An Argonaute phosphorylation cycle promotes microRNA-mediated silencing

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MicroRNAs (miRNAs) perform critical functions in normal physiology and disease by associating with Argonaute (AGO) proteins, which guide to partially complementary sites in messenger RNAs (mRNAs), leading to reduced stability and translation of targeted messages. miRNAs select targets primarily through base pairing of their seed regions, nucleotides 2–7. Consequently, the potential target repertoire for a given miRNA is vast. Multiple sequence features of bona fide target sites distinguish them from non-functional sites with seed complementarity. Nevertheless, recent experiments have demonstrated that the functional pool of targets greatly exceeds the quantity of miRNAs in mammalian cells. While the intrinsic sequence characteristics of miRNA binding sites strongly influence the kinetics of AGO:target interactions, it is currently unknown whether additional, active mechanisms exist that influence miRNA binding to facilitate navigation of the extensive target landscape.

RNA interference (RNAi) screens have been used to dissect the miRNA pathway in invertebrates. Analogous experiments in human cells, however, have been hindered by the fact that the RNAi and miRNA pathways share common molecular machinery. This limitation may be circumvented by recent advances in CRISPR-mediated genome editing, which offers a robust alternative for genetic loss-of-function screens in human cells. Here, we describe the application of CRISPR–Cas9 screening to identify novel regulators of miRNA-mediated silencing. These experiments reveal that the ANKRD52–PPP6C phosphatase complex performs a critical function in the miRNA pathway by dephosphorylating a set of highly conserved amino acids in AGO2. A secondary genome-wide screen revealed CSNK1A1 as the kinase that phosphorylates AGO2 on these sites. This AGO2 phosphorylation cycle is triggered by target engagement and negatively regulates target association, yet is essential to maintain the global efficiency of miRNA-mediated silencing. Transcriptome-wide AGO2 binding studies show that S824–S834 phosphorylation remodels the target pool bound by AGO2 at steady-state. These data reveal a previously unrecognized mechanism that regulates AGO:target interactions to promote miRNA-mediated repression.

CRISPR–Cas9 screen for miRNA regulators

To apply CRISPR–Cas9 screening to interrogate the miRNA pathway, we first generated a cell line expressing a fluorescent reporter of miRNA activity. Enhanced green fluorescent protein (EGFP) transcripts with or without a 3′ untranslated region (UTR) harbouring eight imperfectly complementary binding sites for miR-19, an abundant miRNA, were expressed in the stably diploid cell line HCT116 (ref. 14) (Fig. 1a). The miR-19 reporter line (HCT116EGFP-miR19) but not the control line lacking miR-19 sites (HCT116EGFP), exhibited robust de-repression of EGFP upon infection with a CRISPR lentivirus targeting the essential miRNA biogenesis factor DROSHA or after transfection with an antisense miR-19 inhibitor (Fig. 1b).

A genome-wide CRISPR–Cas9 screen was performed by infecting HCT116EGFP-miR19 and HCT116EGFP cells with a lentiviral library targeting over 19,000 human genes and 1,864 miRNAs. After 14 days of growth, the brightest 0.5% of cells, representing those with deficient miRNA-mediated silencing, were collected (Fig. 1a). Simulations demonstrated that collection of cells in this gate could theoretically yield >150-fold enrichment of highly effective single-guide RNAs (sgRNAs) that target essential genes in the miRNA pathway while still allowing significant enrichment of partly effective guides that incompletely impair miRNA-mediated silencing (Supplementary Table 1). sgRNA
representation in the sorted and unsorted cells was enumerated by high-throughput sequencing and the RNAi Gene Enrichment Ranking (RIGER) algorithm16 was used to identify genes targeted by multiple enriched sgRNAs, representing high-confidence hits (Supplementary Tables 2 and 3). A large number of established components of the miRNA pathway and miR-19 itself were identified as significant hits in HCT116EGFP-miR19 but not HCT116EGFP cells (Fig. 1c, d), establishing the sensitivity of this approach.

We noted two classes of highly ranked hits without a previously defined role in the miRNA pathway: transcriptional regulators (BRD4, CTNNB1, and POU2F1) and interacting components of the serine/threonine protein phosphatase 6 (PPPP6C) complex (ANKRD52 and PPP6C)17. Loss of function of any of these genes measurably de-repressed EGFP in HCT116EGFP-miR19 but not HCT116EGFP cells (Fig. 1e and Extended Data Fig. 1). CTNNB1 and BRD4 promote MYC expression18,19, a known positive regulator of transcription of miR-17-92 (Fig. 1e and Extended Data Fig. 1). POU2F1, in contrast, promotes primiR-17-92, and mature miR-19 levels in ANKRD52–/–, CTNNB1, pri-miR-17-92, and mature miR-19 levels in ANKRD52–/– cells were similarly upregulated in ANKRD52–/– cells (Fig. 2f), demonstrating broader regulation of Argonaute proteins by the ANKRD52–PPP6C complex.

Owing to their central role in miRNA-mediated silencing, we hypothesized that Argonaute proteins may be dephosphorylated by ANKRD52–PPP6C. AGO2 and ANKRD52–PPP6C interacted in an RNA-independent manner (Extended Data Fig. 5a). Phos-tag electrophoresis, a sensitive method for detection of phosphorylated proteins25, revealed that AGO2 migrated as a doublet, with dramatic enhancement of the slowly migrating form in ANKRD52–/– or PPP6C-deficient cells (Fig. 2b). Phosphatase treatment confirmed that the more slowly migrating species corresponded to phosphorylated AGO2 (p-AGO2; Fig. 2c). Deficiency of ANKRD52 or PPP6C in multiple human cell lines similarly led to accumulation of phosphorylated AGO2 (Extended Data Fig. 5b). We also observed enhanced phosphorylation of AGO1 in ANKRD52–/– cells (Extended Data Fig. 5c), suggesting broader regulation of Argonaute proteins by the ANKRD52–PPP6C complex.

Mass spectrometry was used to identify the relevant phosphoresidue(s) in endogenous AGO2. Enhanced phosphorylation within a region of the PIWI domain containing four highly conserved serine residues and a single poorly conserved threonine residue (S824–S834) was detected in ANKRD52–/– cells (Extended Data Fig. 5d). Although close spacing prevented the assignment of additional phosphorylations to specific residues, mass spectrometry using AGO2 alanine mutants allowed definitive identification of phosphorylation at S824 and S831 (Extended Data Fig. 6a, b). Triply phosphorylated peptides spanning S824–S834 were detected, with definitive detection of p-S824 (Extended Data Fig. 6a, b). Triply phosphorylated peptides spanning S824–S834 were detected, with definitive detection of p-S824 (Extended Data Fig. 6a, b). Triply phosphorylated peptides spanning S824–S834 were detected, with definitive detection of p-S824 (Extended Data Fig. 6a, b). Triply phosphorylated peptides spanning S824–S834 were detected, with definitive detection of p-S824 (Extended Data Fig. 6a, b).

ANKRD52 and PPP6C dephosphorylate AGO2

The identification of ANKRD52 and PPP6C as significant hits suggested that phosphorylation regulates the activity of an essential miRNA pathway component. Confirming a general impairment of miRNA-mediated silencing in cells deficient for this phosphatase complex, RNA sequencing (RNA-seq) demonstrated that genes upregulated in AGO2–/– cells were similarly upregulated in ANKRD52–/– cells (Fig. 2a, Extended Data Fig. 3 and Supplementary Table 4). Additional miRNA reporter constructs and endogenous let-7 targets23–24 were also de-repressed by ANKRD52 knockout (Extended Data Fig. 4a–d). The steady-state abundance of representative miRNAs was not decreased (Extended Data Fig. 4e), indicating that the ANKRD52–PPP6C complex does not globally regulate miRNA biogenesis.

