Human nuclear Dicer restricts the deleterious accumulation of endogenous double-stranded RNA

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Dicer is a central enzymatic player in RNA-interference pathways that acts to regulate gene expression in nearly all eukaryotes. Although the cytoplasmic function of Dicer is well documented in mammals, its nuclear function remains obscure. Here we show that Dicer is present in both the nucleus and cytoplasm, and its nuclear levels are tightly regulated. Dicer interacts with RNA polymerase II (Pol II) at actively transcribed gene loci. Loss of Dicer causes the appearance of endogenous double-stranded RNA (dsRNA), which in turn leads to induction of the interferon-response pathway and consequent cell death. Our results suggest that Pol II–associated Dicer restricts endogenous dsRNA formation from overlapping noncoding-RNA transcription units. Failure to do so has catastrophic effects on cell function.

The central role of RNA interference (RNAi) in regulating gene expression is a general feature of eukaryotes. RNAi begins with the formation of dsRNA, which may derive endogenously from overlapping convergent transcription of distinct protein-coding genes or from transcription of antisense noncoding RNA. dsRNA may also enter the cell in the form of an RNA virus and trigger a cytoplasmic antiviral mechanism called the interferon-response pathway. This leads to cellular apoptosis and consequent destruction of virally infected cells. Another major source of dsRNA is inverted-repeat transcription, which results in the formation of RNA hairpin structures. Two RNAi-associated endoribonucleases process hairpin-derived dsRNA in higher eukaryotes. Nuclear hairpin dsRNA is recognized by the RNAse III–type endonuclease Drosha together with the RNA-binding protein DGCR8. Cotranscriptional RNA cleavage at the base of the stem-loop releases hairpin RNA, which is then transported from the nucleus to the cytoplasm. Here, the second RNAse, Dicer, cleaves the dsRNA into short dsRNAs, forming microRNAs or short interfering RNAs (siRNAs) that, together with Argonaute proteins and associated factors, abrogate target mRNA function by either cellular apoptosis and consequent destruction of virally infected cells. Another major source of dsRNA is inverted-repeat transcription, which results in the formation of RNA hairpin structures. Two RNAi-associated endoribonucleases process hairpin-derived dsRNA in higher eukaryotes. Nuclear hairpin dsRNA is recognized by the RNAse III–type endonuclease Drosha together with the RNA-binding protein DGCR8 (ref. 10). Cotranscriptional RNA cleavage at the base of the stem-loop releases hairpin RNA, which is then transported from the nucleus to the cytoplasm. Here, the second RNAse, Dicer, cleaves the dsRNA into short dsRNAs, forming microRNAs or short interfering RNAs (siRNAs) that, together with Argonaute proteins and associated factors, abrogate target mRNA function by either degradation or translational inhibition. Such RNAi is referred to as post-transcriptional gene silencing (PTGS) because it acts on cytoplasmic mRNA. In many eukaryotes, and as exemplified by Schizosaccharomyces pombe, a second RNAi mechanism also exists in which nuclear Dicer generates siRNA associated with a nuclear Argonaute complex called RITS. This complex recruits histone methyltransferase and DNA methyltransferase to target homologous chromatin and generates repressed chromatin structures in a process called transcriptional gene silencing (TGS).

The prevailing view has been that mammalian RNAi relies exclusively on PTGS through the action of microRNAs. Furthermore, mammalian Dicer lacks obvious nuclear-localization signals, and overexpressed GFP-tagged Dicer shows a restricted cytoplasmic localization. This suggests that Dicer has a solely cytoplasmic function. However, it has recently been demonstrated that the C-terminal dsRNA-binding domain of human Dicer possesses a noncanonical nuclear-localization signal, thus implying a nuclear function. Indeed, a number of studies over the past decade have indicated that nuclear RNAi occurs in mammalian cells as it does in other eukaryotes.

