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Maintenance of a Vertebrate Constitutive Heterochromatin Domain by a Dicer-dependent Mechanism

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Abstract

The 16 kb heterochromatin domain between the chicken β -globin locus and the folate receptor gene is used here to study the roles of RNA dependent mechanisms and histone modifications in maintenance of a constitutively heterochromatic structure. Inhibition of HDAC activity is shown to both increase intergenic transcription and render the heterochromatin more accessible to MspI digestion. We show that siRNA-mediated down regulation of the enzyme dicer has similar effects: histone acetylation is increased, transcript levels rise, and the compact chromatin structure becomes more accessible to restriction endonucleases. We also show that the chicken argonaute 2 homolog binds the 16 kb region in a dicer dependent manner and is necessary for a condensed chromatin structure. Heterochromatic domains of this kind, widely distributed in vertebrate genomes, thus appear to be maintained in their condensed form by previously identified and highly conserved mechanisms.

Keywords

Heterochromatin; Dicer; siRNAs

Constitutively heterochromatic domains, though stable, compact, and lacking expressed genes, are transcribed at low levels on both strands; this transcription is essential to maintenance of their heterochromatic state¹. This process has been linked to mechanisms dependent on the enzyme dicer, which processes double stranded transcripts into short interfering RNAs (siRNAs) approximately 22 nts long². These RNAs are incorporated into the nuclear RNA Induced Transcriptional Silencing (RITS) complex that targets the parent DNA sequences and recruits the silencing histone modifications³. RITS is composed of three proteins, chp1, tas3 and the highly conserved ago1⁴. The vertebrate counterpart of RITS has yet to be isolated; however numerous reports implicate a similar mechanism of small RNA induced chromatin modification in vertebrate cells^{5–7}. Dicer has also been shown to process intergenic transcripts from an open chromatin domain and its loss led to an increase in transcription and histone acetylation throughout the region⁸.

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In this paper we make use of the extensively characterized 16 kb constitutive heterochromatin domain that lies between the chicken *folate receptor* gene and the *β-globin* locus (*FR/β-globin* locus). When excised from the nucleus of the chicken erythroid progenitor cell line 6C2, it behaves hydrodynamically as a compact rigid rod, marked by histone modifications associated with heterochromatin, and highly resistant to nuclease digestion^{9–13}. We utilize this domain to investigate mechanisms that maintain the heterochromatic state.

To investigate the possible role of RNA-dependent mechanisms in establishment or maintenance of this extended heterochromatic domain (Figure 1A,B), we searched for transcripts from this region, using one-step reverse transcriptase quantitative PCR (RT-QPCR). Transcripts were observed across the entire region (Fig. 2A). As expected, the transcript from the active *FR* gene (primer site 3.775) was between ~2 and 128 fold more abundant than that from any of the intergenic sites, but ~100 fold less abundant than transcript from the positive control, *gapdh*. Thus, the lowest detectable intergenic RNA from the 16 kb condensed chromatin region was present at levels ~10⁻⁴ that from *gapdh*.

The chromatin structure over the 16 kb condensed chromatin domain is marked by hypoacetylation of histones H3 and H4, and a high level of H3-K9 dimethylation¹⁰. To determine if there was a connection between expression levels of these RNAs and the chromatin state of the 16 kb region we treated the cells with the HDAC inhibitor trichostatin A (TSA). Treatment with 1 μM TSA for four hours resulted in only small changes in RNA levels at all sites studied. However, after overnight incubation with TSA, RNA transcript levels from the heterochromatic region rose significantly (Figure 2B), from ~3 fold (at site 15.850) to ~7 fold (at site 8.989). The nearby open chromatin domains of the *FR* gene exhibited lowered RNA levels after the TSA treatment; there were no significant changes in RNA levels transcribed through HSA (site 5.613), which contains the regulatory sequences for *FR*. However, the boundary element at the 3' end of the 16 kb domain showed a ~4 fold increase in transcript.