Figure 1 | A genome-wide CRISPR–Cas9 screen reveals known and novel regulators of the miRNA pathway. a, Design of CRISPR–Cas9 screen. b, Validation of reporter cell lines. EGFP fluorescence after introduction of lentiCRISPR vectors (top) or antisense miR-19 inhibitors (bottom). c, RIGER analysis of screening results in HCT116EGFP-miR19 (top) or HCT116EGFP cells (bottom). Red dots, known components of the miRNA pathway; blue dots, putative novel regulators. d, Components of the miRNA pathway identified as significant hits. e, EGFP expression in HCT116EGFP-miR19 cells after transduction with lentiCRISPR vectors.
Phosphorylation inhibits target binding

AGO2 S824–S834 is within a structurally unresolved loop of the PIWI domain that is located in the vicinity of the miRNA-target interface. We therefore hypothesized that phosphorylation in this region may reduce miRNA and/or target association. Immunopurification of endogenous AGO2 from ANKRD52+/- or ANKRD52−/− cells demonstrated equivalent miRNA association (Fig. 3a). In contrast, AGO2 target association was significantly reduced in ANKRD52−/− cells, as determined by assessing AGO2 binding to the miR-19 EGF reporter transcript and two established targets of miR-16 and let-7 (Fig. 3b). To confirm these findings, AGO2:miRNA complexes were captured with an RNA oligonucleotide that mimics a target of miR-21, an abundant miRNA in HCT116 cells (Fig. 3c). Whereas unphosphorylated AGO2 was efficiently recovered using this approach, binding of phosphorylated AGO2 to the synthetic target was dramatically decreased (Fig. 3d). Importantly, both phosphorylated and unphosphorylated forms of AGO2 were efficiently recovered by immunoprecipitation using an anti-AGO2 antibody, demonstrating that the relevant phospho-residues were stable under these conditions.

A series of phosphomimetic mutants were generated to identify the specific phospho-residues that impair AGO2 target association (Extended Data Fig. 7a). As expected, none of the mutations measurably decreased miRNA association (Extended Data Fig. 7b). In contrast, target association was significantly impaired by mutation of all five serine and threonine residues in the S824–S834 region to glutamic acid (5XE) and, more importantly, individual mutations or combinations of mutations that mimic definitive phosphorylation sites documented by mass spectrometry (S831E, S828E/S831E, and S824E/S828E/S831E) (Fig. 3e). Notably, mutation of S828 or all serines and threonines in this region to alanine (S828A or 5XΑ, respectively) did not inhibit target interaction. Consistent with an isolated effect of S824–S834 phosphorylation on target binding, tethering AGO2WT, AGO25XE, or AGO25XA to a luciferase transcript using the N-peptide-boxB system resulted in equivalent repression (Fig. 3f). Taken together, these findings establish that phosphorylation of S824–S834 potently and specifically inhibits AGO2:target interactions.

CSNK1A1 is the inhibitory AGO2 kinase

We next sought to identify the kinase that initiates this inhibitory mechanism. Reasoning that loss of function of the kinase would rescue miRNA-mediated silencing in the ANKRD52-deficient state, we performed a secondary genome-wide CRISPR–Cas9 screen in ANKRD52−/− HCT116 EGF-miR19 cells and collected the dimmest 0.5% of cells (Fig. 4a). RIGER analysis revealed four serine/threonine kinases among the top 100 hits: LATS2, CSNK1A1, MTO1, and SRPK1 (Fig. 4b and Supplementary Table 5). Knockout of LATS2 or SRPK1 in the ANKRD52−/− background resulted in minimal to no recovery of EGF repression (Extended Data Fig. 8a, b). mTOR knockout or inhibition with rapamycin moderately rescued EGF repression but did not influence AGO2 phosphorylation (Extended Data Fig. 8a, c–d). In stark contrast, knockout of CSNK1A1 in ANKRD52−/− cells (Extended Data Fig. 9a) fully rescued repression of the EGF reporter without increasing miR-19 levels (Fig. 4c and Extended Data Fig. 9b), greatly reduced AGO2 phosphorylation (Fig. 4d), and restored AGO2 target association (Fig. 4e).

Co-immunoprecipitation demonstrated an RNA-independent interaction between CSNK1A1 and AGO2 (Extended Data Fig. 9c). Moreover, the casein kinase I family, of which CSNK1A1 is a member, prefers previously phosphorylated substrates conforming to the consensus (pS/pT/D/E)-X₂-S/T, with the latter S/T representing the phospho-acceptor site. The five serine/threonine residues within AGO2 S824–S834 all conform to this consensus motif, with S824 and S828 preceded by acidic residues and T830, S831, and S834 having the potential to be primed by hierarchical phosphorylation initiating at S828. Phosphorylation of full-length wild-type AGO2 but not AGO25XA by recombinant CSNK1A1 was robustly detectable in vitro, with or without pre-treatment with phosphatase to remove potential priming phosphorylations (Fig. 4f). To investigate potential hierarchical phosphorylation of these residues, CSNK1A1 kinase assays were performed with a series of phospho-peptides encompassing amino acids 824–834 of AGO2 (Fig. 4g). Unphosphorylated peptide was a poor substrate for CSNK1A1 under these conditions, suggesting that initial phosphorylation of this region is facilitated by contextual features present in full-length AGO2. pS824 alone weakly stimulated further phosphorylation. Prior phosphorylation of S828, however, robustly promoted phosphorylation of S831 (but not T830), while pS831 efficiently primed phosphorylation of S834. Taken together with our earlier data demonstrating a critical role for S828 in phosphorylation of AGO2 in cells (Fig. 2e), these findings support a model whereby initial phosphorylation of S828, and potentially S824, stimulates efficient hierarchical phosphorylation of S831 followed by S834, rendering AGO2 incompetent for target binding until returned to an active state by ANKRD52−/−PPP6C phosphatase activity.

Target binding triggers phosphorylation

Although deficiency of AGO2 S824–S834 phosphorylation was compatible with fully efficient silencing of the miR-19 EGF reporter (Fig. 4c), RNA-seq revealed that genes that are upregulated in AGO2−/− cells were similarly upregulated in CSNK1A1−/−;ANKRD52−/− double knockout cells (Fig. 5a and Supplementary Table 4). These findings suggested that AGO2 S824–S834 phosphorylation is necessary for efficient silencing of endogenous miRNA targets. Further supporting this...
enhanced crosslinking immunoprecipitation (eCLIP) 37. Whereas both AGO2WT and AGO25XA bound primarily to sites within mRNA 3′ UTRs (Fig. 6a, Extended Data Fig. 10b and Supplementary Table 7), AGO25XA, but not AGO2 WT, was strongly impaired (Fig. 6d), consistent with a role for target engagement in facilitating AGO2 phosphorylation.

Phosphorylation remodels AGO2 targeting

Lastly, to investigate why loss of AGO2 S824–S834 phosphorylation impairs miRNA-mediated silencing, we examined the transcriptome-wide target binding profiles of AGO2WT and AGO25XA using enhanced crosslinking immunoprecipitation (eCLIP) 37. Whereas both AGO2WT and AGO25XA bound primarily to sites within mRNA 3′ UTRs (Fig. 6a, Extended Data Fig. 10b and Supplementary Table 7), AGO25XA had more than twice as many detectable binding sites (2,921 CLIP clusters for AGO25XA versus 1,190 clusters for AGO2WT).

Figure 4 | A CRISPR–Cas9 suppressor screen reveals CSNK1A1 as the inhibitory AGO2 kinase. a, Design of CRISPR–Cas9 screen to identify ANKRD52−/− suppressors. b, RIGER analysis with serine/threonine kinases highlighted. c, EGFP expression in ANKRD52−/− HCT116EGFPmiR19 cells transduced with lentisCRIPR vectors targeting CSNK1A1. d, Abrogated phosphorylation of AGO2 in ANKRD52−/−CSNK1A1−/− cells. e, Relative association of mir-19a, mir-19b and EGFP target mRNA with AGO2 assessed as described in Fig. 3a (n = 3 biological replicates). f, In vitro phosphorylation of FH-AGO2 by CSNK1A1 with or without pretreatment with XPP. g, In vitro CSNK1A1-mediated phosphorylation of the indicated peptides. For gel source data, see Supplementary Fig. 1.
While this corresponded to more than twice as many genes bound by AGO2^{5XA}, virtually all genes bound by AGO2^{WT} were also bound by AGO2^{5XA} (Fig. 6b). Thus, loss of AGO2 S824–S834 phosphorylation dramatically expands the target repertoire bound by AGO2 at steady-state. AGO2 immunoprecipitation confirmed enhanced binding of AGO2^{5XA} to representative targets identified by CLIP (Extended Data Fig. 10c).