In light of uncertainties relating to nuclear Dicer, we set out to determine whether Dicer localizes to the nucleus and, if so, what its function is in this cellular compartment. We show that Dicer is detectable in the nuclei of mammalian cells but that its levels appear to be tightly regulated. Furthermore, Dicer interacts with Pol II and chromatin genome wide, and Dicer-binding loci are associated with both dsRNA and small RNAs, interactions presumably related to Dicer activity. We then looked for the nuclear function of Dicer, and we show that its absence correlates with accumulation of dsRNA, which in turn triggers the interferon-response pathway, leading to cellular apoptosis. Thus, we have identified a major nuclear role for Dicer in restricting the formation of dsRNA from convergent transcription. Failure to perform this task leads to interferon activation and cellular apoptosis.

RESULTS

Dicer nuclear location

We used an antibody to Dicer (Abcam 13D6) to investigate the cellular distribution of Dicer in HEK293 cells containing a chromosomally integrated inducible Dicer (official symbol DICER1) short hairpin RNA (shRNA) expression cassette. Western analysis revealed 200-kDa Dicer protein in lysates from HEK293 cells but not after induction of Dicer shRNA (Supplementary Fig. 1a). Complete loss of Dicer cannot be achieved with this system because Dicer itself is needed to process the shRNA that leads to Dicer knockdown.

We next carried out immunofluorescence analysis on these HEK293 cells by using confocal microscopy. As shown in representative cell
Dicer associates with Pol II and specific chromatin regions

To examine the role of nuclear Dicer, we used HeLa-cell nuclear extracts immunodepleted for either Pol II or Dicer. Strikingly, immunodepletion of Dicer caused a reduction in Pol II levels, and, conversely, depletion of Pol II reduced Dicer levels (Fig. 2a). Furthermore, immunoprecipitation (IP) of Pol II from nuclear extracts coimmunoprecipitated Dicer (25% of input signal), and IP of Dicer coimmunoprecipitated Pol II (17% of input signal). In the latter case, the transcriptionally active Pol II O form was coimmunoprecipitated by Dicer, thus indicating that Dicer preferentially associates with elongating Pol II (Fig. 2b). We used antibodies specific to core Pol II (N20) and to the large Pol II subunit’s C-terminal domain (CTD) (8WG16)29.

We next investigated whether the Dicer–Pol II association is dependent on the presence of dsRNA. Pretreatment of nuclear extract with V1 double-strand–specific nuclease caused a substantial reduction of Dicer association with Pol II. As a control, Pol II interaction with the elongation factor Spt5 was unaffected by V1 treatment (Fig. 2c). We confirmed the efficiency and specificity of V1 treatment with in vitro–synthesized RNA.
gene in greater detail. Two probes, one before and one after the
substantially higher. We examined the Dicer-binding
tion in the levels of H3, the reduction in Pol II and Dicer levels was
tested the effect of
chromatin binding is transcription dependent. For a control, we
both enzymes coassociate during transcription and that Dicer’s
Both Pol II and Dicer levels were reduced on chromatin at the four
loci on transcription, we isolated chromatin from HEK293 cells
satellite, rRNA and tRNA DNA sequences were overrepresented
terminal repeat (LTR) elements were underrepresented, whereas
Genomic analysis by chromatin IP–sequencing (ChIP-seq) to
determine whether they were also subjected to TGS effects. Argonaute family
levels (Fig. 3f), thus suggesting dsRNA formation that is restricted
by Dicer. For negative controls, we similarly tested
rDNA. We detected no change in either sense or antisense transcript
by Dicer. For negative controls, we similarly tested
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by Dicer. For negative controls, we similarly tested
rDNA. We detected no change in either sense or antisense transcript

dsRNA (Online Methods), which was digested to completion by V1
tide but not by single-strand–specific T1 RNase (Fig. 2c). Overall,
these biochemical experiments suggest that Dicer interacts with Pol II
through a dsRNA moiety that is also Pol II associated.