To investigate whether the changes in RNA levels observed in response to TSA correlated with changes in epigenetic modifications of histone H4, we performed chromatin immunoprecipitation (ChIP) assays throughout the locus. These assays were designed to determine the absolute fraction of nucleosomes at each site that carry the given modification (Materials and Methods). Unlike methods that determine relative enrichment (concentration of target sequence in the immunoprecipitate/concentration in the input), this allows a direct comparison of cells grown under different conditions, as it takes into account possible changes in genome-wide levels of a histone modification when cells are treated with reagents such as TSA.

The results of this analysis for changes in histone H4 acetylation over the 16 kb heterochromatin domain after treatment with 1 μM TSA are shown in Figure 2C. Each site showed an increase in H4-Ac levels. Of particular note were the 8.989 and 10.35 sites within the condensed chromatin region. Neither of these sites had detectable H4-Ac in untreated cells, but after TSA treatment, H4 acetylation was found at 14% and 8% of the nucleosomes at these sites, respectively. TSA was removed after 4 hours and the cells were allowed to

recover for five days. All sites studied lost H4 acetylation when compared to TSA treated cells, though none returned fully to wild-type levels (Figure 2C).

In an earlier study we showed that treatment of nuclei with the restriction enzyme MspI liberated a largely intact 16 kb chromatin fragment despite the presence of multiple potential cutting sites within that domain, consistent with its compact structure⁹. We asked whether treatment with TSA had further effects on the domain's physical properties. Figure 2D shows the sucrose gradient sedimentation profiles of chromatin obtained after MspI digestion of untreated and TSA treated 6C2 cells. The DNA from each fraction was analyzed by QPCR using a 13.192 primer set roughly in the middle of the 16 kb locus (Figure 1B). The TSA treated chromatin gradient profile demonstrated a clear increase in the fractions containing smaller fragments, consistent with increased chromatin accessibility to MspI.

The presence of intergenic transcription and dynamically regulated heterochromatic regions is reminiscent of the *S. pombe* model of how RNAi regulates heterochromatin¹⁴. To determine whether the RNAi machinery is involved in the regulation of the 16 kb heterochromatin structure, 6C2 cells were transiently transfected with siRNAs designed to knock down chicken *dicer* mRNA (siDicer). *Dicer* mRNA levels were lowered 24 and 48 hours after transfection, but recovered by 72 hours (Figure S1A). However, a western blot revealed only low levels of dicer protein at 72 hours (Figure S1C). Consequently all experiments describing effects of dicer knockdown were carried out 72 hours post-transfection. Results were compared to a mock transient transfection (siMock) (Materials & Methods).

Consistent with RNAi-mediated chromatin structure regulation, Dicer knockdown causes significant increases in RNA levels within the 16 kb region (Figure 3A). The increase in intergenic expression was limited to constitutively heterochromatic regions, and HSA. RNA levels from the folate receptor gene (site 3.775) and HS IV (site 21.365) remained unaffected.

The increase in intergenic transcripts throughout this heterochromatic region suggests that these RNAs are processed by Dicer. However, the effect could be indirect. To determine whether this was a *cis*-effect, we used an RNase protection assay to look for siRNAs derived from any of these heterochromatic loci. Single-stranded RNA probes, against either sense or anti-sense small RNA species at four sites, were hybridized to gel purified 6C2 RNA (ranging from 20 to 40 nucleotides) and digested. The RNAs from siMock cells and siDicer were compared to detect possible small dicer-dependent RNAs.

Small interfering RNAs are generated from the sense strand of both 15.850 (Figure 4B, lanes 9 and 11) and 18.178 (lanes 13 and 15). At both loci, numerous siRNAs between 21 and 30 nucleotides long are present in siMock cells but are either absent or strongly reduced in siDicer. There were no detectable siRNAs originating from the sense strands of 8.989 or 13.192 (Figure 4B, lanes 1–4 and 5–8). No small RNAs were detected from the anti-sense strand of the 16 kb region (data not shown). We also performed a bioinformatic analysis, aligning the RNA probes from Figure 4B against the entire chicken genome (Figure S2C).

We conclude that the only possible genomic origin of protected RNA species is the 16 kb region.

