeric repeat DNA in mouse ES cells which is contested by other group’s work. Existence of a facultative heterochromatin structure and clear lack of knowledge on the roles of Dicer and RNAi machinery in the establishment and maintenance of heterochromatin on human Xi prompted us to study the role of this enzyme. Further, presence of stretches of repetitive sequences in the XIST 5' end and exon-1 (Nesterova et al. 2001; Elisaphenko et al. 2008) and the numerous similarities of sXiRNA with rasiRNA in the constitutive heterochromatin hints the possible existence of Dicer related mechanism in maintenance of Xi heterochromatin.

**Results and Discussion**

To investigate whether small non-coding sXiRNA described in the chapter-1 has any repeat origin, we performed strand-specific northern blots using enriched small RNA extracted from different human cell lineages. By using different truncated radiolabelled riboprobes spanning repeat A, involved in silencing functions of XIST RNA and longest of the repeats, multiple repeats in the D region from XIST exon-1 of human X chromosome were used. Interestingly, no sXiRNA were found complementary to repeat A region (Fig. 3.1), but were readily detected with oligo riboprobes corresponding to repeat D region in human cell lines (Fig. 3.2).

RNAse III family enzyme DICER-1 cleaves long double stranded RNA into small interfering RNAs in the human cell lines (Bernstein et al. 2001; Hutvagner et al. 2001). To test whether DICER-1 has any role in sXiRNA production, we depleted DICER-1 mRNA by transfecting
DICER-1 specific siRNA in the HEK-293 cells and HeLa cells. The reduction of DICER-1 expression by transfection of DICER-1 siRNA was measured at mRNA level via semi quantitative RT-PCR analysis using β-Actin as internal control (Fig.3.3). To determine whether this reduction of DICER-1 is sufficient to disrupt siRNA mediated mRNA silencing, cy3 labeled GAPDH siRNA was co-transfected in the DICER-1 siRNA transfected HeLa cells. A considerable derepression of the GAPDH proteins in the DICER-1 deficient cells compared to control cells verified that DICER-1 level is not sufficient to mediate the depletion of GAPDH mRNA in majority of the Dicer-1 and GAPDH siRNA transfected cells (Fig.3.3, Lower panel). Immunofluorescence and western analysis by using DICER-1 specific antibody, in HEK-293 cells transfected with DICER-1 specific siRNA also showed significant reduction in DICER-1 protein (Fig.3.4). To further verify whether this reduction of DICER-1 protein is sufficient to compromise its endonuclease activity, levels of miR-93, a miRNA present in considerable amounts in HEK-293 cells was analyzed in DICER-1 RNAi cells. Substantial decrease in mature levels of miR-93 (Fig.3.5) indicated a successful functional knockdown of human DICER-1.