Genomic analysis by chromatin IP–sequencing (ChIP-seq) to
detect Dicer association with chromatin isolated from HEK293 cells
revealed that Dicer associates with multiple human loci (Fig. 2d).
We determined the top 12 highest-scoring Dicer–Pol II overlapping
peaks (Supplementary Fig. 2a) and selected four loci with different
mRNA expression levels for detailed study (Supplementary
Fig. 2b). A metagene analysis of the top Dicer peaks showed Dicer
enrichment over transcription start sites (TSSs) and poly(A) signals
(PASs) (Supplementary Fig. 2c). We also investigated repetitive
genomic elements in the Dicer ChIP-seq data. Long or short interspersed
nuclear elements (LINEs and SINEs, respectively) and long
terminal repeat (LTR) elements were underrepresented, whereas
satellite, rRNA and tRNA DNA sequences were overrepresented
(Supplementary Fig. 2d).

To test the dependency of Dicer binding to these Dicer-positive
loci on transcription, we isolated chromatin from HEK293 cells
grown with or without the transcription inhibitor \( \alpha \)-amanitin (Fig. 2c).
Because these four Dicer-positive loci generate dsRNA, we tested
whether they were also subjected to TGS effects. Argonaute family
proteins are required for TGS\(^{26}\), and Ago1 has been shown to direct
siRNA-mediated TGS in human cells\(^{27}\). ChIP analysis revealed Ago1
signals enriched over each locus in comparison to 28S, thus suggesting
Ago1-directed TGS (Fig. 3f). Because TGS leads to repressive
chromatin formation, we also showed enrichment of dimethylated
H3 K9 (H3K9me2) over each Dicer-positive locus, as compared to
28S (Fig. 3g). We normalized H3K9me2 levels to total H3 signals to
rule out variability in nucleosome density. Finally, TGS should lead
to lower transcription levels. Indeed, all loci showed higher Pol II
levels after Dicer knockdown (Fig. 3h), thus implying Dicer-dependent
transcriptional silencing. Overall, our results indicate that Dicer
associated with these loci, together with Ago1, promotes the formation
of repressive H3K9me2 through the processing of dsRNA.
Figure 3 Dicer affects levels of nascent transcripts and TGS. (a) qRT-PCR analysis of sense and antisense transcripts from Dicer-binding loci. Signals are normalized to the levels of sense transcript, set at 1. Error bars, s.d. (n = 3 cell cultures). *P < 0.05 by two-tailed Student’s t test. (b) qRT-PCR analysis of sense transcripts of Dicer-binding loci. Signals are normalized to the levels of sense transcript in normal cells, set at 1. Error bars, s.d. (n = 3 cell cultures). *P < 0.05 by two-tailed Student’s t test. (c) qRT-PCR as in b detecting antisense transcripts. Error bars, s.d. (n = 3 cell cultures). *P < 0.05; **P < 0.12 by two-tailed Student’s t test. (d) qRT-PCR analysis of RNA isolated from Dicer-knockdown HEK293 cells and treated with or without V1 nuclease. Sense-transcript signals are normalized to the levels of transcripts in untreated samples, set as 1. Error bars, s.d. (n = 3 cell cultures). *P < 0.05 by two-tailed Student’s t test. (e) qRT-PCR as in d detecting antisense transcripts. Error bars, s.d. (n = 3 cell cultures). *P < 0.05 by two-tailed Student’s t test. (f) ChIP analysis of Ago1 levels 28S. Error bars, s.e.m. (n = 3 cell cultures). (g) Ratio of H3K9me2 ChIP signal versus H3 as in f. (h) ChIP analysis of Pol II levels on four tested genes in normal and Dicer-knockdown HEK293 cells. Pol II levels are expressed as relative percentage of input normalized to normal cells. Error bars, s.e.m. (n = 3 cell cultures).