The results in Figures 3, 4A and 4B suggest that dicer processes intergenic transcription to generate siRNAs, and that the presence of these siRNAs is involved in regulation of chromatin structure. The function of siRNAs in the establishment and maintenance of heterochromatin has been shown to be mediated by a member of the argonaute family of small RNA binding proteins¹. We therefore investigated the role of chicken argonaute 2 (cAgo2) in the maintenance of the 16 kb heterochromatic region. The chicken Ago2 homolog is not listed in the UC Santa Cruz genome browser. However, alignment of human Ago2 reveals a 29,915 bp genomic region with 99.1% homology to human Ago2 (Figure S3A). A western blot confirms the presence of cAgo2 and shows that the human antibody cross reacts (Figure S4A).

A ChIP assay showed that roughly 2.5% of the chromatin at site 18.178 was immunoprecipitated by Ago2 antibody (Figure 3C), which recognizes only one band when assayed via immunoprecipitation-western blot analysis (Figure S4A). Ago2 was bound at roughly 1% of sites 3.775, 5.613, 10.35 and 15.850. Trace amounts were observed at site 13.192; it was absent from 8.989 and HS IV (21.365). To investigate whether Ago2 binding was dependent on siRNA production, ChIP assays were carried out in siDicer cells (Figure 3C, red bars). Binding was reduced to zero at all sites investigated, except for 15.850, where it was reduced to roughly 25% of siMock levels. Thus, cAgo2 binding to this region requires dicer-dependent siRNA production. We also knocked-down the expression of cAgo2 (Figure S4C). The ChIP of cAgo2 in siAgo2 cells yielded no signals at sites within the 16 kb region (Figure 4C, green bars), confirming that the ChIP data in Figure 3C represent cAgo2 binding.

To investigate the effect of the RNAi machinery on the 16 kb heterochromatin domain, we performed a ChIP analysis as in Figure 2C, and extended the analysis beyond H4-Ac to include H3K9Ac and H3K9(Me)₂. The reduction of Dicer and cAgo2 causes drastic changes in the region's epigenetic profile (Figure 4). Dicer knockdown causes significant increases in H4-Ac at all sites, except the FR gene and 10.350 (Figure 4A, red bars). Similarly, siAgo2 treatment causes an increase at all sites except HSA and 10.350 (Figure 4A, green bars). The increase in H4-Ac is greatest at 18.178 for both siDicer and siAgo2, which is also the peak of cAgo2 binding (Figure 3C).

The effect of siDicer on H3K9(Ac) was smaller than for H4-Ac, with increases at two heterochromatic sites, 8.989 and 10.35 (Figure 4B, red bars). SiAgo2 also caused increases over 10.350, in addition to 13.192 and 18.178 (Figure 4B, green bars). As with H4-Ac, the strongest increase in H3K9(Ac) was over 18.178, the peak site of cAgo2 binding (Figure 3C). We extended our epigenetic analysis from the previously mentioned "active" marks, to a typical "silencing" mark, H3K9(Me)₂. siDicer caused a marked reduction at all sites assayed with the exception of 13.192 (Figure 4C, red bars). Interestingly, siAgo2 had no significant effect on H3K9(Me)₂ levels, similar to the lack of change observed after TSA treatment. (data not shown).

To investigate the role of the RNAi machinery in the regulation of chromatin structure, we asked whether the loss of either Dicer or cAgo2 had an effect on the structure of the 16 kb region (Figure 5). We digested nuclei from three transfected cell lines (siMock, siAgo2 and siDicer) with MspI, and carried out sedimentation experiments like those for TSA treated material in Figure 2D. The sucrose gradients from the siMock treatments were probed at four sites within the putative condensed chromatin domain (Figure 5A). The sedimentation profiles are identical for all probes, demonstrating that the 16 kb fragment is largely intact with little cleavage between site 8.989 and 18.178. (Figure 5A).