Next, to test if DICER-1 dependent cleavage is required for sXiRNA processing and accumulation, northern hybridization of RNA from control or DICER-1 siRNA transfected HEK-293 cells was performed using XIST Exon-1 specific RNA probes. The accumulation of sXiRNA was decreased markedly (<50%) in the DICER-1 deficient cells compared to control siRNA transfected cells (Fig.3.6). Therefore formation of sXiRNA involves DICER-1 processing step. In mouse ES cells, loss of DICER-1 delocalized XIST RNA painting on Xi as exemplified by the loss of stained Xi localized foci in the interphase nuclei (Ogawa et al. 2008). A similar in situ hybridization experiment using fluorescent XIST RNA probe in the human cultured cells showed that depleting DICER-1 do not compromise intense XIST RNA coating on the Xi chromosome/s (Fig. 3.7). Thus DICER-1 has no effect on the XIST localization in female human somatic cells. Heterochromatinization of human Xi is characterized by the recruitment of repressive histone tail modifications as epigenetic marks. Several histone modifiers including macro H2A, histone H3K27 trimethylation, H3K9 dimethylation and also by exclusion of positive histone modifications such as histone H3K9 acetylation (Boggs et al. 1996), H3K4 di+tri methylation and H4K16 acetylation (Jeppesen and Turner 1993) having profound effect on Xi heterochromatin. Similar incorporation of several modified histone isoforms including H3K27 trimethylation (Fig. 3.8), histone variant macro H2A-GFP (Fig. 3.9) on the Xi, observed
as two large foci in majority of interphase nuclei in control siRNA and DICER-1 siRNA transfected HEK-293 cells that have 2 Xi chromosomes. These results suggested that DICER-1 protein and DICER-1 dependent sXiRNA do not have effect on XIST binding or repressive histone modifications on the Xi. Further, accumulation of euchromatic histone modifications, H3K4 di+tri methylation and H3K9 acetylation were also observed in the DICER-1 deficient cells. H3K4 di+tri methylation in DICER-1 deficient cells was increased uniformly throughout the nuclei including Xi chromosome/s (Fig. 3.10). Analysis of H3K27 trimethyl coating as Xi marker, a similar elevation in H3K4 di+tri methylation and to some extent of H3K9 acetylation on Xi was noticed in the DICER-1 repressed nuclei relative to the control cells (Fig. 3.10). The gross elevation in histone H3meK4 methylated protein in entire nuclei was substantiated quantitatively by western blot analysis of total nuclear protein using H3K4 di+tri methylation antibody (Fig. 3.11). To ensure that enrichment of H3K4 methylation on the Xi increases X-linked transcripts in DICER-1 knockdown cells, expression of several X-linked mRNAs were measured by real time RT-PCR analysis. A consistent up regulation of X-linked mRNA (Fig. 3.11) including XIST (Fig. 3.10) in DICER-1 deficient cells relative to control siRNA transfected cells demonstrated that DICER-1 is involved in a post- Xi establishment phase for maintaining the balance of X-linked gene dosage in human somatic cells. As reported above, XIST binding and hypermethylation of histone H3K27 contributed in Xi establishment and their subsequent maintenance is not DICER-1 dependent. These results in human cells were consistent with earlier findings that deletion of DICER-1 has no effect on XIST binding on Xi in the mouse T cells (Cobb et al. 2005), embryonic stem cells(Kanellopoulou et al. 2009) and in embryos itself (Nesterova et al. 2008). However, DICER-1 knockdown resulted in elevation of H3K4 methylation levels on Xi and upregulation of the X-linked gene expression. Some genes (XIST, MospD2, Col4A5 and MAGEH1) are more sensitive than others suggesting a selective and preferential effect of DICER-1 in human dosage compensation. Moreover, ectopic over expression of an in vitro transcribed anti-sense small RNA from repeat D region reduced XIST levels opposite to the effect of functional inhibition of the DICER-1 protein (Fig. 3.12).

Human sXiRNA or its precursor/s were transcribed by polymerase enzyme other than RNA pol II from the XIST locus which is shown to be indispensable for maintenance of dosage compensation by Xi mediated gene repression, genomic stability in the ES and somatic cells. In many ways, human sXiRNA is distinct from their mouse counterpart. Such differences in origin
and function of the sXiRNA might underlie the subtle variations of the dosage compensation mechanism between primate and non-primate mammals considering the gross similarity in their X inactivation process. In human, truncation of TSIX during evolution and its negligible role in Xi imprinting (Vagin et al. 2006; Migeon 2002; Chow et al. 2003; Migeon 2003) supports this view. The origin and nuclear localization of human sXiRNA is strongly analogous to that of rasiRNA (Vagin et al. 2006). Our results indicate that human sXiRNA together with other small RNA functions most likely in chromatin based transcriptional regulation as exemplified by the enhanced methyl H3K4 and acetyl H3K9 binding (Fig. 3.10 & 3.11) on Xi. Contrary to the earlier observations in mice ES cells, our results indicate no role for sXiRNA in the maintenance of XIST or H3K27 marks on Xi. It is likely that general epigenetic marks including XIST RNA painting, once established during embryonic development allow sXiRNA and DICER-1 independent maintenance of Xi silencing. On the other hand, our findings implicate a new role for human DICER-1 in processing repeat associated siRNA (rasiRNA) related species like sXiRNA, from the novel RNA precursors. Further, existence of small XIST RNA of distinct sizes in humans and mouse, this being restricted to certain preferential regions with in XIST locus tends us to explore the existence of low abundant non coding RNA and unidentified sequence specific cleavage and/or stabilization mechanism integrated in the process of small RNA generation from XIC locus in mammals. In summary, these results suggest that sXiRNA play critical role in maintaining X-linked gene balance by a post Xi establishment phase and there by existence of an unexpected link between nuclear RNAi and Xi chromatin state in the human cells. Though our data shows sXiRNA are processed from Xa, it is plausible that low level sXiRNA might also be produced from XIST locus of inactive X chromosome and these small RNA might also be involved in maintaining chromosomal cross talk in trans between XIST alleles of two X chromosomes implicated earlier in control of replication timing as detected by the XIST deletion studies (Diaz-Perez et al. 2006).