Dicer restricts dsRNA accumulation in nuclei

We used a dsRNA-specific antibody (J2) to directly visualize dsRNA accumulation by cellular immunofluorescence. To confirm J2 specificity, we tested its in vitro binding to dsRNA versus a hairpin premicroRNA and single-stranded RNA (ssRNA) (Online Methods). As shown by IP experiments, dsRNA bound efficiently to J2 antibody (43.7% of input signal), and binding was greatly reduced with either a pre-microRNA–like hairpin RNA (4.7% of input signal) or ssRNA (8.9% of input signal), each of similar lengths (Fig. 4a). Although some pre-microRNA–like hairpin RNAs and ssRNAs were precipitated, this was in oversaturated experimental samples. These results confirmed that J2 is dsRNA selective. Furthermore, J2-immunoprecipitated dsRNA was highly sensitive to dsRNA-specific V1 RNase versus single-strand–specific S1 nuclease (Fig. 4b).

Immunofluorescence experiments on normal HEK293 cells gave little dsRNA signal in either nucleus or cytoplasm. In contrast, Dicer-knockdown cells showed a clear accumulation of dsRNA signal, especially around the nuclear envelope but also within the nuclear compartment (Fig. 4c). We tested the effect of Drosha knockdown on dsRNA accumulation, because loss of Dicer might have indirect effects on dsRNA accumulation through loss of microRNA production. We knocked down Drosha expression in HEK293 cells, either by transfection with a plasmid expressing shRNA against DROSHA mRNA (Fig. 4d) or by DROSHA-specific siRNA treatment (Supplementary Fig. 4a). In both cases, Drosha expression was reduced to about 10% of expression in normal cells, as shown by immunofluorescence (Fig. 4d).

To ensure that at this level of Drosha knockdown there is an increase
in primary microRNAs, we analyzed the levels of pri-miR15a and showed that its levels doubled upon DROSHA shRNA–directed knockdown (Supplementary Fig. 4b). Even though Drosha levels were substantially reduced in these knockdown cells, no detectible accumulation of J2-specific dsRNA was apparent. This suggests that accumulation of dsRNA through Dicer knockdown is not due to the accumulation of pre-microRNA through loss of Dicer activity. We also tested whether loss of Dicer induces dsRNA accumulation through general cellular stress. Treatment of HEK293 cells with acivicin, an inhibitor of γ-glutamyl transpeptidase, failed to induce dsRNA accumulation (Fig. 4c). Finally, we tested the effect of Dicer loss on a cell type unrelated to HEK293, ES cells derived from Dicer-knockout mice28. In these ES cells, we again detected nuclear Dicer signals that were lost in knockout cells (Supplementary Fig. 5). Notably, Dicer accumulated in the nucleoli of ES cells, and this possibly reflects the different expression patterns of this cell type. Nonetheless, we demonstrate the presence of nuclear Dicer in a different mammal and cell type. We also confirmed that these Dicer-knockout cells display a clear J2-specific dsRNA signal in both the nucleus and cytoplasm.

We sequenced dsRNA isolated from normal and Dicer-knockdown cells treated with T1 nuclease to remove ssRNA by genomic RNA sequencing (RNA-seq; Fig. 5). As shown for specific loci (Fig. 5a; 5′-flanking region of KPNA2) and genome wide, there is a remarkable correspondence between Dicer-associated loci and dsRNA. Moreover, 59% of loci showing Dicer and Pol II co-occurrence (Online Methods) were also associated with dsRNA (Fig. 5b). Levels of dsRNA detected in Dicer-depleted cells, as compared to normal cells, indicate that in most cases dsRNA accumulates upon Dicer depletion at sites where Pol II, Dicer and dsRNA colocalize (Fig. 5b). The median length of dsRNA peaks, in both normal and Dicer-depleted cells, was approximately 150 nt (Fig. 5d), and a detailed numerical analysis of dsRNA levels at Dicer-associated loci shows that dsRNA levels often increase substantially after Dicer knockdown (Supplementary Fig. 6a–c). Repetitive genomic elements including LINEs, SINEs, LTRs and satellites are significantly underrepresented in the dsRNA data set. In contrast, rRNAs and tRNAs are significantly overrepresented among dsRNA peaks (Supplementary Fig. 6d). Metagene analysis of the top dsRNA peaks (Online Methods) showed an accumulation before TSSs and a broader distribution around PASs (Supplementary Fig. 6e).