A significant expansion of binding sites would be expected to reduce the pool of AGO2^{5XA} that is available to interact with and silence any given individual target transcript. Indeed, targets of wild-type AGO2 with 8-, 7-, or 6-nucleotide miRNA seed complementarity showed reduced binding by AGO2^{5XA} (Fig. 6c). This finding offers a plausible explanation for the global deficit in miRNA-mediated silencing observed in AGO2^{5XA}-reconstituted cells (Fig. 5b). Nevertheless, silencing of some transcripts, including the miR-19 EFGP reporter, was not impaired by loss of AGO2 S824–S834 phosphorylation (Fig. 4c). We speculated that transcripts that are efficiently repressed by AGO2^{5XA} represent particularly strong targets that are able to compete effectively for AGO2 binding against the expanded AGO2^{5XA} target pool. Consistent with this hypothesis, genes whose repression was rescued by expression of AGO2^{5XA} in AGO2^{-/−} cells exhibited greater wild-type AGO2 CLIP coverage (Extended Data Fig. 10d), indicating that they are stronger miRNA targets.

Finally, we sought to identify features of transcripts that are preferentially bound by AGO2^{5XA}. Interestingly, the frequency of AGO2^{5XA} crosslinking to seed matches was equivalent between clusters uniquely identified by AGO2^{5XA} CLIP and clusters that were common to AGO2^{WT} and AGO2^{5XA} (Extended Data Fig. 10e). This suggested that the additional sites identified by AGO2^{5XA} CLIP are bona fide miRNA binding sites that are uniquely detected owing to a prolonged AGO2:target interaction that occurs in the absence of the phosphorylation cycle. In light of this, we reasoned that transcript half-life may influence the relative binding of AGO2^{5XA} versus AGO2^{WT}, since association with slowly decayed transcripts would provide greater opportunity for AGO phosphorylation relative to transcripts that are rapidly decayed. Transcripts bound by AGO2^{5XA} were classified on the basis of published transcrptime-wide half-life measurements. Indeed, transcripts with long half-lives exhibited greater binding by AGO2^{5XA} relative to AGO2^{WT} than transcripts with short half-lives (Extended Data Fig. 10f).

Discussion

This study describes the application of genome-wide CRISPR–Cas9 screening coupled with a fluorescent reporter to interrogate the miRNA pathway. An in-depth analysis of the ANKR522–PP6C phosphatase complex, identified as a major regulator of the miRNA pathway through this approach, unexpectedly reveals that continual transient phosphorylation of AGO2 is required to maintain the global efficiency of miRNA-mediated repression. Our experiments demonstrate that target engagement by AGO2 stimulates its hierarchical, multi-site phosphorylation by CSNK1A1 on a series of highly conserved residues (S824–S834) (Extended Data Fig. 10g). Although this impairs target binding, dephosphorylation by ANKR522–PP6C allows AGO2 to engage new targets. Inactivation of this cycle strongly inhibits global miRNA-mediated repression.

Given that AGO2 S824–S834 phosphorylation impairs target association, why is continual phosphorylation/dephosphorylation of these residues necessary for efficient miRNA activity? Transcriptome-wide analyses of AGO2 targets suggest that this silencing deficit is attributable to a dramatic expansion of the target repertoire bound by non-phosphorylatable AGO2 at steady-state, effectively reducing the pool of active AGO2 on a per-target basis. Multiple potential mechanisms may contribute to this effect. First, it is possible that AGO:target interactions are much more stable in vivo than suggested by in vitro measurements. In this case, AGO could potentially persist on a target longer than is necessary to trigger a productive silencing interaction. Given that target levels greatly exceed miRNA levels in mammalian cells, active disassembly of AGO:target complexes via S824–S834 phosphorylation would thereby effectively increase AGO availability to silence additional targets. Structural studies have revealed AGO2 conformational changes induced by target engagement. While S824–S834 are unresolved in existing AGO structures, it is possible that target binding exposes these sites to the activity of CSNK1A1, providing a timing mechanism that limits the duration of target interaction. This model is consistent with our finding of enriched binding of non-phosphorylatable AGO2 to transcripts with long half-lives since the steady association of AGO2 with slowly decayed transcripts would provide a greater opportunity for CSNK1A1 phosphorylation relative to miRNA targets that are rapidly degraded. AGO phosphorylation might be further stimulated...
by the successful recruitment of additional silencing factors such as the CCR4–NOT deadenylase complex and/or DDX6, indicating the completion of a productive silencing interaction. An alternative, non-mutually exclusive model to account for greater association of AGO2 with specific targets posits the existence of additional features, such as sites for RNA binding proteins, that promote AGO phosphorylation, thus actively disfavoring stable AGO association. This would effectively focus AGO activity on a subset of the possible targets in a cell, facilitating their productive repression. Further characterization of the distinguishing features of targets that most efficiently promote AGO2 phosphorylation will help resolve these uncertainties.

In the context of these models, the conservation pattern of these phosphorylation sites may be informative. In particular, while the relevant serines are conserved in Drosophila AGO1 (Fig. 2d), which carries out miRNA-mediated repression, these sites are absent in Drosophila AGO2, which performs siRNA-mediated target slicing. Single-molecule studies have documented accelerated AGO dissociation following target cleavage8,9, potentially obviating the need for phosphorylation to facilitate Drosophila AGO2 target disengagement. On the other hand, the deep evolutionarily conservation of these phosphorylation sites in Argonaute proteins that perform miRNA-mediated repression strongly suggests that this phosphorylation cycle is a broadly used mechanism to optimize miRNA activity in diverse species. In addition, the potent inhibitory effect of S824–S834 phosphorylation would provide a powerful mechanism to regulate the global activity of the miRNA pathway by upstream signalling pathways. This possibility highlights the importance of future studies to examine this newly discovered Argonaute phosphorylation cycle in diverse developmental, physiological, and pathophysiological contexts.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Cell culture. Cell lines used in this study were obtained from American Type Culture Collection (ATCC) and cultured under standard conditions. HCT116 cells were authenticated by karyotyping. All cell lines were confirmed to be free of mycoplasma contamination.

Construction of EGFP miRNA reporters. EGFP was PCR amplified from EGFP-hA2O (Addgene catalogue number 21981) and cloned into pMSCV-Puro (Clontech) using the BglII and XhoI restriction sites. The puromycin resistance cassette was then removed by EcoRI and ClaI digestion and replaced with an insert containing eight imperfect miR-19 binding sites (modelled from ref. 39), synthesized as a gBlock (IDT) (sequence in Supplementary Table 8). For the EGFP-only reporter, the puromycin resistance cassette was removed by EcoRI and ClaI digestion followed by re-ligation after filling-in overhangs.

Reporters for miR-16 and miR-200c were generated by replacing the puromycin cassette in the pMSCV-Puro vector containing EGFP by digesting with EcoRI and ClaI and ligating in oligonucleotides containing single miRNA binding sites (sequences in Supplementary Table 8). Multiple cloning cycles were performed using MfeI and Clal to generate the final reporters containing eight total binding sites.

Generation of HCT116 reporter cell lines. MSCV-EGFP, MSCV-EGFP-miR-19, MSCV-EGFP-miR-16, and MSCV-EGFP-miR-200 retrovirus was generated by first seeding 6 × 10^5 cells per well in a six-well dish. The following day, cells were transfected using 1 μg of plasmid (MSCV-EGFP or MSCV-EGFP-miR-19, 3 μl of FuGEnE HD (Promega), and 200 μl Opti-MEM (Thermo Fisher)) per well according to the manufacturer’s instructions. Media were changed the next day. Two days after transfection, media were collected and passed through a 0.45 μm SFCA sterile filter. Recipient HCT116 cells were transduced overnight at a multiplicity of infection (MOI) of approximately 0.2 using media supplemented with 8 μg/ml polybrene (EMD Millipore). Cells expressing EGFP were enriched by FACS and single-cell clonal lines were derived.