As reported earlier, DICER-1 depleted mouse ES cells (Ogawa et al. 2008) were more amenable to short term chromatin changes by recruiting epigenetic markers and activation of the Xi genes, while absence of such changes in human somatic cells may be due to recalcitrant nature of differentiated cells. In consistent with this hypothesis, many genes in human Xi escape dosage compensation in the somatic tissues (Carrel and Willard 2005). Therefore existence and accumulation of the sXiRNA in human ES cells or analogous studies will be useful for
determining their specific functions in Xi establishment and also to provide a new platform for investigating role of nuclear RNAi in heritability and maintenance of higher order Xi chromatin structure in primates.
Small RNA isolated from HeLa, S2 and WI-38 cell lines was resolved on 15% denaturing PAGE and subsequently blotted on to Hybond N+ membrane. UV cross linked blots were hybridized over night with 32P labeled RNA probes derived from repeat A region within XIST Exon-1. Hybridized blots were washed at 60°C for an hour with SSC and SDS. Blots were exposed to Fuji phosphor imaging screens 16hrs-30hrs (inset) and imaged using Fuji phosphor imager.
Fig 3.2. Small RNA were isolated from HeLa, S2, HEK293 and WI-38 celllines was resolved on 15% denaturing PAGE, blotted on to Hybond N+ membrane. UV cross linked blots were hybridized over night with $^{32}$P labelled RNA probes derived from repeat D region with in XIST Exon-1. Hybridized blots were washed at 60°C for an hour with SSC and SDS. Blots were exposed to Fuji phosphor imaging screens 16hrs-30hrs(inset) and imaged using Fuji phosphor imager.
Fig 3.3.100nM of either unrelated control siRNA or human DICER1 specific siRNA was used to specifically down regulate human DICER1 expression in HEK-293 and HeLa cells. Five days after transfection, total cellular RNA was isolated from HEK-293 cells, and reverse transcription was performed using random primers. Equal amounts of cDNA from control siRNA or DICER1 siRNA treated samples was analyzed by polymerase chain reaction using gene specific primers as indicated in the figure. PCR was also performed directly with isolated RNA using DICER1 specific primers to check for DNA contamination(-Ve RT).

Lower Panel: Silencing of GAPDH by using specific siRNA is inhibited in the HeLa cells depleted of DICER1 by using siRNA. GAPDH protein levels were observed by immunofluorescence in HeLa cells transfected with GAPDH siRNA in the presence and absence of DICER1 protein. GAPDH antibody(Green), Nuclei(Blue), GAPDH siRNA(Red).
Fig 3.4. 100nM of either unrelated control siRNA or human DICER1 specific siRNA was used to specifically down regulate human DICER1 expression in HEK-293 cells. Five days after transfection, cells were fixed with paraformaldehyde and endogenous levels of DICER1 protein was estimated by immunofluorescence using mouse anti-Dicer1 antibody. Nuclei were counter stained with DAPI. (DICER1, Green; DAPI, Red)

