Nuclear phosphorylated Dicer processes double-stranded RNA in response to DNA damage

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The endoribonuclease Dicer is a key component of the human RNA interference pathway and is known for its role in cytoplasmic microRNA production. Recent findings suggest that noncanonical Dicer generates small noncoding RNA to mediate the DNA damage response (DDR). Here, we show that human Dicer is phosphorylated in the platform–Piwi/Argonaute/Zwille–connector helix cassette (S1016) upon induction of DNA damage. Phosphorylated Dicer (p-Dicer) accumulates in the nucleus and is recruited to DNA double-strand breaks. We further demonstrate that turnover of damage-induced nuclear, double-stranded (ds) RNA requires additional phosphorylation of carboxy-terminal Dicer residues (S1728 and S1852). DNA damage-induced nuclear Dicer accumulation is conserved in mammals. Dicer depletion causes endogenous DNA damage and delays the DDR by impaired recruitment of repair factors MDC1 and 53BP1. Collectively, we place Dicer within the context of the DDR by demonstrating a DNA damage-inducible phosphoswitch that causes localized processing of nuclear dsRNA by p-Dicer to promote DNA repair.

Introduction

The endoribonuclease Dicer is a key component of the RNAi pathway. Dicer processing generates 20–25-nt-long miRNA from a stem-loop precursor miRNA (Chendrimada et al., 2005; Haase et al., 2005). Mature miRNA are loaded onto the Argonaute-containing, RNA-induced silencing complex to target complementary mRNA for degradation or inhibition of translation (Filipowicz et al., 2008; Meister, 2013; Ha and Kim, 2014). Canonical RNAi modulates gene expression by posttranscriptional gene silencing in the cytoplasm to regulate development, tumor suppression, and metabolism (He and Hannon, 2014). Canonical RNAi and miRNA biogenesis and involve noncanonical modes of RNAi processes. Recent findings suggest that noncanonical Dicer generates small noncoding RNA to mediate the DNA damage response (DDR). Here, we show that human Dicer is phosphorylated in the platform–Piwi/Argonaute/Zwille–connector helix cassette (S1016) upon induction of DNA damage. Phosphorylated Dicer (p-Dicer) accumulates in the nucleus and is recruited to DNA double-strand breaks. We further demonstrate that turnover of damage-induced nuclear, double-stranded (ds) RNA requires additional phosphorylation of carboxy-terminal Dicer residues (S1728 and S1852). DNA damage-induced nuclear Dicer accumulation is conserved in mammals. Dicer depletion causes endogenous DNA damage and delays the DDR by impaired recruitment of repair factors MDC1 and 53BP1. Collectively, we place Dicer within the context of the DDR by demonstrating a DNA damage-inducible phosphoswitch that causes localized processing of nuclear dsRNA by p-Dicer to promote DNA repair.

A growing body of evidence suggests that additional functions for Dicer proteins exist, which are independent of miRNA biogenesis and involve noncanonical modes of RNAi processes. In fission yeast, nuclear Dcr1 facilitates transcriptional gene silencing of centromeric, heterochromatic repeats and repression of integrated transgenes by targeting dsRNA formed at actively transcribed loci (Provost et al., 2002; Volpe et al., 2002; Bühler et al., 2006). Dcr1 further promotes the release of RNAPII at termination regions of both highly transcribed protein-coding genes and antisense transcription units of rRNA and ribosomal RNA loci to resolve replication stress (Zaratiegui et al., 2011; Castel et al., 2014). Human nuclear Dicer modulates RNA PI transcription of coding and noncoding transcription units. Dicer stimulates RNAPII transcription at a subset of hormone-responsive promoters in complex with IFN-inducible, dsRNA-dependent protein kinase A activator and steroid-receptor RNA activator (Redfern et al., 2013), as well as silencing of the secreted frizzled-related protein 1 (SFRP1) gene in cholangiocarcinoma cells (Cheng et al., 2017). We showed previously that human Dicer localizes to the nucleus to process endogenous (endo)-dsRNA derived from overlapping transcription units. In the absence of Dicer, unprocessed nuclear endo-dsRNA translates to the cytoplasm and triggers IFN-mediated apoptosis (White et al., 2014). Formation of dsRNA around intronic polyadenylation sites recruits Dicer to chromatin to promote alternative polyadenylation (Neve et al., 2016). Dicer also generates endo-siRNA from dsRNA formed at terminator elements of protein-coding genes to guide heterochromatin formation. This leads to RNAPII pausing and promotes transcription termina-
function of phosphorylated nuclear Dicer in the promotion of turnover of damage-induced dsRNA. Our data suggest a direct role of Dicer in repairing DNA damage in close proximity to DSBs.

The tumor suppressor p53 is an integral component of the DDR and activates the DICER1 locus, loss of p53impairs Dicer expression (Su et al., 2010; Muller et al., 2014). This led us to test Dicer levels in human HEK293 cells subjected to DNA damage-inducing agents Etoposide (Eto; Hande, 1998), hydrogen peroxide, phleomycin, methyl methanesulfonate (MMS), or γ-irradiation. Surprisingly, Dicer expression was not significantly affected in HEK293 cells after continuous drug incubation (Fig. S1 A) or induction and repair of DNA damage (Fig. S1 B). Ser139 phosphorylation of the histone variant H2A.X (γH2A.X) was used as a marker for DNA damage.

We speculated that DNA damage might alter posttranslational modifications of Dicer. To assess changes in Dicer phosphorylation in response to DNA damage, we performed [32P]orthophosphate in vivo metabolic labeling before immunoprecipitation of endogenous Dicer in wild-type HEK293 cells (Fig. 1 A). We detected 5–10-fold induction of various damage-inducible and phosphatase-sensitive bands migrating at ~250 kD. We further observed a shift in migration of Dicer, but not immunoglobulin heavy chain by 6.2% on Phos-tag gels after immunoprecipitation of tandem affinity purification (TAP)–tagged Dicer from cells treated with Eto (Fig. 1 B).