The co-occurrence of dsRNA and Dicer genome wide, and the fact that dsRNA accumulates upon Dicer depletion, suggest that dsRNA is processed into siRNA. For three of the four Dicer-positive loci tested in these studies (Figs. 2 and 3), we detected siRNAs above background levels, using a chemical cross-linking northern blot technique. Furthermore, these weak siRNA signals were reduced after Dicer knockdown (Fig. 6a). No RNA-dependent RNA-polymerase activity has been observed in mammals, unlike in S. pombe and plants, in which...
Loss of Dicer triggers the interferon response and apoptosis

Mammalian somatic cells (though not stem cells) possess a potent antiviral mechanism, the interferon–response pathway, triggered by cytoplasmic dsRNA. Interferon leads to cellular apoptosis, which in turn eliminates virally infected cells. Because loss of Dicer leads to accumulation of dsRNA, we tested whether this also results in an interferon-induction response. In HEK293 cells grown for 1 or 2 weeks under Dicer shRNA-induction conditions, two key proteins in the interferon-response pathway, TLR3 (ref. 35) and PKR1 (ref. 9), were clearly elevated by Dicer knockdown, on the basis of quantitative western blotting (Fig. 7a and Supplementary Fig. 7a). We also confirmed PKR1 activation by Dicer knockdown, using immunofluorescence and the PKR1-specific antibody (Supplementary Fig. 7b). Additional interferon-induced proteins, INF-β and OAS1, were also strongly upregulated in Dicer-knockdown cells (Fig. 7b), as was...
ADAR1, an enzyme involved in dsRNA editing, and which, in its long form, is interferon inducible (Fig. 7c). To control for the possibility of off-target effects, we examined the levels of OAS1 and TLR3 in Dicer-depleted cells, using an siRNA targeted to a different sequence on Dicer mRNA. Again, induction of the interferon response was evident, suggesting that this is a Dicer-specific effect (Fig. 7d).

For a negative control, we analyzed the levels of TLR3 after shRNA-directed knockdown of Dicer, endogenous dsRNA accumulates, and establishment of H3K9me2. In cells lacking Dicer, endogenous dsRNA accumulates, thus resulting in interferon induction and consequent cell death.

**DISCUSSION**

Dicer has a well-established role in siRNA biogenesis and, in mammalian cells, it has been considered to be a purely cytoplasmic protein. In contrast, we detected Dicer in the nucleus, but when Dicer was overexpressed from an episomal plasmid it was transported into the nucleus only if the endogenous protein had also been depleted. Possibly, a chaperone (either protein or potentially RNA) is required for the nuclear localization of Dicer. This proposed chaperone is under active investigation. The presence of nuclear Dicer in mammalian cells it has been considered to be a purely cytoplasmic protein. The capacity for such transcripts to anneal and generate dsRNA is commonplace across the mammalian genome. Repetitive sequence elements such as LINEs and SINEs that often derive from retroposons are also transcriptionally active and may generate dsRNA. Hence dsRNA synthesis is a possible outcome for large tracts of the human genome. The connection between Dicer and SINE-derived dsRNA has been made by studies on the molecular basis of macular degeneration in the human retina. SINE RNA was detectible as dsRNA, and signals were evident in diseased eyes. Importantly, loss of Dicer was directly associated with this defect. These results resonate with our studies on Dicer knockout and dsRNA accumulation. Reduced Dicer levels have also been observed in cancer cell lines, as well as in cells derived from aging animals, thus possibly implying a breakdown in the normal turnover of dsRNA in such cells.