We repeated this analysis on nuclei from siAgo2 cells (Figure 5B). The chromatin in the smaller peak (fractions 8–10) is now increased relative to that from fractions 14–15 (Figure 5B); the amount of intact 16 kb material in fractions 14–15 (~17%) is correspondingly decreased. Thus, the accessibility of the 16 kb chromatin fragment is increased in siAgo2 cells. Similar experiments with MspI digested siDicer nuclei (Figure 5C) reflect a more open, accessible structure in siDicer than in siAgo2 (Figure 5B vs. 5C). Although the sedimentation profiles are different, the amounts of full-length chromatin are the same, roughly 50% of that in siMock, for the two knock-down treatments (Figure 5D). We also probed the gradients for the open chromatin region at the DNaseI hypersensitive site HS IV (21.365) (Figure 5E). The sedimentation profile of HS IV is shifted towards the top of the gradient relative to sites within the heterochromatin domain for all of the transfected cell types. However, both siAgo2 and siDicer treatments cause the chromatin surrounding HS IV to become even more accessible to MspI digestion than the heterochromatic regions (Figure 5E).

These results demonstrate that the highly condensed heterochromatic domain is transcribed at very low levels that can be raised by TSA treatment, increasing regional H4-Ac levels and causing the chromatin to adopt a more open structure. This structure is dynamic: removal of TSA returns acetylation levels towards the unperturbed state. This dynamic interplay between active and inactive chromatin shows that even silent heterochromatin is not inert and can be reactivated by altering the balance between activating and silencing factors.

Reducing the levels of *dicer* mRNA (and protein) results in a completely altered chromatin landscape across the locus, accompanied by an increase in transcriptional activity at multiple sites, a loss of small RNAs, lowered H3K9(Me)₂ levels, increases in H4-Ac and H3K9(Ac) levels, and a loss of chromatin bound cAgo2. Similar changes in histone acetylation levels are seen when Ago2 levels are reduced. Furthermore, the loss of dicer and/or cAgo2 results in opening of the compact heterochromatic structure, revealed by its increased accessibility to digestion by MspI. Our model for the *in vivo* maintenance of this region is illustrated in Figure S5.

The work presented here implicates cAgo2 and endogenous siRNAs in regulating the transcriptional activity, epigenetic marking and physical nature of a constitutive heterochromatin domain. Ago2 is not likely to bind chromatin alone. It is more likely that members of the vertebrate counterpart of the RITS complex and the siRNAs are bound together with Ago2. The siRNAs are likely to help localize Ago2 to the proper site on chromatin and other protein cofactors probably recruit histone modifying enzymes needed to

maintain this domain as transcriptionally silent. Future work will be needed to identify the cofactors that bind to chromatin along with Ago2 and the role each of them plays. It will also be important to determine how many other constitutive heterochromatic sites in chicken and other vertebrate genomes are controlled by similar mechanisms.

Materials and Methods

Tissue Culture and Transient Transfection Assay

The chicken 6C2 cell line used in this study was cultured in previously published conditions⁹. Cells were transfected using Lipofectamine 2000 (Invitrogen # 11668-027) according to manufacturer's instructions. For RT-QPCR, ChIP and RNase protection assays, the protocol was scaled up to a 20 mL volume of transfected cells. 40 mL of culture media was added to the transfected cells after 4 to 6 hours of incubation. For the digestion of 6C2 chromatin with MspI, as shown in Figure 5, the transfection was scaled up linearly such that 1.2L of cells were harvested after 3 days of transfection. RNAi mediated knockdown was carried out with a custom siRNAs designed by Ambion (siMock: AM4635, "silencer negative control". siDicer: AM16706, siRNA ID# 296655. siAgo2: 4390818, siRNA ID # s109013).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-QPCR)

RNA was harvested from roughly 10 million 6C2 cells using the Qiagen RNeasy kit according to manufacturer's instructions. An additional on-column DNase step was used according to kit instructions. RNA was eluted in 50 μ L dH₂O, diluted to 200 ng/ μ L and subjected to an additional DNase step using TURBO DNA-freeTM, (Ambion) according to manufacturer's instructions. The sample was diluted 100-fold and utilized for RT-QPCR.