Generation of knockout cell lines using CRISPR–Cas9. Heterogeneous knockout cell populations were generated using lentiCRISPR v2 (Addgene catalogue number S2961) or lentCRISPR-hygro. lentCRISPR-hygro was constructed by replacing the puromycin resistance open reading frame (ORF) in lentiCRISPR v2 with a hygromycin resistance ORF. A silent mutation was introduced into a BsmBI restriction site within the hygromycin resistance ORF to prevent fragmentation of the vector when cloning sgRNA oligonucleotides. sgRNA sequences (Supplementary Table 8) were cloned as described previously. An sgRNA targeting an irrelevant gene (PPID) or a non-targeting guide were used as negative controls.

To generate active lentivirus, 6 × 10^5 293T cells were first seeded in six-well dishes and transfected the following day using a 5:3:2 ratio of lentiviral CRISPR:psPAX2 (Addgene catalogue number 12260):pMD2.G (Addgene catalogue number 12259) using FuGEnE HD and 1 μg of total plasmid per well. Media were changed the next day. Two days after transfection, media were collected and passed through a 0.45 μm SFCA sterile filter. Media containing the virus were diluted 1:1 with fresh media and used to transduce recipient cells overnight in a final polybrene concentration of 8 μg/ml. Media were changed 24 h later, and cells were split into fresh media containing 1 μg/ml puromycin 48 h after transduction.

To generate clonal knockout lines, single-cell cloning was performed after infection with lentiCRISPR v2, lentCRISPR-hygro, or after transient transfection of PX330 (Addgene catalogue number 42230) targeting the gene of interest. lentCRISPR v2-derived clones were used in Figs 2d, e and 3a, 5a, and Extended Data Figs 2c, 4e, 4f, 5c, 5d, and 6b. A lentCRISPR-hygro derived ANKRDS2–/− clone was used in Fig. 2e. PX330-derived clones were used in Figs 2a, 3a–e, 4a–f, 5a–e and 6a–c and Extended Data Figs 2e, 6c, 6g, 7, 8c, d, 9a, b, and 10a–c. A lentCRISPR-v2-derived clone was used in Figs 3a, 3b, 4c, 4e, 4f, 5a–e and 6a–c and Extended Data Figs 2c, 2d, 6c, 6g, 7, 8c, d, 9a, b, and 10a–c.

Transfection with miR-19 inhibitors. Three hundred thousand reporter cells were seeded well in six-well dishes. Cells were transfected the following day with a mixture of inhibitors for miR-19a and miR-19b at 5 nM each (MIRIDIAN microRNA HairpinGenes, GE Dharmacon) using Lipofectamine RNAiMAX (Thermo Fisher). Fluorescence was assessed by flow cytometry 48 h after transfection.

Genome-wide CRISPR–Cas9 screening. Lentiviral sgRNA library production. The human GeCKO v2 library was obtained from Addgene (catalogue number 1000000048) and amplified according to the provided instructions. Plasmid was purified from bacterial pellets using a Qiagen Plasmid Maxi Kit. Active lentivirus was prepared in 293T cells by first seeding 3.2 × 10^6 cells per 10-cm dish. GeCKO library A and library B were prepared independently using 15 dishes per library. The human GeCKO v2 library was obtained from Addgene (catalogue number 52961) or lentiCRISPR-hygro. lentiCRISPR-hygro was constructed by replacing the puromycin resistance open reading frame (ORF) in lentiCRISPR v2 with a hygromycin resistance ORF. A silent mutation was introduced into a BsmBI restriction site within the hygromycin resistance ORF to prevent fragmentation of the vector when cloning sgRNA oligonucleotides. sgRNA sequences (Supplementary Table 8) were cloned as described previously. An sgRNA targeting an irrelevant gene (PPID) or a non-targeting guide were used as negative controls.

To generate active lentivirus, 6 × 10^5 293T cells were first seeded in six-well dishes and transfected the following day using a 5:3:2 ratio of lentiviral CRISPR:psPAX2 (Addgene catalogue number 12260):pMD2.G (Addgene catalogue number 12259) using FuGEnE HD and 1 μg of total plasmid per well. Media were changed the next day. Two days after transfection, media were collected and passed through a 0.45 μm SFCA sterile filter. Media containing the virus were diluted 1:1 with fresh media and used to transduce recipient cells overnight in a final polybrene concentration of 8 μg/ml. Media were changed 24 h later, and cells were split into fresh media containing 1 μg/ml puromycin 48 h after transduction.

To generate clonal knockout lines, single-cell cloning was performed after infection with lentiCRISPR v2, lentCRISPR-hygro, or after transient transfection of PX330 (Addgene catalogue number 42230) targeting the gene of interest. lentCRISPR v2-derived clones were used in Figs 2d, e and 3a, 5a, and Extended Data Figs 2c, 4e, 4f, 5c, 5d, and 9a, b. A lentCRISPR-hygro derived ANKRDS2–/− clone was used in Fig. 2e. PX330-derived clones were used in Figs 2a, 3a–e, 4a–f, 5a–e and 6a–c and Extended Data Figs 2c, 2d, 6c, 6g, 7, 8c, d, 9a, b, and 10a–c.

Transfection with miR-19 inhibitors. Three hundred thousand reporter cells were seeded well in six-well dishes. Cells were transfected the following day with a mixture of inhibitors for miR-19a and miR-19b at 5 nM each (MIRIDIAN microRNA HairpinGenes, GE Dharmacon) using Lipofectamine RNAiMAX (Thermo Fisher). Fluorescence was assessed by flow cytometry 48 h after transfection.

Sequencing library preparation. Methods to prepare PCR ampfion libraries for deep sequencing were adapted from a previously published protocol. All primer sequences are provided in Supplementary Table 8. For unsorted cells, an initial PCR was performed using 5 μl of gDNA as input. A second PCR was performed using an equal volume of pf 7.9-buffer saturated phenol, followed by phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform. Twenty micrograms of glycogen (Roche) and 1.5 ml of 100% ethanol were added to each tube and DNA was precipitated at −80 °C for 1 h followed by centrifugation at 18,000g for 10 min at 4 °C. Pellets were washed with 1 ml of 75% ethanol, dried, and resuspended in 21 μl of water by incubating at 37 °C for a minimum of 4 h. DNA concentration was determined using a Qubit dsDNA BR assay kit (Thermo Fisher).

To facilitate maximum recovery of gDNA from the sorted cells, a previously described method was used with the following modifications: sorted cell pellets were resuspended in 500 μl of lysis buffer, consisting of 460 μl of STE buffer (1 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0), 100 mM NaCl) supplemented with 10 μl of 0.5 M EDTA, 10 μl of proteinase K (10 mg/ml in TE buffer containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), and 20 μl of 10% SDS. Pellets were digested overnight at 55 °C while shaking at 1,000 r.p.m. on a Thermomixer (Eppendorf). The following day, 5 μl of 2 mg/ml RNase A was added to each tube and incubated at 37 °C for 1 h while shaking at 1,000 r.p.m. Extractions were performed with an equal volume of pf 7.9-buffer saturated phenol, followed by phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform. Twenty micrograms of glycogen (Roche) and 1.5 ml of 100% ethanol were added to each tube and DNA was precipitated at −80 °C for 1 h followed by centrifugation at 18,000g for 10 min at 4 °C. Pellets were washed with 1 ml of 75% ethanol, dried, and resuspended in 21 μl of water by incubating at 37 °C for a minimum of 4 h. DNA concentration was determined using the Qubit dsDNA BR assay kit.

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discarded. Beads were washed twice with 200 μl of 70% ethanol and then dried for approximately 12 min. Bound DNA was eluted from the beads using 40 μl of water.

Next-generation sequencing. Before sequencing, all DNA libraries were analysed using a Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent). Library concentration was then determined by qPCR using a KAPA Library Quantification Kit for Illumina platforms. All samples were sequenced on an Illumina HiSeq 2500 or a NextSeq 500 with 75 bp single reads. Approximately 15 million to 20 million reads were sequenced per library.