A key aspect of nuclear Dicer function in mammalian cells is likely to relate to its TGS function as described in other eukaryotes. Much of the mammalian somatic-cell genome is encased in repressive heterochromatin and can be classified as either constitutive or facultative. Constitutive or pericentric heterochromatin is associated with repetitive elements such as satellite sequence and is generally defined by CG methylation and H3 K9 trimethyl marks that, in turn, recruit heterochromatic proteins such as HP1 isoforms. Among the methyltransferases responsible for this mark are Suv39h1 and Suv39h2, because their gene deletion in transgenic mice results in loss of pericentric heterochromatin. Recent studies have associated the establishment of this class of heterochromatin with features of actively transcribed DNA such as transcription-factor occupancy. Several reports have indicated that this category of repressed chromatin displays low-level transcriptional activity resulting in dsRNA synthesis. Facilitative heterochromatin may be a more dynamic repressed state, and in this case it is associated with different H3 K9 dimethyl marks, which are probably added by the heterodimeric methyltransferase G9a-GLP. Several studies have indicated that this type of repressed chromatin can be induced at localized positions by exogenous siRNA or may exist endogenously, acting to regulate gene transcription and transcript RNA processing. We suggest, on the basis of identification of H3 K9 dimethyl marks in Dicer-associated loci, that there is a Dicer-mediated conversion of locally synthesized dsRNA into an RNAi response through Ago1 that, in turn, sets up and maintains heterochromatin structure.

Finally, we showed that Dicer knockdown perturbs the normal cycle of low-level dsRNA synthesis and Dicer-dependent TGS, resulting in
accumulation of dsRNA throughout the cell. We predict that dsRNA initially formed in the nucleus escapes into the cytoplasm and triggers the interferon response leading to cellular apoptosis (model in Fig. 8). Numerous studies have shown that Dicer loss has severe consequences to the cell. Thus, mice lacking Dicer fail to develop beyond the embryonic stage. However, although ES cells derived from these knockout mice are viable (albeit growth impaired), ES cells lack the interferon-response pathway, thus explaining their viability. In contrast, this study shows that for cultured mammalian somatic cells, loss of Dicer leads to dsRNA accumulation and consequent apoptosis. We therefore predict that a major role of nuclear Dicer is to set up the right balance between heterochromatin and euchromatin. Failure of this process through Dicer knockout results in dsRNA accumulation. This results in a ‘last-ditch’ process of cellular apoptosis to eliminate misregulated cells that would otherwise lead to cell pathologies such as cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Genomic ChIP-seq and RNA-seq data have been deposited in the Gene Expression Omnibus database under accession number GSE56648.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We are grateful to E. Johnson for help with transmission electron microscopy and scanning electron microscopy experiments. This work was supported by grants from the Wellcome Trust (091805/Z/10/Z to N.J.P) and E.P. Abraham Trust (to N.J.P) and by a Medical Research Council Career Development Award (MR/K006606/1 to M.G.) and a L’Oreal-UNESCO Woman in Science UK and Ireland award (to M.G.).

AUTHOR CONTRIBUTIONS

E.W. and M.G. performed all the experimental analyses. M.S. and K.K.-G. performed bioinformatics analyses of the ChIP-seq and RNA-seq data. E.W. M.G. and N.J.P. designed the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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9. M.G. and N.J.P. designed the experiments and wrote the manuscript. E.W. and M.G. performed all the experimental analyses. M.S. and K.K.-G. performed bioinformatics analyses of the ChIP-seq and RNA-seq data. E.W. M.G. and N.J.P. designed the experiments and wrote the manuscript.
ONLINE METHODS

Primers. Primers used in this work are shown in Supplementary Table 1.

Tissue culture. Human HEK293, Dicer-kd/2b2 (ref. 27) and mouse Dicer knockout ES28 cell lines were maintained under standard conditions. All cell lines were tested for mycoplasma contamination. Induction of shRNA was carried out by addition of 10 µg/ml of doxycycline for 7, 10 or 14 d. Unless otherwise stated, cells were treated with doxycycline for 10 d. Transfections of plasmids expressing shRNA directed against Drosha or PKR1 (ref. 48) were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions every 72 h for a total of 10 d. Transcription inhibition was carried out by addition of 2.5 µg/ml α-amanitin for 48 h. Stress induction was carried out by addition of 2 µg/ml acivicin for 48 h. Transient transfections were performed with Lipofectamine 2000 (Invitrogen).