To assess the subcellular distribution of Dicer upon DNA damage, we used subcellular fractionation of HEK293 cells (Fig. 1 C) and a previously characterized phospho-specific Dicer antibody (p-DCR-1), which recognizes two conserved phospho-serine residues (S1728 and S1852; Drake et al., 2014). We detected a two- to threefold increase in p-DCR-1, but not total Dicer signal in damaged nuclei after immunoprecipitation of endogenous Dicer (Fig. 1 D) or TAP-tagged Dicer (Fig. S1 C). We confirmed specific enrichment of TAP–tagged Dicer in cells lackingendo-Dicer by comparison with background in noninduced cells (Fig. S1 D). Using confocal microscopy, we detected several p-DCR-1 spots after incubation with Eto (Fig. S1 E). To monitor the specificity of the p-DCR-1 antibody, we made use of a conditional Dicer-knockdown cell line (Schmitter et al., 2006). Depletion of Dicer was confirmed by staining with the 13D6 antibody, which recognizes total Dicer. Moreover, p-DCR-1 foci were only visible in the nuclei of damaged, wild-type, but not Dicer-depleted, HEK293 cells upon incubation with Eto (Fig. 1 E) or hydrogen peroxide (Fig. S1 F). Next, we applied γ-irradiation and detected a wave of nuclear p-DCR-1 staining concomitant with induction and clearance of γH2A.X using time kinetics. Phosphorylation of H2A.X was strongly induced after 30 min and remained high up to 3 h after irradiation (Fig. 1 F). In contrast, p-DCR-1 did not stain cells treated with osmotic stress (0.1× PBS, 10× PBS) or hydroxyurea (HU; Fig. S1 G). Hydroxyurea induces γH2A.X originating from stalled replication forks (Ward and Chen, 2001) and stimulates phosphorylation of ataxia telangiectasia mutated (ATM)/ATM-related (ATR) kinase substrates (Fig. S1 H), suggesting that nuclear p-DCR-1 foci are primarily caused by DSBs. We further measured proliferation of damaged HEK293 cells and monitored expression of cellular markers of proliferation (Ki-67) and apoptosis (cleaved poly-ADP-ribose polymerase, PARPc) to rule out that nuclear Dicer activity is primarily caused by induction of apoptosis, as demonstrated previously in Caenorhabditis elegans (Nakagawa et al., 2010). Unlike staurosporine (STS), an apoptosis-inducing kinase inhibitor (Kabir et al., 2002), incubation with DNA-damaging agents for 2 h did not significantly alter proliferation (Fig. S1 I) or expression of Ki-67 or levels of PARPc, but induced γH2A.X (Fig. S1 J). Surprisingly, we could not detect significant damage-induced...
Figure 1. Phosphorylation and nuclear accumulation of Dicer upon DNA damage in HEK293 cells. (A) Detection of phosphorylated (autoradiograph, p-Dicer) or total Dicer (immunoblot, A-2) immunoprecipitated with 13D6 from whole cell extracts (WCE) after 32P-orthophosphate metabolic labeling in the absence or presence of calf intestine phosphatase (CIP). CIP signals, silver stain; Eto., etoposide; H2O2, hydrogen peroxide; IgG, immunoglobulin heavy chain. Immunoblot signals were quantified using ImageJ. (B) Immunoblot showing Dicer-TAP migration by Phos-tag SDS-PAGE immunoprecipitated from whole cell extracts (WCE). IgG, immunoglobulin heavy chain; #, unspecific signal; migration units relative to wells. The entire gel is shown. (C) Immuno-bLOTS showing total Dicer (A-2) in subcellular fractions. CP/NP, cytoplasmic/nuclear fraction; fractionation marker: Rad21 and H3, nucleoplasm/chromatin.
changes in the subcellular localization of Dicer with antibodies that recognize the total Dicer pool in immunoblotting (A-2) and confocal imaging (13D6) experiments. We conclude that a fraction of the cellular Dicer pool is responsive to DNA damage and accumulates in the nucleus upon phosphorylation.

**Recruitment of phosphorylated Dicer (p-Dicer) to DNA DSBs**

Recent findings indicate that Dicer promotes DNA repair by generating site-specific, small regulatory RNA in close proximity to DSBs in various organisms (Francia et al., 2012; Michalik et al., 2012; Wei et al., 2012). To assess involvement of human Dicer at DSBs, we used the AsiSI-ER U2OS cell line, which harbors the recombinant endonuclease, AsiSI, which is fused to the estrogen receptor (ER) ligand-binding domain (Iacovoni et al., 2010). Treatment with 4-hydroxytamoxifen (4OHT) triggers nuclear translocation of AsiSI-ER and induces DSBs at AsiSI target sites (CCGATGC, nonmethylated), which allows sequence-specific analysis of DSB-associated proteins. First, we confirmed inducible γH2A.X chromatin immunoprecipitation (ChIP) signals at two previously characterized AsiSI sites (DS1, chr1:88993018–88993227; CCBL2/RBMX1 promoter; DS2, chr6:89638287–89638451, LYRM2 intron 1; Caron et al., 2012; Fig. 2 A, left). The human genome contains 1,231 predicted AsiSI-ER targets sites in both genic and intergenic regions (Fig. 2 A, right). We detected strong, 4OHT-induced γH2A.X ChIP signals at DS1/2 and up to 1-kb distant from DS1 in AsiSI-ER U2OS cells, but not in wild-type U2OS cells or at a nontargeted, exonic GAPDH control locus (Fig. S2 A). In line with DNA-damaging agents, we confirmed a 4OHT-dependent induction of γH2A.X, but not Dicer levels, by immunoblotting (Fig. 2 B) and partial colocalization of Dicer with γH2A.X-positive damage foci (Fig. 2 C).

Our p-Dicer-1 data suggest that Dicer localizes in close proximity to γH2A.X in damaged nuclei. To test recruitment of Dicer to DSBs, we used ChIP analysis at DS1/2 using the 13D6 antibody. Strikingly, we detected a four- to sixfold increase in Dicer occupancy upon induction of DSBs at DS1/2 (Fig. 2 D, left). Dicer recruitment peaked ~500-nt distant from DS1 (Fig. 2 D, right) and was sensitive to preincubation with recombinant, dsRNA-specific RNase III (Fig. 2 E) as well as depletion of endogenous Dicer by transiently transfected shRNA (Fig. S2, B and C). To assess Dicer chromatin occupancy at DSBs globally, we used Dicer ChIP-seq analysis in AsiSI-ER U2OS cells. Meta-gene analysis revealed genome-wide Dicer association with γH2A.X-positive, AsiSI-restricted DSBs at genic loci, such as the TRIM37 promoter (Fig. 2, F and G; and Fig. S2 D) upon 4OHT incubation. Dicer levels were not increased at various AsiSI sites in control HEK293 cells (Fig. S2 E). Dicer occupancy was increased two- to threefold at restricted genic AsiSI target sites, but was also detectable at intergenic loci (Fig. S2, F and G) upon DNA damage induction. During cell division and nuclear membrane disassembly, a fraction of the AsiSI enzyme can leak into the nucleus in absence of 4OHT to target highly accessible AsiSI sites. This phenomenon can cause a certain “damage-like phenotype” in ~4OHT conditions, especially in genome wide analyses. We conclude that Dicer is recruited to DSBs in a dsRNA-dependent manner.