Sequence assembly and analysis

Reference file for all sgrRNAs in the library was acquired from Addgene, and identical sgrRNAs targeting more than one protein-coding gene were removed. Demultiplexed FASTQ files were mapped to the reference file using Bowtie 2 requiring unique alignments with no mismatches. Normalized read counts were calculated as described previously. Screen hits were identified using RIGER with the following parameters: log(fold-change ranking), 1 × 10^4 permutations, second-best rank (SBR) scoring algorithm.

Co-immunoprecipitation assays. For all co-immunoprecipitation assays, 3.2 × 10^6 293T cells were seeded 1 day before transfection. Cells were transfected using FuGENE HD with 10 μg of total plasmid. Media were changed the following day. Cells were harvested 48 h after transfection. Cells were washed once, scraped in PBS, and lysed on ice for 10 min in 1 ml of lysis buffer composed of 25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM DTT, and a protease inhibitor cocktail (complete EDTA-free, Roche). Lysates were spun at 10,000 g for 10 min. Supernatants were collected and diluted with 0.5 volumes of fresh lysis buffer. One and a half microlitres of immunoprecipitation antibody (anti-FLAG or anti-HA (Cell Signaling catalogue number 2637S)) were added to each sample and incubated at 4 °C for 30 min. Thirty microlitres of washed Dynabeads Protein G (Thermo Fisher) were added to each sample and incubated for 6 h. RNase A (Thermo Fisher) was added to a final concentration of 20 μg/ml where indicated. Samples were washed four times in ice-cold lysis buffer. Fifty microlitres of 2 × Laemmli sample buffer were added to each sample and aliquots were used for western blot analysis.

Western blot antibodies. Antibodies used for western blotting included anti-HA (2367S, Cell Signaling), anti-V5 (46-0705, Invitrogen), anti-AGO2 (SA2400085, Sigma), anti-GAPDH (2118S, Cell Signaling), anti-α-tubulin (T6199-200UL, Sigma), anti-BRD4 (13440S, Cell Signaling), anti-CTNNB1 (9587S, Cell Signaling), anti-POU2F1 (8157S, Cell Signaling), anti-ANKRD52 (A302-372A, Bethyl), and anti-CSNK1A1 (sc-6477, Santa Cruz).

Phos-tag SDS–PAGE electrophoresis. SDS–PAGE gels (7%) were supplemented with Phos-tag AAL solution (Wako) according to the manufacturer’s recommendations. Gels were run at 100 V in an XCELL SureLock Mini-Cell (Invitrogen) until the dye front completely exited the gel. Gels were incubated in transfer buffer for 1 h and standard western blotting procedures were subsequently followed.

Mass spectrometry. Endogenous AGO2 was purified from ANKRDS52−/− and ANKRDS52−/− HCT116 cells. AGO2−/− cells were used as a control. Ten million cells were seeded per 15-cm dish, and eight dishes were used per cell line. AGO2 was immunoprecipitated using methods adapted from an established protocol with 100 μl of Dynabeads Protein G loaded with 18 μg of anti-AGO2 antibody (SA2400085, Sigma) per purification. Immunoprecipitation eluates were resuspended in 5 × Laemmli sample buffer. HCT116 cells were transfected with FH-AGO2 constructs (WT, T380A, S824A/T380A) and were stably expressed using MSCV-puro in ANKRDS52−/− cells. Ten million cells were seeded per 15-cm dish, and eight dishes were used per cell line. Media were changed 48 h later. Cells were scraped in PBS 72 h after plating. Lysates were generated using methods similar to the co-immunoprecipitation assays, with the exception that a phosphatase inhibitor cocktail (PhosStop, Roche) was included and lysate supernatants were diluted with one volume of lysis buffer. Proteins were immunoprecipitated using 100 μl of Dynabeads Protein G loaded with 20 μg of anti-Flag antibody (F1804, Sigma). Beads were rotated at 4 °C for 3 h. Beads were washed five times in lysis buffer. Proteins were eluted using 70 μl of 2 × Laemmli sample buffer per 100 μl of beads. Purified AGO2 proteins were separated by SDS–PAGE and stained using InstantBlue (Expedeon). Gel slices containing AGO2 bands were reduced by DTT, alkylated by iodoacetic acid, and digested with trypsin (Trypsin Gold, Promega).

The digestion was stopped by trypsin and followed by peptidase extraction in acetonitrile. Extracted peptides were desalted by C18 ZipTip (Millipore). Peptide mixtures were separated by C-18 resin (100 Å, 3 μm, MICHROM Bioresources) in-house packed into a silica capillary emitter (100 μm ID, 1 mm resin length). LC gradient was generated by a Dionex Ultimate 3000 nanoLC system (Thermo Scientific), with mobile phase A: 0.1% formic acid and B: 0.1% formic acid in acetonitrile. Mobile phase gradient: 2% B at 0–15 min, 30% B at 81 min, 35% B at 85 min, 40% B at 87 min, 60% B at 95 min, 80% B at 96–107 min and 2% B at 108–120 min. Flow rate: 600 nl/min at 0–13.5 min, 2500 nl/min at 13.5–120 min. Peptide eluents were sprayed online with a nano-electrospray ion source (Thermo Scientific) at spray voltage of 1.5 kV and capillary temperature of 250 °C. High-resolution MS analysis was performed on a QExactive Quadrupole-Orbitrap Hybrid mass spectrometer (Thermo Scientific), operating in data-dependent mode with dynamic exclusion of 30 s. Full-scan MS was acquired at an m/z range of 300–1650, resolution of 70,000, and automatic gain control target of 3 × 10^4 ions. The top 15 most intense ions were subsequently selected for higher-energy collisional dissociation fragmentation at resolution of 17,500, collision energy of 27 eV, and automatic gain control target of 1 × 10^4 ions. Proteome data analysis used Mascot (Matrix Science) and Proteome Discoverer (1.4, Thermo Scientific). The raw data were searched against the human proteome database (Uniprot, UP000005640) plus common contaminants. Static modifications were cysteine carbamidomethylation; variable modifications were serine or threonine phosphorylation, methionine oxidation, and glutamine or asparagine deamination. Precursor mass tolerance was 20 p.p.m. and fragment mass tolerance, 0.05 Da. The maximum number of misalignment sites allowed was 2. After peptide identification, precursor ion intensities were quantified manually in Xcalibur using extracted ion chromatogram.

