PRMT5 methylome profiling uncovers a direct link to splicing regulation in acute myeloid leukemia

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Protein arginine methyltransferase 5 (PRMT5) has emerged as a promising cancer drug target, and three PRMT5 inhibitors are currently in clinical trials for multiple malignancies. In this study, we investigated the role of PRMT5 in human acute myeloid leukemia (AML). Using an enzymatic dead version of PRMT5 and a PRMT5-specific inhibitor, we demonstrated the requirement of the catalytic activity of PRMT5 for the survival of AML cells. We then identified PRMT5 substrates using multiplexed quantitative proteomics and investigated their role in the survival of AML cells. We found that the function of the splicing regulator SRSF1 relies on its methylation by PRMT5 and that loss of PRMT5 leads to changes in alternative splicing of multiple essential genes. Our study proposes a mechanism for the requirement of PRMT5 for leukemia cell survival and provides potential biomarkers for the treatment response to PRMT5 inhibitors.

Arginine methylation is an ubiquitous protein posttranslational modification in mammals, catalyzed by the protein arginine methyltransferase (PRMT) family that transfers a methyl group from S-adenosylmethionine to the guanidine nitrogen atom of arginine. There are three forms of methylated arginines in mammals: $\omega-N^\text{methyl}-L-$arginine, asymmetric $\omega-N^\text{methyl},N^\text{dimethyl}-L-$arginine and symmetric $\omega$-$N^\text{dimethyl},N^\text{dimethyl}-L-$arginine, and the PRMT enzymes are classified depending on the type of modification they generate. Functionally, protein arginine methylation is known to affect binding interactions. The bulky methyl groups can prevent access to the potential hydrogen bond donors in arginine known to affect binding interactions. The bulky methyl groups can thereby inhibit protein interactions. At the same time, arginine methylation is known to facilitate the interaction with Tudor domains on proteins.

PRMT5 has recently emerged as a promising cancer drug target, and three PRMT5 inhibitors are currently in clinical trials for a range of solid and blood cancers (https://clinicaltrials.gov). PRMT5 was shown to act as an oncprotein in multiple malignancies, conferring aggressiveness and promoting cell proliferation. Moreover, several recent reports have demonstrated a selective sensitivity of cancers with 9p21 deletion to the knockdown (KD) of PRMT5, due to synthetic lethality with the deleted MTAP (methylthioadenosine phosphorylase) gene. Since 9p21 is a very frequent deletion present in about 14% of all cancers, PRMT5 inhibition represents an exciting therapeutic strategy for cancers with, in particular, this chromosomal aberration.

PRMT5 belongs to the class II arginine methyltransferases, as it catalyzes monomethylation and symmetrical dimethylation of arginines on proteins. It acts in a complex with WDR77 (also known as MEP50 and WD45), responsible for proper orientation of the PRMT5 substrates. Several nuclear and cytoplasmic substrates of PRMT5 have been reported, which are involved in different cellular processes, including transcription, DNA damage response, splicing, translation and cell signaling. However, further studies are required to understand the mechanism by which PRMT5 contributes to tumorigenesis and normal cellular physiology. In this study, we aimed at identifying substrates regulated by PRMT5, which are essential for cancer cell proliferation.

Results

The catalytic activity of PRMT5 is required for proliferation of MLL-AF9-rearranged AML cells. To assess the requirement for PRMT5 expression in AML cells, we used CRISPR interference (CRISPRi) and CRISPR knockout (CRISPRko) (Extended Data Fig. 1a). For CRISPRi, the cells were transduced with a lentivirus carrying a lentiviral backbone with a packaging vector (vector only) or an AML-specific sgRNA expressing a catalytically dead Cas9 (cdCas9) protein fused to a KRAB repression domain. On the transduction of the THP-1-cdCas9-KRAB cells with two independent single-guide RNAs (sgRNAs) complementary to the PRMT5 transcription start site, efficient PRMT5 gene repression was observed (Extended Data Fig. 1b,c). This led to decreased levels of global symmetrical arginine dimethylation (Extended Data Fig. 1d) as well as substantial cell proliferation defects (Extended Data Fig. 1e). A similar effect was observed using MOLM-13-cdCas9-KRAB (Extended Data Fig. 1f,g). Using a similar setup, we also confirmed the requirement of the PRMT5 co-factor WDR77 for the growth of AML cells (Extended Data Fig. 1h,i). The requirement for PRMT5 for cell proliferation was also validated in human THP-1, MOLM-13, MONOMAC-6 and mouse MLL-AF9-wtCas9 leukemia cells using the CRISPRko system (Extended Data Fig. 1j). Taken together, these
Identification of new PRMT5 substrates in human AML cells.