Next, we assessed whether DNA damage signaling induces Dicer phosphorylation at residues S1728 and S1852. Three members of the phosphatidylinositol-3-kinase (PI3K) family, ATM, ATR, and DNA-dependent protein kinase (DNA-PK) govern the response to DNA damage by phosphorylating hundreds of substrates (Kastan and Lim, 2000; Matsuoka et al., 2007; Giglia-Mari et al., 2011; Maréchal and Zou, 2013). We speculated that PI3Ks target Dicer in the DDR. Indeed, preincubation of AsiSI-ER U2OS cells with various kinase inhibitors prevented damage-induced p-DCR-1 foci formation and accumulation of γH2A.X but did not affect Dicer expression (Fig. S3, A and B). Similarly, p-DCR-1 foci were largely diminished after depletion of the DNA-PK catalytic subunit (Fig. S3, C and D). We conclude that DSBR induced Dicer phosphorylation at residues S1728 and S1852 is dependent on PI3K signaling.

**Phosphorylation of Dicer residue S1016 is necessary and sufficient for nuclear localization**

More than 30 phosphoresidues have been detected for human Dicer (http://www.phosphosite.org). To analyze DNA damage-induced Dicer phosphorylation in detail, we used comparative phosphoproteomics of total Dicer immunoprecipitated from HEK293 nuclei. In total, we detected seven phosphorylated Dicer residues. A single serine residue in the Dicer platform—PAZ–connector helix S1016 was increased threefold upon DNA damage, whereas unmodified Dicer peptides did not change (Fig. 3 A, Fig. S4, and Tables S1 and S2). These findings suggest that a subset of Dicer is phosphorylated upon DNA damage at residue S1016. Surprisingly, we could not detect phosphorylation of serine residues 1728 or 1852 phosphopeptides, which correspond to the p-DCR-1 epitope.

To assess the relevance of Dicer phosphorylation for subcellular localization, we created RFP-tagged nonphosphorylatable (residues S1016A, S1728/1852A) or phosphomimetic (S1016D, S1016E) Dicer mutants and expressed them in wild-type HEK293 cells (Fig. 3 B and Fig. S5 A). Although RFP-Dicerα and RFP-Dicerδ localized mostly in the cytoplasm in nondamaged cells, RFP-Dicerα, but not RFP-Dicerδ, displayed increased nuclear accumulation upon Eto treatment in a subset of cells. In contrast, RFP-Dicerβδ displayed consistent nuclear localization. Surprisingly, the RFP-Dicerβδδ double mutant remained nuclear (Fig. 3, C and D). We confirmed comparable expression of all RFP constructs in these cells (Fig. S5 B). Similarly, we detected damage-induced nuclear localization of GFP-tagged, wild-type Dicer in a subset of cells (Fig. S5 C).

So far, we assessed RFP-Dicer localization after overexpression of tagged-Dicer and in presence of endo-Dicer. Although detecting clear differences in subcellular localization,
we next aimed to express the RFP-Dicer constructs in Dicer-depleted (Dicer KD) cells to assess damage-induced Dicer localization in the absence of endo-Dicer (Fig. S5 D). Note that the shRNA, which targets endo-Dicer, prevents overexpression of RFP-Dicer constructs in Dicer KD cells, resulting in more physiologic expression levels (compare Fig. S5, B and E). Con-
Consistently, we detected nuclear RFP-Dicer upon damage. When expressing RFP-Dicer\textsuperscript{S1016A} in damaged Dicer KD cells, the p-DCR-1 antibody displayed strong cytoplasmic signals, colocalizing with nonphosphorylatable RFP-Dicer\textsuperscript{S1016A}. In contrast, expression of the RFP-Dicer\textsuperscript{S1728/1852A} double mutant displayed nuclear RFP signal but no detectable p-DCR-1 signal, underscoring the specificity of the p-DCR-1 antibody. We conclude that phosphorylation of residue S1016, but not S1728/S1852, is necessary and sufficient for nuclear accumulation of Dicer.

Damage-induced accumulation of nuclear Dicer is conserved in mammals

Encouraged by DNA damage-induced nuclear Dicer accumulation in HEK293 cells, we next investigated the subcellular localization of endogenously tagged Dicer. Therefore, we used a recently described HA-tagged mouse embryonic fibroblast (MEF) cell line (PMEF::HA-Dicer; Comazzetto et al., 2014). First, we assessed the specificity of the HA antibody and confirmed expression of full length HA-tagged Dicer.
PMEF::HA-Dicer cells by confocal imaging and immunoblotting (Fig. 4 A). Surprisingly, HA staining was detectable in >90% of PMEF::HA-Dicer nuclei, in addition to widespread cytoplasmic staining. The HA reactivity was largely diminished in wild-type MEF cells and generated a single band migrating at ~250 kD after incubation with PMEF::HA-Dicer, but not wild-type, MEF extracts.

Next, we performed an interspecies heterokaryon assay to assess changes in the subcellular localization of mouse HA-Dicer in response to AsiSI-ER–induced DSBs. Co-culture and fusion of human AsiSI-ER U2OS cells with either wild-type or HA-Dicer–expressing MEFs resulted in sporadic formation of interspecies heterokaryons, consisting of a cytoplasmic continuum with both human and mouse nuclei (Fig. 4 B). In absence of 4OHT, we could detect neither significant induction of γH2A.X nor nuclear accumulation of HA-Dicer in human nuclei. In mouse nuclei, γH2A.X levels were also low and accompanied by modest HA staining. Strikingly, addition of 4OHT strongly elevated γH2A.X signals, confirming DSB induction by the AsiSI-ER endonuclease in both human and mouse nuclei. Concomitantly, we detected strong, spotted HA staining in nuclei of both species. HA signals co-localized with γH2A.X–positive foci, suggesting recruitment of mouse HA-Dicer to DSBs. We observed that the AsiSI-ER endonuclease encoded in U2OS cells was also HA tagged. To dissect the contribution of HA-Dicer and AsiSI-ER toward the observed HA staining, we fused wild-type MEF cells with AsiSI-ER U2OS cells, resulting in interspecies heterokaryons devoid of HA-Dicer. We observed no HA signal in mouse nuclei, despite induction of γH2A.X foci in the presence of 4OHT. Similarly, colocalization of γH2A.X foci with HA signals was also greatly reduced in human nuclei. We conclude that the HA staining observed in interspecies heterokaryons mostly represents mouse HA-Dicer and that damage-induced nuclear Dicer localization and recruitment to DSBs is conserved in mammals.