Lower Panel: Total proteins were isolated from HEK-293 cells 5 days after DICER1 specific siRNA transfection and analyzed by western blotting by anti-Dicer1 and β Tubulin antibodies. Marked reduction in DICER1 protein levels were observed upon DICER1 depletion in HEK-293 cells.
Fig 3.5. Total RNA was isolated from control siRNA and DICER1 siRNA transfected cells 5 days after transfection and resolved on 15% urea-PAGE. RNA from gel was stained with ethidium bromide and then blotted on to positively charged Hybond N+ membrane over night using Hoefer wet transfer system. RNA was cross linked to membrane by UV. miRNA-93 levels were detected by labeling oligo 5'-CTACCTGCACGAACAGCATT-3' and hybridizing to blot over night at 37 °C. Densitometric analysis of levels of miRNA-93 exposed to phosphor imaging screen and ethidium bromide stained rRNA levels was made and relative miRNA-93 levels in control siRNA transfected cells and DICER1 siRNA transfected cells was plotted. Amount of mature miRNA93 levels were significantly less in DICER1 transfected cells compared to control siRNA transfected cells.
Fig 3.6. Small RNA was enriched from DICER1 or control siRNA transfected cells after 5 days of transfection. Small RNA was resolved on 15% urea-PAGE and subsequently blotted on to nylon membrane. Blots were probed with radiolabeled oligo riboprobes derived from XIST Exon-1 region (cartoon). After hybridization, blots were washed extensively with SSC/SDS and exposed to phosphor imaging screen for 24 hrs. Significant reduction in the levels of sXiRNA in the DICER1 depleted cells indicated DICER1 is essential for processing of sXiRNA.
Fig 3.7. Control siRNA or DICER1 specific siRNA was transfected into HEK-293 cells to observe the effects of DICER1 depletion on the localization of XIST onto inactive X chromosomes.

100nM of either control or DICER1 specific siRNA were transfected twice on Day1 and 3 into HEK-293 cells and after 5 days of first transfection, cells were fixed with paraformaldehyde and RNA FISH was performed to detect the localization of XIST on Xi by using random primed XIST Exon-1 specific probes. Cells were counter stained with DAPI and scanned under Carl-Zeiss LSM 510 meta confocal microscope under 100X lens (XIST, red ; DAPI, blue) Number of XIST spots were also counted in control and DICER1 knock down cells and histogram was plotted. No difference in the localization of XIST was observed upon depletion of DICER1 from cells of human origin.
Fig 3.8. Control siRNA or DICER1 specific siRNA was transfected into HEK-293 cells to observe the effects of DICER1 depletion on the incorporation H3K27 trimethyl mark onto 2 Xi chromosomes.

100nM of either control or DICER1 specific siRNA were transfected twice on Day1 and 3 into HEK-293 cells and after 5 days of first transfection, cells were fixed with paraformaldehyde and immunofluorescence was performed using 2 different antibodies recognizing the trimethyl mark on H3K27. After washes with PBS, cells were incubated with secondary antibody(White or Red) and subsequently counter stained with DAPI(blue). Total number of nuclei and number of H3K27 foci were counted under Carl-Zeiss fluorescent microscope (Histogram). No significant changes in incorporation of H3K27 mark onto Xi chromosomes was observed upon DICER1 depletion.
Fig 3.9. Incorporation of histone variant macroH2A on to Xi chromatin is unaltered upon DICER1 RNAi.

macroH2A-GFP construct was transfected into cells after twice transfection of control or DICER-1 specific siRNA and cells were fixed and observed after 24 hrs under 100X lens of carl-zeiss LSM 510 meta confocal microscope.
Fig 3.10. 100 nM control or DICER1 specific siRNA was transfected into HEK-293 cells using Hiperfect transfection reagent. 5 days after transfecting twice with same siRNA on day one or day three cells were fixed with 4% paraformaldehyde and immunofluorescence was performed with H3K27 trimethyl(red) or H3K4 di+tri methyl(green) antibodies(Top panel).