Given the essential nature of the enzymatic activity of PRMT5, we performed systematic identification of its substrates by liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based proteomics with the use of an isobaric mass tag labeling approach for quantitation\(^1\), as outlined in Fig. 2a. Ten samples were co-analyzed in the TMT10plex experiment: four biological replicates of cells transduced with a nontargeting sgRNA and three biological replicates of cells transduced with two independent sgRNAs inducing PRMT5 KD. Deep and reliable profiling of proteins and methylated peptides was achieved by two strategies. First, we performed three two-dimensional liquid chromatography separations with a different number of first dimension (high pH reverse phase chromatography) fractions and different lengths of the second dimension (low pH RP) gradient. Second, we enriched for peptides with monomethylated (MMA) and symmetrically dimethylated (SDMA) arginines using immunoaffinity purification.

To ensure high accuracy of quantitation we analyzed the samples on an Orbitrap Fusion Lumos platform using multinox SPS\(^1\), and removed peptide spectrum match (PSM) prone to inaccurate quantitation (with low intensity of reporter ions or high degree of contamination with co-selected peptides). Proteins were included, if their quantitation was based on at least two proteotypic peptides. After application of the filtering criteria we identified 384,765 PSMs and 125,801 peptides from 7,426 proteins (Supplementary Table 1). We found that 2,962 proteins were differentially expressed in both PRMT5 sgRNA samples. For all 2,962 proteins, the change in abundance between the two PRMT5 sgRNA conditions and the control sample was in the same direction (Fig. 2b). Furthermore, PRMT5 and its co-factor WDR77 were the two most downregulated proteins (Fig. 2c). PRMT5 sgRNA-2 induced a better PRMT5 KD (Fig. 2c and Extended Data Fig. 1b,c) and, consistently, higher absolute changes of 83% of regulated proteins, supporting the idea that the observed protein changes are specific. Downregulated proteins were significantly enriched in components of DNA replication and repair pathways, while upregulated proteins were enriched in leukocyte granulation, RNA processing and vesicle transport categories (Fig. 2d).

Combining the two approaches of deep two-dimensional LC–MS and immunoenrichment (Fig. 2a), we identified 1,209 peptides with arginine methylation (Fig. 2e). Relative abundances of modified peptides were divided by their corresponding protein abundances (Fig. 2f and Supplementary Table 1), to ensure that the reported changes reflect site occupancy and not just changes in protein expression. We found that 420 peptides from 183 different proteins were differentially methylated (\(q \leq 0.1\) in both PRMT5 sgRNA conditions). All the observed changes had the same direction in both PRMT5 sgRNA conditions (Fig. 2g). To define a list of potential PRMT5 substrates, we selected proteins with at least one arginine monomethylation or dimethylation site decreased. This resulted in a set of 61 protein, which were enriched in proteins involved in RNA end processing, splicing and binding (Fig. 2h and Supplementary Table 1).

To investigate which of the identified potential targets of PRMT5 could explain its requirement for the leukemic cell growth, we cross-referenced the list of the 61 arginine methylated proteins with a previously published genome-wide CRISPRko screen in human AML cell lines\(^\text{*}\). We selected 17 proteins that were shown to be required for AML cell growth (Extended Data Fig. 3a), with the hypothesis that arginine methylation of these would be essential for their function. Next, for these 17 proteins we identified those directly targeted by PRMT5. We synthesized 25 amino acids parts of the protein sequences containing the identified differentially regulated arginine methylation sites and incubated these peptides with a recombinant PRMT5–WDR77 complex followed by mass spectrometry analysis. Peptides from 12 out of the 17 proteins demonstrated characteristic methylation mass shifts (single or multiples of +14 Da) and, hence, confirmed their proteins as PRMT5 substrates (Fig. 3a and Extended Data Fig. 3b). Most of the substrates contained several arginine methylation sites (for example, SNRPB, SF3B1, PNN, CPSF6, CCT7, ALYREF, RPS10). In addition, 2,962 proteins were differentially expressed in both PRMT5 sgRNA samples. For all 2,962 proteins, the change in abundance between the two PRMT5 sgRNA conditions and the control sample was in the same direction (Fig. 2b). Furthermore, PRMT5 and its co-factor WDR77 were the two most downregulated proteins (Fig. 2c). PRMT5 sgRNA-2 induced a better PRMT5 KD (Fig. 2c and Extended Data Fig. 1b,c) and, consistently, higher absolute changes of 83% of regulated proteins, supporting the idea that the observed protein changes are specific. Downregulated proteins were significantly enriched in components of DNA replication and repair pathways, while upregulated proteins were enriched in leukocyte granulation, RNA processing and vesicle transport categories (Fig. 2d).
we observed a tendency for more arginines in the vicinity of the methylated site, but none directly next to each other, resulting in a potential GRGRGR pattern as a PRMT5 consensus motif (Fig. 3b).