**Processing of damage-induced dsRNA by nuclear Dicer**

We noticed that Dicer ChIP signals were sensitive to RNase III incubation in vitro and speculated that Dicer might recognize damage-induced dsRNA as a substrate in vivo. To assess the effect of Dicer phosphorylation on dsRNA processing, we transfected Dicer KD cells with RFP-Dicerwt, RFP-DicerS1016A, or RFP-DicerS1728/1852A and visualized dsRNA levels using the dsRNA-specific antibody J2. We and others previously have confirmed the specificity of J2 toward long dsRNA (>40 bp), but not hairpin pre-miRNA or single-stranded RNA in vitro and in vivo (Bonin et al., 2000; Weber et al., 2006; White et al., 2014). Although no significant onset of J2 reactivity was detectable in damaged wild-type HEK293 cells (Fig. S5 F), incubation with Eto caused cytoplasmic accumulation of dsRNA in untransfected, Dicer KD cells (Fig. 5 A). Processing of dsRNA was partially restored by expression of RFP-Dicerwt and RFP-DicerS1016A, resulting in a two- to threefold decrease in J2 signal intensity (Fig. 5 B). In contrast, RFP-DicerS1728/1852A failed to process dsRNA and dsRNA levels accumulated in the cytoplasm, resembling mock-transfected cells. A modest accumu-
mulation of nuclear J2 signal was detected in Dicer KD cells transfected with RFP-Dicer\textsuperscript{S1728/1852A} after preincubation with the nuclear export inhibitor leptomycin B (LMB), suggesting that damage-induced dsRNA originates in the nucleus. To confirm that Dicer is specifically required for dsRNA processing at DSBs, we induced sequence-specific DSBs in HEK293 cells by transient transfection of recombinant \textit{Asi}\textsubscript{SI-ER} endonuclease. First, we monitored induction of DSBs in HEK293 cells. 4OHT incubation caused a twofold induction of both \textit{S1981-γH2A.X} by transient transfection of recombinant \textit{Asi}\textsubscript{SI-ER} endonuclease at DSBs, we induced sequence-specific DSBs in HEK293 cells to firmly establish that Dicer is specifically required for dsRNA processing that damage-induced dsRNA originates in the nucleus. To completely test the Dicer requirement for dsRNA processing, we assessed the cell cycle distribution of Dicer KD cells (Fig. S5 G). Quantitation of the cell-cycle distribution revealed no significant change after Dicer depletion. Instead, we observed that Dicer depletion caused prolonged phosphorylation of DNA damage-responsive kinases ATM and Chk1, as well as delayed clearance of phosphorylated ATM/ATR substrates and \textit{γH2A.X} levels (Fig. 6 C). Moreover, the combination of Dicer depletion with hydrogen peroxide caused an additive increase in \textit{γH2A.X} levels. We conclude that accumulation of \textit{γH2A.X} levels upon Dicer depletion primarily represents induction of DNA damage and that the DDR is delayed in the absence of Dicer.

To test the relevance of Dicer residues S1016, S1728, and S1852 for the DDR in absence of endo-Dicer, we generated a human A549 Dicer knockout cell line (ΔDicer) using CRISPR/Cas9 and validated both the loss of Dicer expression and the accumulation of \textit{γH2A.X} in ΔDicer cells (Fig. 7 A). Next, we transfected wild-type A549 cells with pbABE::\textit{Asi}\textsubscript{SI-ER} plasmid to test for induction of DSBs. Indeed, we observed a wave of \textit{γH2A.X} induction, peaking 2 h after removal of 4OHT (Fig. 7 B), and time-dependent formation of damage foci, positive for DSB repair factors MDC1 (Fig. 7 C, percentage of MDC1 foci–positive cells) and 53BP1 (Fig. 7 D, percentage of 53BP1 foci–positive cells). Next, we co-transfected pbABE::\textit{Asi}\textsubscript{SI-ER} and RFP-Dicer constructs into ΔDicer cells. Similar to wild-type A549 cells, we observed formation of MDC1- and 53BP1-positive foci after 4OHT incubation and complementation with RFP-Dicer\textsuperscript{S1016A} (Fig. 7, E and F, showing percentage of foci-positive cells; for quantification of foci intensity signal, see Fig. S5 H). Reassuringly, nuclear RFP-Dicer\textsuperscript{S1016A} partially colocalized with damage foci. After transfection of RFP-Dicer\textsuperscript{S1016A} or RFP-Dicer\textsuperscript{S1728/1852A}, however, recruitment of both MDC1 and 53BP1 to the damage foci was largely impaired. We also observed morphological changes, arguably caused by increased cellular stress, in ΔDicer cells complemented with nonphosphorylatable RFP-Dicer mutants. Finally, we confirmed comparable expression of RFP-Dicer constructs in ΔDicer cells (Fig. 7 G). We conclude that wild-type, nuclear Dicer, phosphorylated both at residues S1016 and S1728/S1852, promotes recruitment of DNA repair factors MDC1 and 53BP1 to DSBs.

Collectively, we unravel a damage-inducible Dicer phosphoswitch to engage a subset of cellular Dicer in nuclear dsRNA processing in close proximity to DSBs to promote the DDR (Fig. 7 H).

**Discussion**

Our data provide novel insights into Dicer function during the DDR. We identify a damage-inducible phosphoswitch at human Dicer residue S1016, which is required for nuclear accumulation of Dicer. The damage-induced redistribution of a subset of the cellular Dicer pool parallels the Dicer translocation phenotypes observed in \textit{S. pombe} and \textit{C. elegans} upon heat stress (Woolcock et al., 2012) and developmental stimuli (Beshore et al., 2011; Drake et al., 2014), respectively. We further demon-
strate phosphorylation of Dicer residues S1728/S1852 promotes the turnover of damage-induced dsRNA. The accumulation of Dicer in damaged nuclei is conserved in mammals. We postulate that the presence of nuclear phosphorylated Dicer promotes the DDR, arguably by processing of damage-induced dsRNA to mediate an RNA-dependent DDR.

Multiple phosphorylation events regulate nuclear accumulation and activity of human Dicer

Our data suggest that Dicer S1016 phosphorylation may represent a molecular switch that triggers nuclear accumulation. How does S1016 affect Dicer localization? S1016 resides in the platform–PAZ-connector helix cassette, a species-specific sequence that separates the 2-nt 3′-overhang-binding pocket within the PAZ domain and a phosphate-binding pocket within the platform domain (Tian et al., 2014). S1016 residue is conserved between humans and Drosophila melanogaster but is altered from serine to asparagine in C. elegans (Fig. S4, box). No canonical PAZ domain was identified in S. pombe Dcr1. Thus, phosphorylation of the connector helix may have evolved as a regulatory principle for higher eukaryotes to alter Dicer localization or function. The S1016 residue is located at ∼3.2 Å distance to a co-crystallized small RNA substrate and may...
contribute to dsRNA end recognition (MacRae et al., 2007). The platform–PAZ domain forms a tightly connected, head-like structure in close proximity to the RNase III domains, which are located in the body of the Dicer enzyme (Lau et al., 2012). Thus, S1016 phosphorylation may induce structural rearrangements and thereby also affect the dsRNA binding and processing activity of the dsRBD and RNase III domains in trans. It is tempting to speculate that phosphorylation of S1016 reduces the affinity of human Dicer for its cognate pre-miRNA substrate toward noncognate dsRNA, which is produced in the nucleus and may serve as an anchor to prolong nuclear localization.