Each one-step RT-QPCR assay was carried out in a volume of 50 μ L, containing 10 μ L of RNA sample, 25 μ L of Taqman 2X master mix, 10 μ L of 4.5 μ M forward and reverse primers and 5 μ L of 2.25 μ M Taqman probes. The amplification was performed on the ABI Prism 7900 HT sequence detection system and the data was analyzed on the Sequence Detection System 2.2 software. In the case of untreated or mock transfected 6C2 cells, RNA levels are presented as the cycle threshold (Ct) value for each primer set without normalization. In the case of TSA treatment or *dicer* knockdown, RNA levels were compared to the RNA levels of the untreated or mock transfected cells after normalizing each sample preparation to *gapdh* levels. It was observed that the *gapdh* levels do not change with TSA treatment or siRNA transfection. A fold difference was calculated using the formula $2^{(Ct(\text{untreated}) - Ct(\text{treated}))}$ and the geometric mean $\pm$ the 95 % confidence interval was calculated for each primer set.

Chromatin immunoprecipitation (ChIP)

ChIP assays of histone modifications (H4-Ac; upstate 06-866, H3K9(Ac); upstate 07-352, H3K9(Me)2; upstate 07-441) were designed to calculate the absolute percentage of loci that are precipitated in each treatment. A fixed excess amount of antibody was titrated with increasing amounts of input chromatin. If, at any point in the titration, f is the fraction of total sites available to bind the antibody, and y is the total mass of input chromatin for each

point, then the total amount of immunoprecipitated chromatin, $P = fy + B$, where B is the background binding. We plot $P/y_{\max}$ vs $y/y_{\max}$, where $y_{\max}$ is the maximum amount of chromatin used in the titration. The plot is linear for a range of input chromatin levels, with a slope corresponding to the fraction of DNA sites that are authentically bound by the antibody.

Approximately 10^6 6C2 cells in 25 mL growth medium were fixed with 0.4% formaldehyde for 10 minutes at room temperature. Cross-linking was quenched with 2.75 mL of 1.375M glycine. The cells were pelleted in a clinical centrifuge, incubated in hypotonic buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM DTT, 1 mM aprotinin, 1mM leupeptin and 1mM pepstatin) for 10 minutes on ice and suspended 25 times in a dounce homogenizer. The lysed cells were pelleted in a clinical centrifuge and resuspended in 1mL of lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1mM EDTA, 1% TritonX-100, 0.1% Na deoxycholate, 1 mM aprotinin, 1 mM leupeptin and 1 mM pepstatin) for 10 minutes on ice. With the exception of the data in Figure 2C, the nuclear extract was subjected to sonication with a diagenode Bioruptor with a 15 mL sonication tip at 4°C, for 20 minutes, cycling on and off every 30 seconds on high. The extract was pelleted to remove any insoluble debris. One tenth of the total extract was removed from the supernatant and the DNA was purified (proteinase K digested for one hour at 65°C, followed by phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation). This aliquot was electrophoresed on a 1% agarose gel to determine the size of the sonicated chromatin and the DNA concentration. The average chromatin fragment size in these experiments was generally in the range of 2kbp. The remainder of the chromatin extract was frozen and aliquots were taken for each ChIP. Chromatin for the experiments in Figure 2C was prepared similarly using a Misonix 3000 1/16'' microtip (power level 1, 30 seconds on, 30 seconds off) for 5 minutes of total sonication time.

For each ChIP experiment, binding reactions were done with three different chromatin input amounts corresponding to 10 μ g, 5 μ g and 2.5 μ g. The three reactions were generated by diluting 20 μ g of chromatin 10-fold with dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2mM EDTA, 1% TritonX-100) and preclearing (to minimize background binding) by adding to 100 μ L of a protein A or G agarose bead slurry (depending on animal the antibody was generated in.) The beads were pelleted and the chromatin supernatant obtained was serially diluted 2x, to generate three tubes containing 10 μ g, 5 μ g, and 2.5 μ g of chromatin, respectively. 10 μ g of antibody was added to each tube for an overnight incubation at 4°C. The next day, protein A or G agarose beads were equilibrated in dilution buffer and 100 μ L of 1:1 bead slurry was added to each reaction for 1 hour at 4°C. The beads containing bound chromatin were then harvested with a brief pulse in a microfuge at full-speed, and washed with 1 mL of ice cold wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS). This was repeated 4 times, followed by an additional 2 washes in final wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS). In order to obtain the immunoprecipitated chromatin, the pellet was resuspended in 200 μ L elution buffer (100 mM $NaHCO_3$, 1% SDS) for 15 minutes at room temperature. The beads were pelleted and the supernatant saved. This was repeated one more time to yield a second batch of supernatant. Both supernatants were combined and