Next, we confirmed that 11 out of the 12 identified protein substrates indeed are required for the proliferation of human AML cells (Fig. 3c,d and Extended Data Fig. 3c). Most of these substrates are involved in different aspects of RNA metabolism and, particularly, mRNA splicing (Table 1 and Fig. 3e).

In summary, by combination of proteomics, in vitro methyltransferase assays and genetic studies we have identified 11 proteins as PRMT5 substrates that are also essential for the proliferation of AML cells. These proteins are highly enriched for regulators of RNA metabolism.

**PRMT5 depletion leads to changes in alternative splicing in human AML cells.** Several recent studies reported changes in
alternative splicing as a result of PRMT5 downregulation in glioma, lymphoma, fetal liver cells and several solid cancer cell lines\(^{21-23}\). However, mechanistic insights into which PRMT5 substrates regulate this process are lacking. Since the majority of the essential PRMT5 substrates we identified were previously implicated in RNA binding, processing (including splicing) and transport, we decided to profile differences in alternative splicing between the wild-type and PRMT5 KD AML cells. As before, THP-1-cdCas9-KRAB cells were transduced with a nontargeting sgRNA or an sgRNA against PRMT5, and the cells were subjected to RNA sequencing.

First, we performed a DESeq2\(^{24}\) and edgeR\(^{25,26}\) approaches, which identified 2,974 retained introns (RIs) in the transcriptome of THP-1 cells (Extended Data Fig. 4a and Supplementary Table 2). The retention of these introns was confirmed by Cufflinks\(^{29}\) assembly of full-length transcripts (Extended Data Fig. 4b,c) and, for selected events, by quantitative PCR with reverse transcription (RT–qPCR) (see below). Next, we extended the list of the identified RIs with exons expressed in THP-1 cells, and applied DEXSeq\(^{30}\) and limma-diffSplice\(^{31}\) algorithms to detect the differentially used RIs (diffRIs) and exons following the PRMT5 KD. We found that wild-type and PRMT5 KD cells differ in the usage of 415 RIs (Fig. 4a) and 1,079 exons (data not shown) distributed over 321 and 777 individual genes, respectively. The vast majority of the diffRIs exhibit higher levels in the KD cells, indicating the tendency of the PRMT5-depleted cells to retain introns in RNA molecules (Fig. 4b), which is consistent with a previous report in glioma cells\(^{32}\).

Next, using the limma-diffSplice\(^{28}\) and JunctionSeq\(^{33}\) approaches, we analyzed differential usage of the exon–exon junctions (EEJs) in the transcriptome of THP-1 cells on the PRMT5 KD. We found 719 differential EEJs (diffEEJs, Fig. 4c, Extended Data Fig. 4d and Supplementary Table 2) distributed over 572 individual genes. Among the identified diffEEJs, the majority are annotated by Ensembl, while about 13% of the upregulated and 4% of the downregulated EEJs represent novel EEJs (Fig. 4d). This suggests that rather than inducing aberrant splicing events, PRMT5 loss leads to the differential usage of already known isoforms. If we classify all the identified EEJs according to splicing modes, PRMT5 KD mostly causes single or multiple exon skipping and alternative 5′- and 3′-splice sites selection (Fig. 4e). We observed very small overlap between the differentially spliced and differentially expressed genes (Fig. 4f), suggesting that the detected alternative splicing differences rarely affect total mRNA expression levels.