The Dicer amino-terminal helicase domain is required for discrimination of dsRNA termini and is supposed to regulate substrate specificities in C. elegans and D. melanogaster (Welker et al., 2011). Deletion, insertion mutagenesis, or limited proteolysis of the helicase domain impairs dsRNA processing activity of Dicer but not its binding to dsRNA (Zhang et al., 2002; Ma et al., 2008; Soifer et al., 2008). A recently discovered oocyte-specific mouse Dicer isoform Dicer(O), which comprises a truncated amino-terminal helicase domain, shows enhanced processing activity toward long dsRNA substrate during mouse development but no apparent change in subcellular localization (Flemr et al., 2013). The subcellular localization of mouse Dicer has been proposed to be exclusively cytoplasmic Much et al., 2016. Close inspection of mass spectrometry data provided by Much et al. (2016) revealed that several factors involved in RNAPIII transcription, such as the RNAPII coactivator p15, the transcriptional coactivator TIF1B, and the pre-mRNA processing factor Fip1, are enriched in HA-Dicer immunoprecipitations, suggesting that a fraction of HA-Dicer interacts with RNA metabolic factors in the nucleus of unperturbed cells. Using these HA::Dicer PMEF cells (Comazzetto et al., 2014) in confocal microscopy and an interspecies heterokaryon assay, we detected nuclear accumulation of HA-Dicer upon induction of DSBs in both mouse and human nuclei.

Localization studies using human Dicer constructs suggest that the helicase domain in the full-length protein occludes the Dicer dsRBD in an auto-inhibitory manner (Doyle et al., 2013). Deletion of the helicase domain or duplication of the dsRBD causes prominent nuclear localization of Dicer. Moreover, a cryptic nuclear localization signal was identified in the dsRBD and partial accumulation of wild-type Dicer was observed upon inhibition of CRM1-dependent nuclear export by LMB. We detected accumulation of damage-induced dsRNA
Figure 7. Impaired recruitment of DNA repair factors upon mutation of Dicer in A549 cells. (A) Immunoblots (top) and confocal microscopy (bottom) detecting endogenous Dicer (H212, A-2, 13D6) in wild-type and Dicer knockout (ΔDicer) A549 cells. Ponce., Ponceau S, loading control; #, unspecific signal. (B) Immunoblots detecting γH2A.X levels in wild-type A549 cells after transfection with pBABE::Asi-S1-ER plasmid and 4OHT incubation (2 h pulse). (C and D) Confocal imaging of MDC1 (C) and 53BP1 (D) in wild-type A549 cells after transfection with pBABE::Asi-S1-ER plasmid and 4OHT incubation as indicated. (E and F) Imaging as in C and D, but performed in ΔDicer cells, including transfection of RFP-Dicer constructs. All quantifications represent...
in the absence of Dicer S1728/S1852 phosphorylation. How does phosphorylation of S1728/S1852 promote turnover of dsRNA? The amino-terminal Dicer helicase domain forms a clamp-like structure adjacent to the RNase III active site in the base of the Dicer enzyme (Lau et al., 2012). Phosphorylation of residues S1728/S1852 may cause structural rearrangements that “unfold” the helicase domain, potentially exposing an “unmasked” carboxy-terminal domain for increased dsRNA binding affinity and catalytic activity (Doyle et al., 2013). However, recent data demonstrate that a cytoplasmic amino-terminal deletion mutant of human Dicer efficiently processes exogenous dsRNA substrates in HEK293-derived Dicer knockout cells but fails to accumulate to the nucleus (Kennedy et al., 2015). Collectively, these studies suggest that Dicer is a nuclear-shuttling protein with a relatively short nuclear half life in unperturbed cells. An unmasked carboxy-terminal domain may be necessary but is arguably insufficient for nuclear accumulation of Dicer, which requires additional, damage-induced S1016 phosphorylation.

**p-Dicer processing is linked to DNA repair.** Our data suggest that p-Dicer is localized predominantly in damaged nuclei and targeted by PI3K signaling. However, we detect cytoplasmic p-DCR-1 staining when expressing cytoplasmic RFP-DicerΔ106A mutants in the absence of endo-Dicer, indicating that damage-induced signaling can phosphorylate Dicer in the cytoplasm. We noticed that most p-DCR-1 staining in damaged cells is mutually exclusive to total Dicer staining using 1D6 antibody. We detected S1016, but not S1728/S1852, Dicer phosphopeptides in samples immunoprecipitated with 1D6 by mass spectrometry. This suggests that Dicer phosphorylation at carboxy-terminal residues S1728/S1852, but not S1016, may mask epitope recognition of 13D6 and that the Dicer signal detected by autoradiography or in ChIP experiments contains S1016, but not S1728/S1852, phosphoepitopes. Nevertheless, we detect S1728/S1852 phosphorylated Dicer after immunoprecipitation with H212 or TAP antibodies. We further show that nuclear Dicer is recruited to DSBs in a dsRNA-dependent manner, suggesting that nascent RNA synthesis is induced at DSBs. Given that recruitment of MDC1 and 53BP1 to DSBs is dependent on both Dicer function and DDRNA (Hawley et al., 2017), we hypothesize that DDRNA may be a product of p-Dicer processing. It is currently unclear how dsRNA is assembled upon DNA damage. Intriguingly, DDRNA may also promote changes in chromatin conformation at DSBs through mechanisms that involve Argonaute proteins and recruitment of chromatin-modifying enzymes (Wei et al., 2012; Gao et al., 2014; Wang and Goldstein, 2016). Collectively, these findings suggest that transcription- and p-Dicer-dependent RNA synthesis promote chromatin relaxation at DSBs to generate a “window of opportunity” for recruitment of repair factors engaged in DNA repair.