Cloning, mutagenesis, and expression of cDNA constructs. Sequences of all primers used for cloning are provided in Supplementary Table 8. Flag–HA–AGO2 (FH–AGO2) was PCR amplified from pIRES-neo-Flag/HA AGO2 (Addgene catalogue number 10822) and subcloned into pcDNA3.1+. FH–AGO2 mutants were generated using a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent) or by cloning customized gBlocks (IDT) into the parental pcDNA3.1+ vector containing FH–AGO2 (sequence of all mutants provided in Supplementary Table 8). Stable expression of wild-type or mutant FH–AGO2 was achieved in one of two ways. In one, constructs were subcloned into pMSCV-puro (Clontech). In another, stable expression of AGO2 for RNA-seq and cCLIP experiments was achieved by cloning individual mutants into a modified pLJM1–EGFP vector (Addgene catalogue number 19319) where EGFP was resected using Agel and BsrGI before blunt-end ligations. AGO2 constructs were introduced at the EcoRI cloning site. Flag–HA–AGO2 was subcloned from pIREShneo-Flag/HA AGO1 (Addgene catalogue number 10820) into pMSCV-Pig (Addgene catalogue number 21654). V5-tagged ANKRDS2 (corresponding to NP_775866.2) was constructed by PCR amplification from HCT116 cDNA followed by cloning into pcDNA3.1+. cDNA clones for human PPP6C and CSNK1A1 were obtained from the Invitrogen Ultimate ORF LITE Library (Clone ID IOH7224 and IOH59130, respectively) and subcloned into pCAGIG (Addgene catalogue number 11159) using Gateway LR Clonase (Thermo Fisher). For tethering assays, a 5 × BoS sequence adapted from a previous report was designed as a gBlock (IDT) and cloned in the Xba site of the FH–AGO2 expression construct. In the FH–AGO2 constructs, a gBlock containing the NH peptide sequence with an HA tag was subcloned into pcDNA3.1–FH–AGO2, replacing the Flag–HA tag. To generate control plasmid expressing NH–HA peptide alone, the NH–HA sequence was PCR amplified and cloned into pcDNA3.1+. Expression of FH–AGO2 mutants in ANKRDS52−/− HCT116 EGFP−/− cells was performed using an eLife lentivirus preparation. Active lentivirus was generated using FH–AGO2 mutants (WT, 5X, S828A, and empty vector) cloned into a modified pLJM1 vector with EGFP resected. A viral packaging protocol analogous to that used for the lentCRISPR lentivirus preparations was used. Recipient ANKRDS52−/− HCT116 EGFP−/− cells were transduced at an MOI of approximately 10–50. Cells were selected in puromycin for at least 10 days, before use in flow cytometry experiments (Fig. 2f). AGO2 miRNA and AGO2 mRNA association studies. For experiments involving endogenous AGO2, HCT116 EGFP−/− cells were used. For analysis of FH–AGO2 miRNA or mRNA binding, cells stably expressing the indicated wild-type or
mutant FH-AGO2 protein were first generated by infecting AGO2−/− HCT116 cells with MSCV retrovirus. Then, for each immunoprecipitation sample, 6 × 10^4 cells were seeded per 10-cm dish. Cells were harvested 48 h later by scraping in PBS. Pelleted cells were resuspended in 1 ml of a lysis buffer consisting of 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM DTT, a protease inhibitor cocktail (Complete, EDTA-free, Roche), and 250 μl of Recombinant RNasin Ribonuclease Inhibitor (Promega). Cells were lysed on ice for 10 min. Samples were spun at 10,000g for 10 min. Supernatant fractions were retained. Protein concentration was determined using a Bio-Rad DC Protein Assay Kit, and all samples were adjusted to the same concentration with lysis buffer. Dynabeads Protein G (Thermo Fisher) were prepared by pre-incubating with 1.5 μg of antibody (either anti-Flag (F1804, Sigma) or anti-AGO2 (SAB4200085, Sigma)) and pre-blocking with 0.5 mg/ml BSA, 0.5 mg/ml yeast RNA, and 0.2 mg/ml heparin. Each sample was incubated with 25 μl of prepared Dynabeads Protein G for 3 h at 4°C. Samples were washed three times in lysis buffer. Captured protein was eluted from the beads using either 2.5 mg/ml 3× Flag peptide (Sigma) or 3.5 mg/ml AGO2 peptide (sequence derived from ref. 42, synthesized at the University of Texas Southwestern Protein Chemistry Technology Core) dissolved in lysis buffer. For each immunoprecipitation, qRT–PCR assays were performed to determine input and immunoprecipitation levels for mature miRNAs and mRNA targets of interest. Western blot analysis determined the relative amount of AGO2 in the immunoprecipitation eluate. RNA quantity as a percentage of input was determined for all immunoprecipitation eluates and then normalized to the relative amount of protein captured in each eluate.

AGO2 capture using an mRNA target mimic. Experiments to capture AGO2 loaded with miRNA were adapted from a previously published method36. ANKRDS52−/− and ANKRDS52−/− HCT116 cells were seeded at 1.35 × 10^6 cells per dish in six 15-cm dishes per cell line. Forty-eight hours later, cells from each dish were scraped in PBS, pelleted, and lysed on ice for 10 min in 1 ml of a buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM MgCl₂, 0.5% NP-40, 1 mM DTT, a protease inhibitor cocktail (Complete, EDTA-free, Roche), a phosphatase inhibitor cocktail (PhosStop, Roche), and 250 U/ml Recombinant RNasin Ribonuclease Inhibitor (Promega). Lysates were spun at 10,000g for 10 min and supernatants were further diluted with one volume of lysis buffer. To assess binding of AGO2 to the target mimic, 1.8 μl of each lysate was incubated with 50 μl of washed Dynabeads MyOne Streptavidin C1 (Thermo Fisher) pre-loaded with 300 pmol of wild-type or mutant RNA oligonucleotide (Supplementary Table 8) and pre-blocked with 1 mg/ml BSA, 0.5 mg/ml yeast tRNA, and 0.2 mg/ml heparin. To assess AGO2 phosphorylation after immunoprecipitation, 1.8 μl of each lysate was incubated with 50 μl of washed Dynabeads Protein G (Thermo Fisher) pre-incubated with 5 μl of anti-AGO2 antibody (SAB4200085, Sigma) and pre-blocked as noted previously. Lysates were incubated with beads for 3 h at room temperature. Beads were washed four times in lysis buffer before 50 μl of 2× Laemmli sample buffer was added. Phos-tag electrophoresis was performed on captured protein complexes and on input protein samples subjected to chloroform–methanol precipitation44.

Tethering assays. The 293T cells were seeded in 24-well plates using 7.5 × 10^4 cells per well. Cells were transfected the following day using FuGENE HD and 301 ng of pGL3-Control or pGL3-BoxB, 150 ng of pcDNA3.1−/− HCT116 EGFP-miR-19 cells were harvested 28 h later for western blot analysis. In vitro kinase assays using AGO2 peptides. In vitro co-IP assays were performed using assay conditions adapted from the manufacturer’s recommendations (Recombinant CNPK1A1, PV3880, Thermo Fisher). All reactions were performed in a 50 μl assay volume for 90 min at 30°C. Assay buffer was composed of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2.5 mM DTT, 0.01% Triton X-100, 0.5 mM mg BSA, 0.5 mM EGTA, 0.5 mM Na₂VO₃, 5 mM γ-glycerophosphate, 170 ng of recombinant CSNK1A1 (AP38500, Thermofisher), and 200 μM [γ-32P]ATP (SA = 100–500 c.p.m./pmol). Reactions were incubated at 37°C for 2 h. Beads were separated and mixed with 50 μl of 2× Laemmli sample buffer. SDS-PAGE was performed, and gels were stained using SimplyBlue SafeStain (Invitrogen). 32P signal was detected using a phosphor screen (GE Healthcare) and Typhoon FLA 7000 (GE Healthcare).

Cloning and expression of ciRS-7. The linear form of ciRS-7 was constructed by amplifying the endogenous ciRS-7 locus from human genomic DNA (Roche) by PCR (Phusion Polymerase, Thermo Scientific) using primer sequences described previously36 (Supplementary Table 8). The PCR fragment was then cloned into the HindIII and NotI cloning sites of pcDNA3.1+ (Invitrogen). To generate the ciRS-7 construct capable of circularization, an ~800-bp region upstream of the splice acceptor was amplified using previously described primers36 (Supplementary Table 8) and inserted in the inverse orientation downstream of the linear ciRS-7 sequence at the XhoI cloning site of pcDNA3.1−/−.

The effect of ciRS-7 expression on AGO2 phosphorylation was assessed through co-transfection experiments. Cells were seeded at a density of 9 × 10^4 cells per well in six-well dishes. Cells were transfected according to the manufacturer’s recommendations using Lipofectamine 2000 (Thermo Fisher). Where indicated, each well received 2 μg of plasmid and 10 nM miRNA mimics (miRDIAN miRNA mimics, GE Dharmacon). Cells were harvested 28 h later for western blot analysis.

RNA-seq. Parental HCT116 [EGFP−/−], AGO2−/− [HCT116 EGFP−/−], ANKRDS52−/− [HCT116 EGFP−/−], and ANKRDS52−/− [CSNK1A1−/− HCT116 EGFP−/−] cells were used for RNA-seq. Three independent clonal AGO2−/−, ANKRDS52−/−, and ANKRDS52−/−[CSNK1A1−/−] knockout cell lines and three biological replicates of parental cell lines were sequenced. Five hundred thousand cells were seeded per well in six-well dishes. Cells were harvested 48 h later and RNA was extracted using a RNeasy Mini Kit (Qiagen) with an on-column DNase digestion. Sequencing libraries were generated using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) and run on a NextSeq 500 using a NextSeq 500/550 High Output v2 Kit, 75 cycle (Illumina).