To understand whether there is a link between the differential splicing and the changes in arginine methylation of the splicing regulators identified as PRMT5 substrates, we focused on two factors that are known to bind RNA during the splicing process (SRSF1 (ref. 34) and SFPQ\(^{35,36}\)). We compared the frequency of their binding motifs in 100 nucleotides around the diffEEJs and nondiffEEJs, as well as around the diffRIs and nondiffRIs. The SRSF1 binding motif frequency was significantly higher both at the 5′ and 3′ splice sites of the diffEEJs (Fig. 4g and Extended Data Fig. 4e), but not around the diffRIs (data not shown). The absolute number of SRSF1 motifs per splice site was significantly increased for the differential splicing events of both classes (Fig. 4h,i). Notably, the motifs of two SRSF1 interacting partners, SRSF2 and SRSF3, but not SRSF7, were also significantly enriched at the splice sites of the diffEEJs (Extended Data Fig. 4f,g and data not shown). At the same time, minimal differences in the frequency of the SFPQ motif were observed between the differential and nondifferential splicing events (Fig. 4j and Extended Data Fig. 4h).

To analyze whether changes in alternative splicing induced by the loss of PRMT5 bear similarities in different cell types, we compared the differential splicing events found in AML cells with the ones from a recently published study in U-87 MG glioma cells\(^{37}\). We observed many common differential splicing events using both RI and EEJ analysis (Fig. 4k,l and Supplementary Table 2). Moreover, we found that the SRSF1 motif is significantly enriched in the differential EEJs in U-87 MG cells (Extended Data Fig. 4j–l). Taken together, these findings suggest a potential common mechanism of alternative splicing regulation by PRMT5 via SRSF1 methylation.

In summary, our results show that loss of PRMT5 leads to numerous changes in alternative splicing events in human AML cells; in particular, increased exon skipping and higher frequency of RIs. Motif analysis demonstrates a strong enrichment for the motif of the PRMT5 substrate SRSF1 among the differential EEJs.

**Fig. 2** | Proteome and methylome profiling identify new PRMT5 substrates in human AML cells. a, Outline of the proteome and methylome profiling strategies in THP-1-cdCas9-KRAB cells transduced with a nontargeting sgRNA or two independent sgRNAs against PRMT5 (see Methods for details). High pH RP, high pH reverse phase chromatography. b, Heatmap of 2,962 differentially expressed proteins (q value ≤ 0.05 in both sgRNAs, limma test, with P values adjusted by the Storey method). c, Boxplot representing relative protein abundance of PRMT5 and its co-factor WDR77 in THP-1-cdCas9-KRAB cells transduced with nontargeting sgRNA or two independent sgRNAs against PRMT5. Boxplot summary: outliers (points), minimum (lower whisker), first quartile (lower bound of box), median (horizontal line inside box), third quartile (upper bound of box), interquartile range (box) and maximum (upper whisker). For PRMT5-depleted cells, n = 3 independently transduced samples. For wild-type cells, n = 4 independently transduced samples. The difference between the negative control and each of the KD sgRNA is statistically significant (q < 0.05, limma test, with P values adjusted by the Storey method). d, Gene ontology-based functional classification of 2,962 up- and downregulated proteins in THP-1 cells following PRMT5 KD (two-sided Fisher’s exact test, false discovery rate (FDR)-adjusted P < 0.05). The nodes represent significantly enriched protein sets, node size is proportional to the number of members in a protein set, and color intensity reflects the q value. Edges indicate the protein overlap between the nodes with thicker edges indicating higher degree of overlap. Orange edges illustrate upregulated categories; green, downregulated. Functionally related protein sets are clustered, numbered and named. Blue color in half circles indicates no enriched categories. e, Venn diagram representing the number of methylated peptides identified using the strategies outlined in a, f. An example of normalization of the arginine methylation site quantified against the protein level. 681R and 696R methylation sites of SUTP5H were quantified as decreasing on PRMT5 KD, while the SUTP5H protein itself was, conversely, slightly upregulated. Hence, normalization results in the increased fold change for methylation site quantification. Rme = arginine methylation. Boxplot summary as in c. For PRMT5-depleted cells, n = 3 independently transduced samples. For wild-type cells, n = 4 independently transduced samples. g, Heatmap of 420 differentially methylated sites. (q ≤ 0.1 in both sgRNAs, limma test, with P values adjusted by the Storey method). h, Enrichment map of the gene ontology-enriched protein sets across 61 identified potential PRMT5 targets. Representation and statistics as in d. The proteomics experiments were performed using three independently transduced samples of PRMT5-depleted cells (two independent sgRNAs) and four independently transduced samples of wild-type cells. Source data are available in Supplementary Table 1.
reliably quantified protein levels and 88 of those demonstrated significant changes in protein expression, with the majority, 74, being downregulated on the PRMT5 KD (Fig. 5a). To understand whether differential splicing of some of these genes could be linked to reduced proliferation in KD cells, we selected seven genes with affected splicing: POLD1, POLD2, PPP1R7, PNISR, FDPS, PNKP and PDCD2, which are essential according to published CRISPRko screens\(^{20}\). Using a CRISPRko competition assay, we independently