DNA purified as described above. The Chromatin Immunoprecipitated DNA, resuspended in 200 μ L of dH₂O, was then used as a template in a QPCR reaction carried out as described for the RT-QPCR assay.

The ChIP assay using anti-Ago2 antibody (Abcam ab57113) was carried out as described for the histone modifications, except that the background binding was calculated by measuring the percent of chromatin binding to the protein A beads as well as the total chromatin that was immunoprecipitated in each experiment.

Chromatin digestion followed by sucrose gradient and QPCR analysis

Restriction digests on 6C2 nuclei, chromatin release and analysis by sucrose gradients were carried out as described ⁹.

RNase protection assay

The RNase protection assay was performed by hybridizing a single stranded, homogenously α -³²P-CTP, labeled RNA molecule with the cellular RNA ranging from 20 to 40 nucleotides. The cellular RNA was gel purified using an 20% acrylamide/8M urea gel that was stained with ethidium bromide to detect the RNA. Single-stranded DNA oligonucleotides were used as molecular weight markers. The radiolabeled ssRNA probe was prepared by *in vitro* transcription of the QPCR amplicons using T7 or T3 promoters. The sequence of this amplicon, and thus the probe, was previously published and shown to be unique for this region ¹⁰. Overnight hybridization was carried out at 37°C, and digestion performed as previously published ¹⁵.

Statistics

The RT-QPCR data was processed using the geometric mean, geometric standard deviation and the 95% confidence interval of each sample. The chromatin immunoprecipitation data is analyzed by the mean plus or minus the standard deviation. All calculations were carried out in Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The *folate receptor/β-globin* locus contains a condensed, heterochromatic region flanked by two DNase hypersensitive sites. (A) The *folate receptor* gene is depicted in a blue box at the 5' end of the locus. Immediately downstream is the DNase I hypersensitive site HSA (5.613, marked 'A' in all figures), which likely serves as a promoter and enhancer for *folate receptor* expression as well as an insulator containing barrier function activity. The 16 kb region of condensed chromatin (green rectangle) lies between HSA and the well characterized insulator, 5' HS4, herein referred to as HS4. HS4 is one of four hypersensitive sites found upstream of the four globin genes, which are depicted as blue boxes. The β/ε enhancer is indicated with an arrow pointing down. The locus terminates with a final hypersensitive site, termed 3'HS. (B) An enlarged representation of the region of interest in this study. Primer sets are shown below the locus and are numbered according to their position within the locus; the first base of the *folate receptor* is defined as position 1. The region 5' of HSA, HSA itself and HS4 (HS IV) are open chromatin domains that are enriched for histone H4-Ac and H3-K9(Ac). The condensed chromatin region that lies between the two hypersensitive sites is enriched for H3-K9(Me)₂, fully CpG methylated and is resistant to restriction endonucleases.

Figure 2.