Incorporation of positive histone marks H3K4 di+tri methylation and H3K9 acetylation on to Xi was determined by calculating the relative enrichment in green fluorescence in DICER1 siRNA transfected cells compared to controls(Lower Left histogram)

Total RNA was isolated from DICER1 siRNA twice transfected cells 5 days after transfection and real time analysis was performed using SYBR green to determine the amount of XIST transcripts. XIST RNA levels were found to be considerably increased in the absence of DICER1.(Lower right histogram)
Fig 3.11.100 nM control or DICER1 specific siRNA was transfected into HEK-293 cells using Hiperfect transfection reagent. 5 days after transfecting twice with same siRNA on day one or day three nuclear proteins were isolated and separated on SDS-PAGE gels. Separated proteins were transferred onto Hybond-P membrane and probed with mouse monoclonal H3K4 di+tri methyl specific antibody or after development of signal blots were stripped and reprobed with Histone H3 specific antibody that served as internal control(Top panel). There is a significant increase in the level of H3K4 di+trimethyl histone in DICER1 deficient cells compared to control siRNA transfected cells.

Real time analysis for X linked transcripts indicated a significant increase in some of the X linked transcripts upon depletion of DICER1(Lower panel)
Fig 3.12. Identification of small RNA within repeat D region
Left Panel: Small RNA were enriched from the total cellular RNA of HeLa and HEK-293 cells. Small RNA was resolved on 15% Urea-PAGE and blotted onto nylon membranes. UV cross linked membranes were hybridized with radiolabeled oligo riboprobes prepared from the T7 sequence containing template oligo. Blots were washed at 42°C and exposed to phosphor imager screens for 24 hrs.

Right Panel: Over-expression of sXiD repeat derived sequences in regulating XIST transcripts. *In vitro* transcribed RNA oligos prepared by megascript kit using the following oligotemplate "GTGAATAGACATG-GAAGCCTCCCTGCCCACACTCCACCCCCAATCTCCTATAGTGAGTCG-TATTAATT" were purified through column and transfected into HEK-293 cells. Three days after transfection RNA was isolated and real time PCR was performed using XIST specific primers and GAPDH as internal control. Compared to control siRNA transfected cells sXiD RNA transfected cells showed lesser XIST levels indicating that XIST levels inside cells are regulated by small sXiRNA.
Materials and Methods

Small RNA Northern blot analysis

Small RNA northern analysis was performed essentially as described in chapter-1. Briefly total RNA was extracted from cell lines using trizol reagent (Invitrogen). Low-molecular-weight species was enriched from high molecular weight RNA by salt precipitation or by miRVA NA miRNA isolation kit (Ambion). 10-15 μg of either total or small RNA was loaded into 15% denaturing polyacrylamide gel, and transferred onto a Hybond N+ membrane (Amersham), and fixed by UV cross-linking. Blots were probed with hydrolyzed [α-32P] UTP labeled RNA transcribed by T7 or T3 using Xbal fragment of human XIST exon-1 (kindly provided by B. Chadwick) as template using Maxiscript (Ambion). Blots were washed with 2x SSC and 0.2% SDS at 60°C for 30min and exposed for 12-48 hrs on a Phosphor Imager screen (Fuji). RNA polymerase inhibitor studies were performed by treating 80% confluent HEK 293 cells with either DMSO (sigma) or 10 μg/ml α-amanitin (sigma) dissolved in distilled water or 20 μM of chlorobenzenesulfonamide (Calbiochem) dissolved in DMSO. Cells were harvested after 24h and RNA was isolated and processed as above for northern blot hybridization. Northern blot analysis for estimating mature miR-93 level was performed with 15 μg of enriched small RNA from control and DICER-1 siRNA transfected cells by labeling oligo with [γ-32P] ATP using T4 PNK kit (NEB). EtBr stained rRNA levels were used for normalization as indicated.

Fluorescent In-situ Hybridization and Immunofluorescence

Cells were fixed with 3.7% Paraformaldehyde in PHEM buffer for 10min and permeabilised with 1% triton X 100. Cells were used immediately for staining or stored in 70% alcohol at 4°C until use. XIST RNA FISH was carried out using Cy3 labeled random primed probes that were synthesized using the DECAprime™ II Kit (Ambion) at 37°C overnight as described. 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 10X, 20X and 100X objectives in confocal microscope (Carl-Zeiss). Immunofluorescence was carried out using 1:50 or 1:100 dilutions of following primary antibodies: rabbit or mouse H3K27 trimethyl (Upstate, Abcam), Rabbit H3K9 acetyl (Upstate), mouse H3K4 di, tri methyl (Abcam), mouse DICER-1 (abcam).
1:100 or 1:200 dilutions of Secondary anti rabbit or anti mouse antibodies conjugated to FITC, Cy3 or Cy5 (Jackson) were used (see figure legends for details) and finally counterstained with DAPI or Propidium Iodide and observed under confocal or fluorescent microscope. For calculating the accumulation of H3K4 di+tri methylation or H3K9 acetylation on inactive X chromosome, H3K27 trimethylation was used to select the area of Xi chromosomes of >100 nuclei from different microscopic fields in both control and DICER-1 siRNA transfected cells keeping the parameters constant by using Carl-Zeiss LSM software.