AGO2−/− HCT116 EGFP−/− cells generated using PX330 were reconstructed with either empty pLM1 vector (with EGFP previously repressed), FH-AGO2−/− WT (AGO2−/−), or FH-AGO2−/− SKA (AGO2−/− SKA). Biological triplicates for each cell line were seeded with 5.0 × 10^4 cells per well in six-well dishes. Cells were collected 48 h later, and RNA was extracted using a mirNeasy Mini Kit (Qiagen) with an on-column DNase digestion. Sequencing libraries were generated using a TruSeq Stranded Total RNA with Ribo-Zero Human/Mouse/Rat Low-throughput (LT) kit (Illumina) and run as performed in the previous RNA-seq experiment.

Quality assessment of the RNA-seq data was done using the NGS-QC-Toolkit43 with default settings. Quality-filtered reads generated by the tool were then aligned to the human reference genome hg19 (for AGO2−/−, ANKRDS52−/−, and ANKRDS52−/−[CSNK1A1−/−] RNA-seq experiments) or hg38 (for FH-AGO2 reconstitution experiments) using version 2.0.12 aligner44 using default settings. Read counts obtained from featureCounts44 were used as input for edgeR (version 3.8.6) for differential expression analysis. Genes with FDR < 0.05 were regarded as differentially expressed for comparisons of each sample group.

eCLIP. Cell culture, library preparation, and deep sequencing. AGO2−/− cells or AGO2−/− cells reconstituted with FH-AGO2 WT or FH-AGO2 SKA (AGO2−/− SKA). Biological triplicates for each cell line, duplicate input and immunoprecipitation samples were prepared and sequenced. The RiL19 RNA adaptor (Supplementary Table 8) was used as the 3′ RNA linker for all samples. PAGE-purified DNA oligonucleotides were obtained from Sigma for the PCR library amplification step (Supplementary Table 8).
PCR amplification was performed using between 11 and 15 cycles for all samples. Paired-end sequencing was performed on a NextSeq 500 using a NextSeq 500/550 High Output v2 Kit, 75 cycle (Illumina).

Mapping deep sequencing reads. Adapters were trimmed from original reads using Cutadapt (version 1.9.1)50 with default settings. Next, the randomer sequence from the rand103Tr3 linker (Supplementary Table 8) was trimmed and recorded. TopHat2 (version 2.0.12)51 was used to align mate 2 to hg38. Only the uniquely mapped reads were retained. PCR duplicates were then removed using the randomer information with an in-house script. All reads remaining after PCR duplicate removal were regarded as usable reads and used for cluster calling.

cECLIP cluster calling and annotation. cECLIP clusters were identified using a previously described method52 with the following modifications. Genome coverage by usable reads was determined at nucleotide resolution for each data set, and regions of continuous coverage greater than expected from a Poisson noise distribution were identified ($P \leq 0.001$). For each region, read counts were obtained using Bedtools (version 2.17)53. If 50% of a read overlapped a region on the same strand, it was counted as a read covering that region. For each region, normalization to total usable reads was performed and a fold change between immunoprecipitation and input samples was calculated. Significant CLIP clusters in each data set were defined by (1) the presence of significantly greater coverage in the region than expected by chance on the basis of the Poisson distribution, and (2) log2(fold change) of normalized reads in the cluster was $\geq 2$ comparing immunoprecipitation to input.

The final CLIP clusters for FH-AGO2WT and FH-AGO2SA were identified by first identifying significant clusters present in both experimental replicates. A region was considered to be present in both replicates if it occurred on the same strand and the replicate clusters overlapped by at least one-third of their total length. Significant clusters from both replicates were then merged to define the final cluster length. Lastly, all clusters identified in the AGO2−/− samples were subtracted to generate the final CLIP cluster calls (Supplementary Table 7). Clusters were annotated on the basis of their genomic locations (Ensembl GRCh38.85) if 55% of the cluster overlapped with a given genomic region. If a cluster was assigned to multiple annotations, the annotation was selected using the following priority: CDS exon $\geq$ 3′ UTR $\geq$ 5′ UTR $\geq$ protein-coding gene intron $\geq$ noncoding RNA exon $\geq$ noncoding RNA intron $\geq$ intergenic.

Identification of active miRNA seed families and calculation of CLIP coverage at miRNA binding sites. Active miRNAs in HCT116 were identified using an approach similar to that described previously6 with the following modifications. The top 100 most highly expressed miRNAs in HCT116 cells were identified on the basis of a previously published small RNA sequencing experiment in this cell line49 and collapsed to 66 7-nucleotide seed families with identical sequence from nucleotides 2–8. Eight-nucleotide binding sites for these seeds, defined as in ref. 3, were identified in the 3′ UTRs of all expressed genes (FPKM $> 0$) using seqMap (version 1.0.12)50. The locations were then transformed to genomic coordinates and extended 10 nucleotides upstream and downstream to obtain a seed match region (excluding sites on exon–exon junctions). The numbers of crosslinking sites in these seed match regions for each miRNA seed family in FH-AGO2WT CLIP data were counted, normalized to the total usable reads in each replicate library, and averaged across replicates. To determine the significance cut-off, all possible 8-nucleotide sequences except for known miRNA seeds and those with four consecutive A, C, G, or T nucleotides were used to generate a null distribution.

To quantify CLIP coverage of miRNA binding sites in FH-AGO2WT and FH-AGO2SA CLIP data (Fig. 6c), 8-, 7-, and 6-nucleotide binding sites for all active miRNAs were identified within each class of CLIP cluster. Windows around each site were then extended 10 nucleotides upstream and downstream to obtain a seed match region. The numbers of crosslinking sites within these regions were counted and normalized to the total number of reads in clusters of each class (FH-AGO2SA-unique or FH-AGO2WT/ FH-AGO2SA-common) to derive the CLIP coverage used to draw the CDF plots.

To quantify CLIP coverage of miRNA binding sites in FH-AGO2SA-unique clusters versus FH-AGO2WT/FH-AGO2SA-common clusters (Extended Data Fig. 10e), 8-, 7-, and 6-nucleotide binding sites for all active miRNAs were identified within each class of CLIP cluster. Windows around each site were then extended 10 nucleotides upstream and downstream to obtain a seed match region. The numbers of crosslinking sites within these regions were counted and normalized to the total number of reads in clusters of each class (FH-AGO2SA-unique or FH-AGO2WT/ FH-AGO2SA-common) to derive the CLIP coverage used to draw the CDF plots.

CLIP coverage of FH-AGO2WT rescued versus non-rescued transcripts. Genes whose repression in AGO2−/− cells was rescued by FH-AGO2SA were defined by first identifying the genes that were significantly upregulated in AGO2−/− cells compared with parental HCT116 (FDR $\leq 0.05$), then, among these genes, those that were significantly downregulated in FH-AGO2SA versus AGO2−/− (FDR $\leq 0.05$). All other genes upregulated in AGO2−/− cells were considered not-rescued. The FH-AGO2WT CLIP coverage for each gene in these classes was calculated as the sum of all reads in CLIP clusters in a given 3′ UTR, normalized to total reads in all clusters, divided by the FPKM of the transcript. The final reported CLIP coverage (Extended Data Fig. 10d) is the average of both FH-AGO2WT CLIP replicates.

mRNA half-life analysis. Half-lives of transcripts with FH-AGO2SA CLIP clusters in their 3′UTRs were obtained from a previously published study49. Genes that had half-lives assigned to more than one ReSeq mRNA isoform were removed to avoid ambiguity. Genes in the top quartile of half-lives were defined as having a long half-life ($n = 273$) and genes in the bottom quartile of half-lives were defined as having a short half-life ($n = 274$). The total numbers of CLIP reads in clusters in a given 3′ UTR were obtained for each replicate, and edgeR (version 3.8.6)46 was used to calculate the normalized fold change of CLIP coverage comparing FH-AGO2SA with FH-AGO2WT (Extended Data Fig. 10f).