Intergenic transcription and histone H4-Ac levels within the condensed chromatin region are dynamically regulated in the presence of HDAC inhibitors. (A) A RT-QPCR analysis showing RNA levels for each of the primers sets used in the study; data for primer set 10.350 are not shown as no RNA was detectable. The ordinate axis depicts the cycle at which RNA levels crossed an arbitrarily set threshold in the linear range of the amplification on a log₂ scale (Ct). The average (40 – Ct) value ± the standard deviation for at least 3 independent experiments is shown. (B) The fold change of the RNA levels is shown after 17 hours of 1 μM TSA treatment. The ordinate axis is a linear representation of the fold change in RNA levels given by the formula $2^{(Ct(WT)-Ct(TSA))}$. The RNA levels in each experiment were normalized to *gapdh*. The geometric mean ± the 95% confidence interval for three independent experiments is shown. (C) A ChIP assay depicting the percent of nucleosomes containing the H4-Ac modification in both wild type cells (blue), cells treated with 1 μM TSA for 4 hours (red), and cells that were given 5 days to recovery after removal of the TSA (green). Each data point shows the average of 3 or 6 independent ChIP experiments along with the standard error. ** p < 0.01; * p < 0.05. (D) Nuclei from 6C2 cells either untreated (WT) or treated with TSA for 16 hours (TSA) were digested with MspI, floated out of the nuclei, sedimented on a sucrose gradient and analyzed by QPCR⁹.

Figure 3.

Dicer and cAgo2 are involved in regulation of transcription within the 16kb heterochromatin domain. (A) A RT-QPCR assay depicting the fold change of RNA levels at 72 hours post-transfection. The increase was calculated using the formula $2^{(Ct(mock)-Ct(dicer\ kd))}$ as in Figure 2B and values shown are the geometric mean $\pm$ 95% confidence interval. (B) 8M urea- 20% acrylamide gel showing the results of an RNase protection assay using the sense strand of the amplicons 8.989 (lanes 1–4), 13.192 (lanes 5–8), 15.850 (lanes 9–12) and 18.178 (lanes 13–16). 20–40 nt RNA from siMock (lanes 1, 5, 9, and 13), or siDicer (lanes 3, 7, 11, and 15) cells was gel-purified, hybridized overnight with a radiolabeled sense strand of the indicated amplicon and digested with RNases A and T1 as described in the methods section. As a control for incomplete digestion of each ssRNA probe, the samples were split and one-half was boiled to denature the probe and digested. The complete absence of any bands in lanes 2, 4, 6, 8, 10, 12, 14, and 16 demonstrates that the probes are completely digested. (C) A ChIP assay depicting the binding of cAgo2 throughout the 16 kb locus. The binding of cAgo2 to chromatin was measured in both siMock (blue), siDicer (red) and siAgo2 (green) and is presented as the percent of total sites that are immunoprecipitated using an anti-human Ago2 antibody.

Figure 4.

Dicer knockdown completely alters the histone modification landscape of the *folate receptor/β-globin* locus. Each ChIP assay used the primer sets depicted at the bottom of each graph and the schematic representation of the locus. SiMock (blue), siDicer (red) and siAgo2 (green) cells were harvested 72 hours post-transfection. Chromatin immunoprecipitations were carried out using the following antibodies, (A) H4-Ac, (B) H3-K9(Ac) and (C) H3-K9(Me)₂. The ChIP data was quantified and the ordinate axis represents the percent of total sites that contain the indicated epitope. The statistical significance of each locus was determined by a students t-test using graphpad software, by comparing both the siDicer and the siAgo2 treatment to the siMock. The p values are indicated by the asterisk above each data point, ** p < 0.001; * p < 0.05.

Figure 5.

Condensed chromatin becomes accessible to nuclease cleavage 72 hours post dicer knockdown. All gradients were probed at loci 8.989, 13.192, 15.850, 18.178 and HS IV (21.365). The first four loci are all contained within the heterochromatin domain and HS IV is a DNase I hypersensitive site (Figure 1). The nuclei from either siMock (A), siAgo2 (B) or siDicer (C) were digested with MspI and the resulting chromatin fragments were allowed to diffuse out of the nuclei. The percent of total chromatin found in each fraction is listed on the ordinate axis. The fraction number for each gradient is listed below the graph. The detailed sedimentation patterns of digests from siAgo2 and siDicer cells differ substantially. The extent of degradation by Msp I is very sensitive to the amount of open structure: a few more open sites will lead to noticeably more degradation.

(D) The total amount of chromatin contained in the peak fractions (14 and 15), which represents the intact 16 kb fragment, for each locus was averaged and presented plus or minus the standard deviation. (E) The same gradients that are shown in (A), (B), and (C) were probed for the HS IV (site 21.365).