**Materials and methods**

**Tissue culture, cell lines, cloning, and transfection**

Mammalian cells were cultured in DMEM (Sigma-Aldrich) with 10% FBS (Thermo Fisher Scientific) at 37°C and 5% CO₂. Expression of recombinant HEK293T-REx cell lines 293-control_sh (Control KD), 2.B (endo-Dicer knockdown), and 1.3 (endo-Dicer knockdown and Dicer-TAP knock-in; Schmitter et al., 2006) was induced with doxycycline (3 µg/ml; Sigma-Aldrich) for 2–5 d. Wild-type U2OS or A5SI-SR U2OS cells (a gift from the Esashi Laboratory, Centre for Regenerative Medicine, Edinburgh, Scotland, UK) were induced with 4OHT (300 nM; Cayman Chemical) for 2–4 h. Wild-type MEF or PMEF::HA-Dicer PMEF cells (a gift from the O’Carroll Laboratory, Centre for Regenerative Medicine) were cultured at low passages (<20 passages). For site-directed mutagenesis, pTagRFP-Flag-HA-linker-huDicer plasmid (10 ng, a gift from M. Drozdz, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland), harboring wild-type, RFP-tagged Dicer, was amplified using site-specific primers and Phusion HF high-fidelity polynuclease (New England Biolabs, Inc.). For primers, see Table S3. Parental plasmids were digested with 5 U DpnI (Promega) overnight at 37°C, transformed in XL-1-Blue competent cells using heat shock (42°C, 45 s), amplified and purified using the QiAprep spin mini prep kit (Qiagen). Mutations were confirmed by Sanger sequencing. Transient transfections of HEK-tagged A5SI-SR-encoding pBabe plasmid (a gift from the d’Adda di Fagagna Laboratory, Milan, Italy; Iaconov et al., 2010), shRNA-encoding Dicer knockdown plasmid (Mission shDicer NM_030621; 10271413MN; Sigma-Aldrich), GFP::RFP-Dicer plasmids (pTagEGFP-Flag-HA-linker-huDicer and pTagRFP-Flag-HA-linker-huDicer, a gift from Maciek Drozdz), or mutations thereof were performed using Lipofectamine 2000 (Invitrogen), polyethyleneimine (Sigma-Aldrich), or TransIT-2020 (Mirus Bio) according to the manufacturer’s instructions. siRNA sequences were as follows: siControl (ON-TARGET plus, D-001810-01-05; scrambled sequence; GE Healthcare); siDNA-PKC₅, 5'-GGGGCGCAUAUCGUACUGAADTDT-3' (Sigma-Aldrich); a gift from the Gromak Laboratory, Sir William Dunn School of Pathology, Oxford, England, UK).

CRISPR/Cas9 genome editing in human A549 cells was used as described (Ran et al., 2013). A gRNA sequence specifically targeting exon 4 in the DICE₁ gene (5'-CCCTCATAAATTCTCGATAGGGG-3') was designed and ligated into the hSpCas9-2A-Puro pX459 V2.0 vector (Addgene), expressing Cas9 and puromycin resistance for delivery of the complete CRISPR/Cas9 system. To generate a clonal A549 cell line lacking expression of Dicer (ΔDicer), wild-type A549 cells expanded from single cells were transfected with 10 µg of the CRISPR/Cas9 constructs using the Neon Transfection System (Invitrogen), according to the manufacturer’s instructions. Electroporation settings were as follows: voltage, 1,230 V; pulse width, 30 ms; pulse number, 2; cell density, 5 × 10⁶ cells/ml. Puromycin (1 µg/ml) was added to cells 24 h after transfection. Puromycin selection was performed for a total of 48 h after transfection, refreshing the puromycin media after the initial 24-h treatment. Puromycin-resistant cells were grown to confluence and clonally selected. PCR with locus-specific primers (forward, 5'-CAAAAAGGTTCATATATCTAGTACTACT-3'; reverse, 5'-ATAATATGGCTGTTGGGATCT-3') was used to

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*The percentage of foci positive cells, n = number of cells analyzed. * P < 0.05; error bars, means ± SEM of three biological replicates. |G| Immunoblot detecting expression of RFP-Dicer constructs in the absence or presence of 4OHT. |H| Model for DNA damage-induced redistribution of the cellular Dicer pool. In undamaged cells (control), Dicer is a predominantly a cytoplasmic protein that shuttles to the nucleus sporadically and is rapidly exported back to the cytoplasm (CP). In the presence of DSBs, the DNA damage response (DDR) targets a small fraction of the cellular Dicer pool by arguably sequential phosphorylation of serine residues S1016 (green) and S1728/S1852 (blue), which causes accumulation in the nucleoplasm (NP) and recruitment to DSBs. Phosphorylated Dicer (p-Dicer) binds and processes dsRNA, which may be produced by RNAPII transcription at lesions to promote the DDR. Phosphorylation of Dicer at S1016 may also alter the import/export rate.
amplify a 650-bp region around the CRISPR target site and to verify mutation of the Dicer1 gene. TIDE analysis was performed using the TIDE Software online webtool (http://tidecalculator.nki.nl/).

**Chemicals and antibodies**

Cells were treated with the following chemicals: DMSO (0.1%, Control; Sigma-Aldrich), STS (3 µM; LKT Labs), Eto (25 µM; Sigma-Aldrich), H2O2 (500 µM; Sigma-Aldrich), phleomycin (5 mg/ml; Cayman Chemical), MMS (500 µM; Sigma-Aldrich), HU (2 mM; Sigma-Aldrich), LMB (5 nM; Cayman Chemical), ATM inhibitor Ku-55933 (5 µM; Sigma-Aldrich), ATR inhibitor VE-821 (1 µM; Sigma-Aldrich), and P3K inhibitor LY294002 (5 µM, New England Biolabs, Inc.) and osmotic stress (0.1x or 10x PBS) for 2 h, unless stated differently. Cells were exposed to γ-irradiation for up to 10 min, equivalent to doses up to 10 gY.