![Diagram](image_url)
**Fig. 3 | Validation of the essential PRMT5 substrates.** a. Distributions of abundances of unmethylated and methylated peptide forms after the incubation with or without recombinant PRMT5-WDR77 complex. Only the peptides belonging to the confirmed PRMT5 substrates are shown here. b, PRMT5 methylation motif predicted using an iceLogo tool. The y axis represents the difference between the frequency of an amino acid in a sample set and the reference set (human proteome). c, CRISPRi competition assays to confirm essentiality of CCT4, CCT7, PNN, SNRPB, SRSF1, TAF15, SUPT5H, SFPO, RPS10 and CPSF6. THP-1-cdCas9-KRAB cells were transduced with the sgRNAs against the genes of interest and the percentage of sgRNA-transduced (BFP-positive) cells was measured over time. An sgRNA targeting POLR1D was used as a positive control and a nontargeting sgRNA was used as a negative control. d, CRISPRko competition assays to confirm essentiality of ALYREF and WDR33. THP-1-wtCas9 cells were transduced with lentiviruses expressing the sgRNAs against the genes of interest and the percentage of sgRNA-transduced (BFP-positive) cells was measured over time. An sgRNA targeting MCM2 was used as a positive control and a nontargeting sgRNA was used as a negative control. e, Enrichment map of the gene ontology-enriched protein sets across 11 validated essential substrates of PRMT5 (two-sided Fisher’s exact test, FDR-adjusted *P* < 0.05). Nodes represent significantly enriched protein sets, node size is proportional to the number of members in a protein set and color intensity depends on the *q* value. Edges indicate the protein overlap between the nodes with thicker edges indicating higher overlap between the nodes. Functionally related protein sets are clustered, numbered and named. The experiments in a, c, d were repeated twice with similar results. Source data for a are available in Supplementary Table 1. Source data for c, d are available online.
confirmed their essentiality in human AML cells (Fig. 5b). The downregulation on protein level ranged from 25 to 50% (Fig. 5c), but since the tandem mass tag (TMT)-based proteomics experiments might be subject to ratio suppression, the differences could be more pronounced. Using RT–qPCR, we validated all the identified differential splicing events in these genes (Fig. 5d,e), and the results strongly correlated with the RNA-sequencing values (Fig. 5f). In these selected events, we also observed an increased frequency of SRSF1 binding motifs around the differential splicing sites (Extended Data Fig. 5a,b).

To illustrate the potential functional consequences of the identified differential splicing events in the selected essential genes, we present two examples (Fig. 5g,h). For PDGDC2, differential splicing leads to early alternative transcription termination and, thereby, potential loss of a C-terminal domain (Fig. 5g). For PNP, a differentially RI between exons 5 and 6 of the gene contains a premature translation termination codon, which likely leads to nonsense-mediated mRNA decay (NMD) and/or protein truncation (Fig. 5h).

To test whether alternative splicing of PNP leads to NMD of its mRNA, we treated the cells, transduced with either a negative control or a PRMT5 sgRNA, with emetine, a drug inhibiting NMD. This led to stabilization of the PNKP mRNA containing the RI with a more pronounced effect in the case of PRMT5 KD (Fig. 5i). This confirms that differential alternative splicing of PNKP, induced by the PRMT5 KD, leads to the NMD of its mRNA.

In summary, PRMT5 depletion leads to changes in alternative splicing and protein downregulation of several essential genes in human AML cells. This likely contributes to the requirement of PRMT5 for cell survival.