Data availability. All high-throughput sequencing data generated in the course of this study (CRISPR–Cas9 screens, RNA-seq, eCLIP) have been deposited in Gene Expression Omnibus under accession number GSE89946. All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Knockout of candidate miRNA regulators in HCT116<sup>EGFP</sup> cells. Flow cytometry analysis of EGFP in HCT116<sup>EGFP</sup> cells after transduction with lentiCRISPR vectors targeting the indicated genes.
Extended Data Figure 2 | **BRD4, CTNNB1, and POU2F1 positively regulate miR-19 expression.** a, Model depicting how each gene may promote expression of the miR-17-92 cluster. b–d, Western blot analysis confirming loss of expression of the indicated gene in HCT116 knockout clones. Asterisk indicates non-specific band. For each protein, all lanes came from the same blot but irrelevant lanes were removed. e–g, qRT–PCR assays demonstrating reduced expression of MYC (e), pri-miR-17-92 (f), or mature miR-19a/b (g) in BRD4−/−, CTNNB1−/−, or POU2F1−/− cells. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 3 | Western blot analysis confirms loss of protein expression in AGO2 and ANKRD52 HCT116 clonal knockout lines. For both AGO2 (a) and ANKRD52 (b) western blots, all lanes came from the same blot but irrelevant lanes were removed. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 4 | General impairment of miRNA-mediated silencing in ANKRD52−/− cells. a, b, Flow cytometry analysis of EGFP expression in HCT116 cells stably expressing reporters for miR-16 (a) or miR-200 (b) after transduction with lentiCRISPR vectors targeting ANKRD52 or expressing a non-targeting sgRNA. c, qRT–PCR showing de-repression of established let-7 targets (DICER1 or HMGA2) in AGO2−/− or ANKRD52−/− cells. *P < 0.05, **P < 0.01, two-tailed Student’s t-test comparing AGO2−/− or ANKRD52−/− to parental. (n = 3 biological replicates, each assayed in triplicate.) d, qRT–PCR analysis of DICER1 and HMGA2 in non-transfected (NT) HCT116EGFP-miR-19 cells or after transfection with miR-19 antisense oligonucleotides (Anti-miR-19) confirms that these transcripts are not regulated by miR-19. Upregulation of the EGFP miR-19 reporter transcript served as a positive control in this experiment. (n = 3 biological replicates, each assayed in triplicate.) e, qRT–PCR was performed for the indicated miRNAs and expression levels were normalized to U6 snRNA (n = 2 biological replicates, each assayed in triplicate).
Extended Data Figure 5 | The ANKRD52–PPP6C complex interacts with and dephosphorylates AGO proteins. a, Co-immunoprecipitation of Flag–HA-AGO2 (FH-AGO2) with V5-ANKRD52 or V5-PPP6C with or without RNase A treatment. b, Phos-tag electrophoresis demonstrating AGO2 hyperphosphorylation in multiple ANKRD52/PPP6C-deficient cell lines. c, Phos-tag western blot analysis of Flag–HA-AGO1 (FH-AGO1) stably expressed in ANKRD52+/− and ANKRD52−/− HCT116 cells. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 6 | Identification of multiple definitively phosphorylated residues in the S824–S834 region of AGO2 by mass spectrometry. 

**a** Full-scan mass spectra zoomed to the region for the AGO2 815–837 peptide. The unphosphorylated and multiply phosphorylated precursor ions are shown in red. Peak labels indicate the mass-to-charge ratios and the charge state. The singly charged ion with grey label (top panel) does not correspond to peptides 815–837. Data at two close elution time points are shown for ANKRD52−/− to illustrate the unphosphorylated (0P), singly (1P), doubly (2P), and triply (3P) phosphorylated peptides. 

**b** Quantification of the indicated endogenous AGO2 phosphopeptides relative to unphosphorylated peptide as determined by mass spectrometry. Labels 1P, 2P, or 3P respectively denote singly, doubly, or triply phosphorylated peptides spanning residues 815–837 of AGO2. Superscript indicates peptide charge state. ND, not detected. 

**c** MS/MS spectra demonstrating phosphorylation of endogenous AGO2 at S824 in ANKRD52−/− cells. Red bars denote site-determining ions. 

**d, e** MS/MS spectra demonstrating phosphorylation of FH-AGO2 (T830A) at S824 and S828 (d) or phosphorylation of FH-AGO2 (S824A/T830A) at S828 and S831(e) in ANKRD52−/− cells.
Extended Data Figure 7 | Phosphomimetic mutants of FH-AGO2 do not exhibit reduced miRNA association. a, Western blots showing expression of the indicated FH-AGO2 mutants. Within each panel (top, middle, bottom), all lanes came from the same blot but irrelevant lanes were removed. b, miRNA association of wild-type or mutant FH-AGO2 assessed as described in Fig. 3a (n = 4 biological replicates, each assayed in triplicate). For gel source data, see Supplementary Fig. 1.
Extended Data Figure 8 | Analysis of serine/threonine kinases identified in the CRISPR–Cas9 suppressor screen. a, b, Flow cytometry demonstrating EGFP expression in HCT116<sup>EGFP-miR19</sup> (a) or HCT116<sup>EGFP</sup> cells (b) after transduction with lentiCRISPR vectors targeting the indicated genes. c, Flow cytometry demonstrating EGFP expression in HCT116<sup>EGFP-miR19</sup> cells treated with the indicated dose of rapamycin. NT, not treated. d, Phos-tag western blot analysis of AGO2 in ANKRD52<sup>−/−</sup> cells after treatment with rapamycin. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 9 | Functional characterization of CSNK1A1 and AGO2 target binding mutants. a, Western blot analysis confirms loss of CSNK1A1 expression in HCT116 ANKRD52<sup>−/−</sup>;CSNK1A1<sup>−/−</sup> clonal knockout cells. All lanes came from the same blot but irrelevant lanes were removed. b, miR-19 expression normalized to U6 expression, assessed by qRT–PCR, in cells of the indicated genotypes (n = 4 biological replicates, each assayed in triplicate). c, Co-immunoprecipitation of V5-CSNK1A1 with FH-AGO2, with or without RNase A treatment. d, miRNA association of FH-AGO2 assessed as in Fig. 3e (n = 4 biological replicates, each assayed in triplicate). *P < 0.05 comparing mutant to wild-type AGO2, two-tailed Student’s t-test. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 10 | See next page for caption.
Extended Data Figure 10 | Generation and eCLIP analysis of AGO2−/− cells reconstituted with AGO2WT or AGO25XA. a, Western blot showing equivalent expression of FH-AGO2WT and FH-AGO25XA at physiological levels. For gel source data, see Supplementary Fig. 1. b, Distribution of AGO2 binding sites determined by eCLIP. c, Validation of targets identified by eCLIP using FH-AGO2 pull-down assays performed in reconstituted AGO2−/− cells. Experiment was performed as in Fig. 3a except anti-Flag antibody was used for immunoprecipitation (n = 3 biological replicates, each assayed in triplicate). *P < 0.05, **P < 0.01, one-tailed Student’s t-test comparing FH-AGO25XA with FH-AGO2WT. NS, not significant. d, FH-AGO2WT CLIP coverage (normalized total number of reads in clusters in a given 3′ UTR divided by the FPKM) of genes whose AGO2-mediated repression is or is not rescued by FH-AGO25XA. e, The 8-, 7-, or 6-nucleotide binding sites for active miRNAs in HCT116 were identified within FH-AGO2WT/FH-AGO25XA-common CLIP clusters or FH-AGO25XA-unique CLIP clusters in 3′ UTRs. CDF plots show CLIP coverage for each class of site (normalized number of crosslinking events within ten nucleotides of each site). NS, not significant, assessed by Kolmogorov-Smirnov test. f, CDF plot showing the fold change in CLIP coverage comparing FH-AGO25XA to FH-AGO2WT for transcripts with long half-lives (top quartile) versus those with short half-lives (bottom quartile). g, Summary of the newly defined AGO2 phosphorylation cycle. Target engagement triggers the hierarchical, multi-site phosphorylation of AGO2 by CSNK1A1, which inhibits target binding. The ANKR52–PPP6C phosphatase complex dephosphorylates these residues, allowing AGO2 to engage new targets. Continual phosphorylation/de-phosphorylation of AGO2 through this cycle is necessary to maintain the global efficiency of miRNA-mediated silencing.