**Reverse Transcription – PCR (Real time)**

Semiquantitave RT-PCR analysis was carried out from the RNA isolated by Trizol method from control RNA and anti-sXi RNA transfected cells or from control siRNA and DICER-1 siRNA transfected cells. DNA was removed from RNA samples by using turbo DNA free (Ambion). cDNA synthesis was carried out by using superscript II according to manufacturer's instructions. SYBR green real time PCR analysis was performed using the following primers with 18S rRNA as internal control in 7900 HT sequence detection system (Applied Biosystems). Transcript levels were calculated according to the $2^{\Delta \Delta Ct}$ method. Following primer sets were used for amplification of different amplicons.

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence</th>
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<tr>
<td>XIST1F</td>
<td>AGCTCCTCGGACAGCTGTAA</td>
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<tr>
<td>XIST1R</td>
<td>CTCCAGATAGCTGGCAACC</td>
</tr>
<tr>
<td>GAPDH1F</td>
<td>TGAAGGTCGGAGTCAACGGATTTG</td>
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<tr>
<td>GAPDH1R</td>
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<tr>
<td>ZCCHC12R</td>
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<tr>
<td>SMARCA1F</td>
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Western blot analysis

For western blotting, HEK-293 cells were collected 5 days after transfecting twice with control or DICER-1 siRNA; washed with ice cold PBS and lysed in RIPA buffer. Protein concentration was estimated by Bradford method and equal amounts of heat denatured total protein or nuclear protein (for histones) was resolved in 8% or 12% SDS-polyacrylamide gels respectively. The blots were transferred on the PVDF membranes. Membranes were probed with mouse anti-DICER-1 antibody (1:200 Abeam) or anti-Histone H3 di+tri methyl K4 antibody (1:300 abcam) and then treated with respective secondary antibodies (1:500, Promega) and developed using chemiluminescence substrates (Perkin-Elmer). The blots were subsequently reprobed in the same manner with either anti-beta actin antibody (1:300 abcam) or anti-Histone H3 antibody (1:300 upstate) for loading control.

sXiD RNA transfection

sXiRNA transfection from Repeat D region, 3 μg of in vitro transcribed and gel purified repeat D RNA was transfected into 60% confluent HEK 293 cells plated in 6 well plates by using hiperfect transfection reagent according to manufacturers protocol and processed for RNA isolation using Trizol reagent. The following sequences were used to synthesize sXiRNA from repeat D. Equimolar mixtures of both DNA oligos were mixed and annealed and used for in vitro transcription by megascript-T7 kit (Ambion) according to manufacturer’s instructions.

T7 oligo – 5’ –AATTTAATACGACTCACTATAGG-3’
sXiRNA oligo from repeat D –
5’GTGTAATAGACATGGAAGCCTCCCCTGCCACACTCCACCCCCAATCTCTCATAGTGAGTCGTATTAAATT-3’

siRNA and plasmid transfections
The following siRNA sequences were used to transfect cell lines using hiperfect (qiagen) reagent according to manufacturer’s instructions.

Dicer siRNA: UGCUUGAAGCAGCUCUGGAdTdT
Control siRNA: AGGUAGUGUAUACGCUUGdTdT

293 cells were plated at 60% confluency and transfected twice on alternative days and RNA was isolated after 5 days or trypsinized and replated in chamber slides after second transfection. macroH2A tagged to GFP (kind gift from Dr. Barbara Panning) was transfected using lipofectamine 2000(Invitrogen).

References