Arginine methylation of SRSF1 is functionally important for cell survival. Next, we tested whether PRMT5-mediated arginine methylation of an identified substrate regulates its function. To do this, we generated arginine-to-lysine mutants (R-to-K) of the identified PRMT5-mediated methylation sites in the essential substrates and assessed their functionality by performing rescue experiments of the corresponding gene KD or knockout. For this, we first established stable cell lines overexpressing either the wild-type or mutant versions of the substrates and then delivered the sgRNAs to deplete the corresponding endogenous gene. As shown in Fig. 6a, the R-K mutant proteins of nine substrates (SNRPB, SFPQ, CCT4, SUPT5H, PNN, CPSF6, ALYREF, CCT7 and RPS10), demonstrated comparable performance to the wild-type version of the gene, suggesting that arginine methylation on those sites is not essential for their function and therefore cell survival (Fig. 6a).

Overexpression of SRSF1 on top of the endogenous levels caused cell death in four different human AML cell lines, demonstrating tight regulation of its levels. Thus, we could not use the same strategy for the rescue experiments as above. To address this challenge, we transduced the THP-1-cdCas9-KRAB-2A-mCherry cells with an SRSF1 wild-type or triple R-to-K mutant cDNA simultaneously with the SRSF1 KD sgRNA construct, also driving the blue fluorescent protein (BFP) expression. This approach provides an opportunity for some KD cells to establish wild-type SRSF1 levels and survive. Then, 14 d after transduction we sorted out the BFP and mCherry double positive (dCas9 and sgRNA-positive) cells from each condition and assessed the source of their SRSF1 expression by RT–qPCR. As expected, cells transduced with the wild-type SRSF1 transgene exhibited KD of the endogenous SRSF1 and relied on the exogenous SRSF1 expression for their growth, indicating complete rescue (Fig. 6b). At the same time, the surviving mutant- and

**Table 1 | A list of the validated essential PRMT5 substrates**

| Substrate name | Description |
|----------------|-------------|
| ALYREF         | Aly/REF export factor |
| SUPT5H*        | Transcription elongation factor |
| CPSF6          | Cleavage and polyadenylation specific factor 6 |
| SNRPB*         | Small nuclear ribonucleoprotein polypeptides B and B1 |
| SFPQ*          | Splicing factor proline and glutamine rich |
| PNN            | Pinin |
| SRSF1          | Serine and arginine-rich splicing factor 1 |
| WDR33          | Pre-mRNA 3′ end processing protein |
| RPS10*         | Ribosomal protein S10 |
| CCT4           | T-complex protein 1 subunit delta |
| CCT7           | T-complex protein 1 subunit eta |

*| published PRMT5 substrates.

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**Fig. 4 | PRMT5 depletion leads to changes in alternative splicing in human AML cells.**