The following primary antibodies were used: anti-Dicer (13D6, ab14601, mouse; Abcam); anti-Dicer (A-2, sc-136891, mouse; Santa Cruz Biotechnology, Inc.) and anti-Dicer (H212, sc-30226, rabbit; Santa Cruz Biotechnology, Inc.); anti-p-DCR-1 (gift from S. Arur’s laboratory, MD Anderson Cancer Center, Houston, TX; Drake et al., 2014); anti-α-tubulin (YL1/2, ab6160, rat; Abcam); anti-Y2A.X (S139, 05–636, mouse; EMD Millipore); anti-GFP-tag (GT859, GTX628528, mouse; GeneTex Inc.); anti-RFP-tag (RF5R, MA5–15257, mouse; Thermo Fisher Scientific); anti-Rad21 (05–636, mouse; EMD Millipore); anti-J2 (10010200, mouse; SCICAON); anti-ATM (2C7, sc-23921, mouse; Santa Cruz Biotechnology, Inc.); anti-p-ATM (S1981, ab81292, rabbit; Abcam); and anti-53BP1 (H-300, sc-22760, rabbit; Santa Cruz Biotechnology, Inc.); anti-Grp75 (JG1, ab2799, mouse; Abcam), and anti-histone H3 (ab1791, rabbit; Abcam); anti-HA (3F10, rat; Roche); anti-p-ATM/ATR substrates mix (SxS, D23H2/D695S, 9670, rabbit), anti-cleaved PARP (5625, rabbit), anti-pChk1 (S345, 133D3, rabbit), and anti-pChk1 (S317, D12H3, rabbit; Cell Signaling Technology); anti-Ki-67 (S6, ab16667, rabbit; Abcam); and anti-DNA-PKcs (18–2, ab13922, mouse; Abcam), and anti-p-p53 (3C10; Santa Cruz Biotechnology, Inc.) and anti-DNA-PKcs (S321, D12H3, rabbit; Cell Signaling Technology; anti-β-actin (ACTB, S47, 21C7, mouse; Santa Cruz Biotechnology, Inc.) and anti-DNA-PKcs (3A2, ab13922, rabbit; Abcam). Secondary antibodies (Invitrogen) were incubated in PBS/0.15% FBS at 4°C in PBS/0.15% FBS for 2 h at 4°C. Primary antibodies were incubated overnight at 4°C in PBS/0.15% FBS. Cells were washed in PBS/0.1% Triton X-100 (3 min, three times). Alexa Flour 488–, 555–, or 647–conjugated secondary antibodies (Invitrogen) were incubated in PBS/0.1% FBS at 4°C in PBS/0.15% FBS. Cells were washed in PBS/0.1% Triton X-100 (3 min, three times). Nuclei were counterstained and mounted with DAPI-containing Mowiol (EMD Millipore). Samples were imaged by epifluorescence and confocal microscopy (BX61 and FV1000; Olympus) using equal exposure times. For epifluorescence microscopy, samples with 1.5-thick coverslips were imaged using a 60× 1.35 NA oil immersion objective lens and a CoolSNAP HQ2 camera (Roper Technologies). Image Z stacks, comprising 12 images, 0.2 µm apart, were collected and maximum projected to give a single image for each color channel.

For confocal imaging, samples with 1.5-thick coverslips were imaged using an an FV1000 confocal system on an Olympus IX-81 mi-
croscope with photomultiplier tube detectors and Olympus PlanApo N, 60x/1.35NA lens at RT. DAP-containing Mowiol (EMD Millipore) was used as the imaging medium. DAPI; Alexa Fluor 488, 539, and 635 (Thermo Fisher Scientific); RFP; and eGFP channels were used for acquisition with Olympus Fluoview software. ImageJ software (Schindelin et al., 2012) was used for further processing of the images. For quantification of γH2A.X-positive cells, >200 wild-type and Dicer KD cells were counted and scored as γH2A.X-positive, if they comprised five or more γH2A.X spots. For RFP-Dicer wild-type and mutants, >50 transfected cells were counted for each construct and analyzed with ImageJ software. For dsRNA, >50 cells from each sample were analyzed with ImageJ software. Co-localization was quantified with an RGB-profile (ImageJ). All quantifications represent several cells that have shown phenotype or percentage of positive cells (see figure legends for details; n, number of cells).

For heterokaryon formation, wild-type or recombinant MEF cells expressing wild-type or endogenously tagged HA-Dicer (PMEF::HA-Dicer) were grown to 70–80% confluency. AsI-SI-ER U2OS cells were seeded on top of the MEF layer before membrane fusion. Mixed-cell populations were grown in the presence of cycloheximide (50 µg/ml) for 4 h before fusion. For heterokaryon formation, cells were washed with warm 1× PBS, incubated with 100 µl warm PEG-3000 solution (50% w/vol in PBS) for 2 min, and washed with 1× PBS five times. Heterokaryons were cultured for 4 h in cycloheximide-containing medium in the presence or absence of 4OHT before fixation. Alexa Fluor 647 phalloidin (Thermo Fisher Scientific) was used to stain the cytoskeleton.

ChIP
ChIP analysis was performed as previously described (Neve et al., 2016). AsI-SI-ER U2OS cells were fixed with 1% formaldehyde (10 min, 37°C). Formaldehyde was inactivated by the addition of glycine to a final concentration of 0.125 M (10 min, 37°C). Cells were washed twice with 5 ml ice-cold PBS and then scraped into 15-ml tubes. Samples were centrifuged for 5 min at 1,600 rpm at 4°C. Cells were resuspended in 500 µl of cell lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, and 1× protease/phosphatase inhibitor cocktails; Roche) and incubated on ice for 10 min. Nuclei were collected by centrifugation twice with 5 ml ice-cold PBS and then scraped into 15-ml tubes. Sam-
ples were centrifuged for 5 min at 13,000 rpm at 4°C to remove cell debris. The supernatant was discarded by the addition of 2.5 volumes IP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 0.5 mM PMSF, 0.8 µg/ml pepstatin A, 1 µg/ml leupeptin, and 1× protease/phosphatase inhibitor cocktails; Roche). The supernatant was used as the immunoprecipitation reaction. The reaction mixture was incubated at 37°C. Specific antibodies (5 µg/100 µg chromatin) were added to 30 min and aliquoted into various IP samples. RNA digestions were performed using RNase A, 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl, pH 6.5, and 2 µl of 10 mg/ml proteinase K; then, incubating at 65°C overnight. DNA was purified by phenol/chloroform extraction and recovered in distilled H2O. Signals represent the mean of at least three biological repeats expressed as the percentage of input, as ratios, or as fold-change relative to controls. For primers see Table S4.

Genomics and bioinformatics analysis
Genomics and bioinformatics analysis ChIP-seq (White et al., 2014) data were mapped with Bowtie 2 (version 2.2.5) after trimming of the first poor-quality nucleotide with Cutadapt (version 1.8.3). Duplicate reads were removed with Samtools (version 1.1).

HEK293 Dicer ChIP-Seq data were taken from White et al. (2014). Adapter and contaminating sequences were identified with fastQC (version 0.11.5; Available online at http://www.bioinformatics.babraham.ac.uk/projects/fastqc; Babraham Bioinformatics) and were trimmed in single-end mode using Cutadapt (version 1.8.3). These sequences include 5′-AGATCGAAGAGCTCAGTATCGGTCTT CTGGTG-3′, 5′-TCGATATCCCGTCTCTCG-3′, and 5′-CTGTA GCACCATCAA-3′. Only reads of more than 10 nt were kept and mapped with Bowtie 2 (version 2.2.5). Duplicate reads were removed with Samtools (version 0.1.19). Coverage bigWig graphs were computed with deepTools 2 bamCoverage. The profile around the AsI-SI sites was computed with deepTools 2 computeMatrix reference-point and normalizing to the library read count.

Data were visualized with ggplot2 (http://www.ggplot2.org/) in R software (http://www.R-project.org/) by applying a 1,000-nt rolling mean to the trimmed signal mean (2% of most-extreme values trimmed from both ends). The rolling mean was computed with the roll_mean function, and the rolling SD was computed with the roll_sd function from the ReppRoll package.