Immunofluorescence and microscopy. Dicer nuclear localization was analyzed by immunofluorescence and FRAP experiments, with anti-Dicer 13D6 (Abcam, ab14601), according to standard protocol with a confocal microscope (Olympus). Anti-PKR1 (Abcam, ab28943), anti-Drosha (Abcam, ab12286) and J2 antibody (Scicon, 10010200) were also used in immunofluorescence experiments. All presented images show signals at one specific z axis. All antibodies were used by immunofluorescence and FRAP experiments, with anti-Dicer 13D6 (Abcam, ab1791). 5 µg of antibody was used per reaction, and validation for all antibodies except anti-Ago1 is provided on the manufacturer’s website. Immunoprecipitated, nonprecipitated and input DNA was analyzed by qRT-PCR. For ChIP-seq, the eluted ChIP DNA was used for library preparation and cluster generation with Illumina kits, according to the manufacturer’s instructions. After passing Solexa CHASTITY quality filter, the reads were mapped to the human genome (hg19) with BOWTIE, allowing a maximum of mismatches. 990,363 uniquely mapped reads were obtained. 1,957 enriched regions were identified by peak calling with MACS V1.4.0 with the default P-value threshold of 10−5. For downstream analysis, only 119 high-confidence, top-scoring regions (score >100) were considered, as referenced in the manuscript as ‘Dicer ChIP-seq peaks’. For analysis, genomic coordinates of hg19 RefGene transcription start sites (TSSs) and gene bodies were downloaded from the UCSC table browser (http://genome.ucsc.edu/). Promoters were defined as 2 kb regions flanking TSSs 1 kb upstream and downstream. Gene bodies were defined as generic regions excluding previously defined promoters. Terminators were defined as regions 5 kb downstream of annotated polyadenylation sites (PASs) that do not overlap with any promoter or gene body. Distal promoters were defined as regions between 1 kb and 5 kb upstream of a promoter, not overlapping with any of the previous categories. The rest of the genome was considered intergenic. The peak sum coordinate determined the annotation. The coordinates of Pol II–binding regions (raw signal as well as called peaks) in the HEK293 cell line were obtained from ENCODE transcription factor–binding sites by ChIP-seq from Stanford/Yale/USC/Harvard (GEO accession GSE31477)49. Dicer and Pol II overlap was calculated in two different ways. In the first case, Dicer ChIP-seq peaks were eliminated from comparison if the signal was lower than an input control from HEK293 cells, and the remaining 49 peaks were assessed for overlap on the basis of Pol II ChIP-seq signal, which also displayed a higher level of signal than that of the input control. In the second case, overlap of Dicer and Pol II peaks was calculated with PeakAnalyzer 1.4, with 105 random data sets as a control (used for Supplementary Fig. 2).

RNA analysis. RNA isolation was carried out with TRIzol reagent (Invitrogen) and reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen) with gene-specific primers. RNA to prepare dsRNA, ssRNA and miRNA was in vitro transcribed from PCR-generated templates of either exon 2 of the human β-globin gene or the mir22 sequence, with T7 or T3. RNA was then gel-purified and for dsRNA and miRNA was hybridized by denaturation at 95 °C and then cooled slowly to allow RNAs to anneal before being used for IP with 1 µg J2 antibody (Scicon, 10010200). The binding percentage numbers (Fig. 4a) are based on quantification of the input and the IP signal from 100% of input (lane 2) and calculated as follows: (IP/(input × 10)) × 100. Digestions with V1, T1 and S1 (Ambion) were carried out according to the manufacturer’s specifications. For RNA-seq, total RNA was treated with T1 nuclease according to the manufacturer instructions, and remaining dsRNA was purified and used for library preparation with NERNext Small RNA Library Prep Set for Illumina, according to the manufacturer’s instructions. RNA reads were mapped to the human genome as described for ChIP-seq. Peaks derived from normal and Dicer-depleted cells were cross-compared: multiple peaks in one data set that overlapped with only one peak in the other were combined into one peak. This was done iteratively until no change was observed within the data sets. For downstream analysis, we imposed a cutoff of 1,500 in both dsRNA samples, and only the remaining peaks were considered (dsRNA top peaks). Metagene analysis was carried out as described for ChIP-seq, on the defined dsRNA top peaks. The box plots for dsRNA top peaks display points as outliers (red crosses) if they are larger than q3 + 1.5(q3 − q1) or smaller than q1 − 1.5(q3 − q1), where q1 and q3 are the 25th and 75th percentiles (the blue box), respectively. The default whisker length of 1.5 corresponds to approximately ±2.7σ and 99.3% coverage if the data are normally distributed. The plotted whisker extends to the adjacent value, which is the most extreme data value that is not an outlier. Loci where Dicer and Pol II colocalized were then overlapped with dsRNA peaks to find co-occurrence in Perl 5.14.2 (Fig. 5b). Small-RNA mapped binary data was downloaded from the UCSC genome browser for IMR90 cells52. Any signal above 0 was considered as presence of small RNA. Peaks were overlapped with the dsRNA peaks data set and with the Dicer ChIP-seq top (118) peaks data set to find co-occurrence in Perl 5.14.2 (Fig. 6c). siRNA isolation was carried out with PEG precipitation and separated with 20% PAGE, transferred with a semidry blot apparatus and chemically cross-linked (with EDC53) before being probed with 32P-labeled PCR products of tested gene loci. Probes were labeled with the DECAprime kit (Ambion).

Protein analysis. Immunoprecipitation and immunodepletion experiments were performed on nuclear extracts, with specific antibodies (Pol II 8WG16 (Abcam, ab24758) and Dicer 13D6 (Abcam, ab14601)). 10 µl of antibody was used per reaction, and validation of the antibodies in immunoprecipitation is provided on the manufacturer’s website. Western blot experiments were performed according to standard protocols with the following antibodies: anti–Pol II 8WG16 (Abcam, ab24758), anti–Pol II N20 (Santa Cruz Biotechnology, sc-899), anti-Dicer 13D6 (Abcam, ab14601), anti–β-actin (Sigma-A3853), anti-Spi5 (Millipore, ABE443), anti–TLR3 (Millipore, 06-008), anti–PKR1 (Millipore, 07-151), anti–IFN-β (Abcam, ab6979), anti–OAS1 (Abcam, ab82666), anti–ADAR1 (Abcam, ab88574), anti-Drosha (Abcam, ab12286), anti–α-tubulin (Sigma, T5168) and anti–Grp75 (Abcam, ab2799). Antibodies were used at 1:500 dilution except anti–α-tubulin, which was used at 1:10,000. Validation for all antibodies is provided on the manufacturer’s websites.

Scanning electron microscopy. Cells grown on glass cover slips were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate, pH 7.2, and this was followed by secondary fixation in 1% osmium tetroxide. Samples were dehydrated sequentially in 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol and dried with a Critical Point Dryer. Finally, cells were gold-plated and viewed under the SEM.

Transmission electron microscopy. Cells grown on cover slips were fixed as described for SEM. Dehydration was followed by epoxy-resin infiltration and embedding. Resin blocks with covers on top were polymerized overnight at 60 °C. To remove cover slips, sample blocks were submerged in liquid nitrogen. Blocks with samples were cut for ultrathin sections (70 nm) and transferred to a 200 copper-mesh grid. Grids were poststained with 2% uranyl acetate and lead citrate, then viewed under the TEM.

Flow cytometry. Normal and Dicer-, Drosha- or PKR-depleted cells were labeled with annexin V APC (Ebioscience) and 7-AAD (Ebioscience) and then measured by flow cytometry according to the manufacturer’s instructions.
Relative levels of pulldown were measured by ImageQuant software and are expressed as a percentage of input.

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