We used γH2A.X and H2A.X ChIP-seq data from Yata et al. (2014). The log2 ratio of γH2A.X/H2A.X was computed in 10-kb bins with deepTools 2 bamCompare, with read count normalization. From this ratio, peaks were called with a custom script by using MAT LAB (http://www.mathworks.co.uk/matlabcentral/fileexchange/25500 -peakfinder; MathWorks). Using Perl programming language, peaks were extended to either side until at least eight bins had ≤ 0 signal. Peaks ≤ 40 kb long were discarded. AsI-SI sites overlapping those peaks were ranked according to the log2(γH2A.X/H2A.X) signal in a (AsI-SI – 25 kb, AsI-SI +25 kb) window. AsI-SI sites ≤ 10 kb apart were summarized into one with the highest log2(γH2A.X/H2A.X) signal in the 50-kb window. The top 200 of these AsI-SI sites were considered as efficiently cut upon damage induction. The remaining AsI-SI sites were also ranked according to log2(γH2A.X/H2A.X) signal in the 50-kb window. 200 AsI-SI sites within 500 nt of a gene (RefSeq V9 – hgs38) with the lowest log2(γH2A.X/H2A.X) signal were considered as not cut upon damage induction to serve as the negative control. For Dicer signal ratio box plots between induced and noninduced cells at γH2A.X-positive or γH2A.X-negative sites (Fig. S2 D), we used 99 cut AsI-SI sites, as annotated in Aymard et al. (2014). The ratio was computed via deepTools 2 bamCompare with read count normalization.

Code description is as follows: (a) peakf.m: MATLAB code to find peaks in 1-kb γH2A.X/H2A.X data (uses publicly available peakfinder.m code http://uk.mathworks.com/matlabcentral/fileexchange/25500 -peakfinder-x0-sel-thresh-extrema-includeendpoints- interpolate-); (b) peak_mathlab.pl: Perl code to further process, summarize, and exclude peaks found by peakf.m code; (c) AsI-SI-gamma_signal.pl: Perl code to compute γH2A.X/H2A.X cumulative signal in the peaks output by D, 10:1 TE buffer, pH 8.0. Immune complexes were eluted with 500 µl IP elution buffer (1% SDS, 0.1 M NaHCO3) for 30 min on a rotating wheel. Reversal of cross-links was performed by adding 0.3 M NaCl, 3 µg/ml RNase A, 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl, pH 6.5, and 2 µl of 10 mg/ml proteinase K; then, incubating at 65°C overnight. DNA was purified by phenol/chloroform extraction and recovered in distilled H2O. Signals represent the mean of at least three biological repeats expressed as the percentage of input, as ratios, or as fold-change relative to controls. For primers see Table S4.
Mass spectrometry
For mass spectrometry analysis, SDS-PAGE–purified IP samples were digested in the gel with trypsin. Peptides were analyzed on a nano ultra-HPLC system coupled to a QExactive mass spectrometer (Thermo Fisher Scientific). Phosphopeptides were purified by C18 reverse-phase chromatography and were enriched using titanium dioxide columns before analysis.

In detail, endo-Dicer was purified form subcellular fractions of HEK293 cells. Samples were separated by SDS-PAGE and cut in gel slices. For in-gel tryptic digestion, slices were briefly washed with 50% ACN and dried in 100% ACN at 37°C for 10 min. Dried slices were incubated with 2% Tris (2-carboxyethyl) phosphine diluted in 100 mM triethylammonium bromide (TEAB) at RT for 30 min. Tris (2-carboxyethyl) phosphine was removed, and slices were incubated in 50 mM 2-chloracetamide, diluted in 100 mM TEAB in the dark at RT for 30 min. 2-Chloracetamide was removed, and slices were dried in 100% ACN at 37°C for 10 min. ACN was removed, and trypsin (500 ng/IP), diluted in 50 mM TEAB, was added. Slices were digested at 37°C overnight. Supernatants were collected and reduced to small volumes on a speedvac for several hours. Peptides were loaded on C18 columns. Columns were sequentially equilibrated with 100% ACN and 0.1% trifluoroacetic acid (TFA). Peptides were loaded and washed with 0.1% TFA. Peptides were sequentially eluted with 50% ACN and 0.1% TFA, transferred to glass vials, and dried on a speedvac.

Peptides were resuspended in 5% formic acid and 5% DMSO and then trapped on a C18 PepMap100 precolumn (300 µm inner diameter × 5 mm, 100 Å; Thermo Fisher Scientific) using 0.1% formic acid in water at a pressure of 500 bars and analyzed on an Ultimate 3000 ultra-HPLC system (Thermo Fisher Scientific) coupled to a QExactive mass spectrometer (Thermo Fisher Scientific). The peptides were separated on an in-house packed analytic column (360 μm × 75 μm inner diameter packed with ReproSil-Pur 120 C18-AQ, 1.9 μm, 120 Å; Dr. Maisch GmbH) and then electrosprayed directly into a QExactive mass spectrometer (Thermo Fisher Scientific) through an EASY-Spray interface. The peptides were then eluted using a linear gradient (length: 60 min, 7–28% solvent B [0.1% formic acid in ACN], flow rate: 200 nL/min). Raw data were acquired on the mass spectrometer in a data-dependent mode. Full-scan, mass spectra were acquired in the Orbitrap (scan range 350–2000 m/z, resolution 70,000, AGC target 3 × 106, maximum injection time 100 ms). After mass spectrum scans, the 20 most-intense peaks were selected for higher-energy collisional dissociation fragmentation at 30% of normalized collision energy. The higher-energy collisional dissociation spectra were also acquired in the Orbitrap (resolution 17,500, AGC target 5 × 106; maximum injection time, 120 ms) with first-fixed mass at 180 m/z.

Generated raw data files were processed using MaxQuant (version 1.5.0.35; Max Planck Institute of Biochemistry), integrated with the Andromeda search engine, as previously described (Cox and Mann, 2008; Cox et al., 2011). To identify protein groups, peak lists were searched against human database (Swiss Prot, version 04/13) as well as a list of common contaminants by Andromeda. Trypsin with a maximum number of missed cleavages of 2 was chosen. Acetylation (protein N-term, i.e., only the amino terminus of the protein), oxidation (M), and phosphorylation (S, T, and Y) were used as variable modifications, whereas carbamidomethylation (C) was set as a fixed modification. A protein and posttranslational modification false-discovery rate of 0.01, a minimum score of 40, and a localization probability of >0.7 for phosphopeptides were set. Match between runs was applied.

The authors declare no competing financial interests.

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Author contributions: K. Burger and M. Gullerova performed all molecular biology and imaging experiments. M. Schlackow performed bioinformatics analysis. M. Potts established the CRISPR/Cas9 Dicer knockout cell line. K. Burger and M. Gullerova designed the experiments and wrote the manuscript. S. Hester and S. Mohammed performed mass spectrometry analysis.