- a. DEXSeq and limma-diffSplice algorithms show the differential usage of 415 RIs in the transcriptome of THP-1 cells following PRMT5 KD (more than two fold changes, P < 0.002, q < 0.05).
- b. Volcano plot demonstrating the differential usage of RIs in the transcriptome of THP-1 cells on PRMT5 KD (total 109,530 events). RIs were included in differential analysis as expressed exons, and 336 diffRIs are shown using dark orange squares. The vertical dashed lines represent two-fold differences between the PRMT5 KD and wild-type cells and horizontal dashed line shows the FDR-adjusted q value threshold of 0.05. These results were generated using DEXSeq algorithm, and very similar results were observed with limma-diffSplice approach.
- c. Volcano plot demonstrating the differential usage of EEJs in the transcriptome of THP-1 cells on PRMT5 KD (total 76,434 EEJs). The 430 DiffEEJs are shown by dark orange squares. The vertical dashed lines represent two-fold differences between the PRMT5 KD and wild-type cells, and horizontal dashed line shows the FDR-adjusted q value threshold of 0.05. These results were generated using limma-diffSplice algorithm.
- d. Most of the identified nondiffEEJs or diffEEJs are annotated in the Ensembl database (GRCh38.p7 assembly of human genome, release 85, July 2016), and only small fractions of EEJs are new junctions. The following numbers of EEJs were analyzed: nondiffEEJs, 76,004; diffEEJs, log(fold change (FC)) < -1.184; diffEEJs, log(FC) > 1.246).
- e. Classification of nondiffEEJs and diffEEJs according to main modes or types of alternative splicing. The identified EEJs were divided in three subsets (left to right): (1) nondiffEEJs, (2) diffEEJs with prevalence in the control cells (log(FC) < -1) and (3) diffEEJs with prevalence in the cells with PRMT5 KD (log(FC) > 1). This classification was carried out using Ensembl-based models of hypothetical nonalternative preRNAs of human genes. Numbers show the percentage of EEJs assigned to a particular mode of alternative splicing.
- f. Venn diagram demonstrating minimal overlap between the lists of differentially expressed genes (genes diffExpr), genes with diffRIs (genes diffRIs) and genes with diffEEJs (genes diffEEJs).
- g. Density diagrams of SRSF1 motif frequency at the 5′ and 3′ splice sites of the differential and nondifferential EEJs (dynamic thresholding). h. Median absolute numbers of SRSF1 motifs in differential and nondifferential EEJs (h) and RIs (i) (fixed thresholding, two-sided Mann–Whitney U-test). Boxplot summary (hi): outliers (diamonds), minimum (lower whisker), first quartile (lower bound of box), median (horizontal line inside box), third quartile (upper bound of box), interquartile range (box) and maximum (upper whisker). The following numbers of splicing events were compared: nondiffEEJs, 76,004; diffEEJs downregulated, 184; diffEEJs upregulated, 246; nondiffRIs, 1,540 and diffRIs, 336.
- j. Density diagrams of SFPQ motif frequency at the 5′ and 3′ splice sites of the differential and nondifferential EEJs (dynamic thresholding). k. Venn diagrams of the overlapping lists of the differentially used EEJs (k) and RIs (l) identified in THP-1 and U-87 MG cells on PRMT5 KD. The splicing analysis experiments were performed using three independently transduced samples of each sgRNA. Source data are available in Supplementary Table 2.
Stuffer-transduced BFP and mCherry double positive cells demonstrated no efficient KD of the endogenous SRSF1 (Fig. 6c), demonstrating that for these samples we could only recover the cells that escaped the effect of SRSF1 CRISPR interference. This shows that mutant SRSF1 cannot substitute for the wild type and confirms that arginine methylation of SRSF1 by PRMT5 is important for its function. Thus, we propose that PRMT5 loss leads to cell death, at least in part, by affecting the function of SRSF1.

PRMT5 depletion affects the binding of SRSF1 to mRNAs and proteins. The same three amino acids in SRSF1 (R93, R97 and R109) were previously shown to be important for its proper cellular localization and activity. However, SRSF1 localization was affected only on mutating these sites to alanines and not lysines, suggesting the importance of positive charge rather than methylation. To analyze this in THP-1 cells, we performed nuclear-cytoplasm fractionation with and without PRMT5 and compared the amounts of...
SRSF1 in different fractions. As a result, we did not observe a change in SRSF1 localization after PRMT5 KD (Extended Data Fig. 6a,b). We also performed experiments in HeLa cells, similar to the ones presented in Sinha et al., and found that when triple-flag-tagged wild-type SRSF1, triple R-to-K or triple R-to-A SRSF1 mutants were mildly overexpressed, all three demonstrate predominantly nuclear localization (Extended Data Fig. 6c). Some localization changes of the mutants could be observed only on increased overexpression (Extended Data Fig. 6d). Since mutant SRSF1 relocalization occurs only at high overexpression levels and mostly for the triple R-to-A mutant, it is unlikely that the primary effect of SRSF1 methylation is to retain it in the nucleus.

To further investigate the effect of PRMT5 on SRSF1 function we analyzed changes in SRSF1-bound mRNAs and proteins on PRMT5 depletion. First, we performed SRSF1 RNA immunoprecipitation (RIP) in cells transduced with either a negative control sgRNA or sgRNAs against PRMT5 or SRSF1. Efficient immunoprecipitation was verified by western blotting (Extended Data Fig. 7a). SRSF1 KD samples did not yield detectable RNA amounts after RIP, in contrast to negative control and PRMT5 KD samples, indicating highly specific immunoprecipitation (Extended Data Fig. 7b). Next-generation sequencing of the pulled down RNA reliably quantified mRNAs of 13,754 genes, of which 4,459 were significantly differentially bound between the negative control and PRMT5 KD conditions (q < 0.05) (Fig. 7a and Supplementary Table 3). There was a clear and significant enrichment of the differentially spliced genes among the genes that mRNAs were differentially bound by SRSF1 on PRMT5 KD (chi-square P = 2.2 x 10^-11) (Fig. 7b,c), suggesting that alternative splicing of these is caused by altered interaction with SRSF1. As an example, five out of seven essential alternatively spliced genes described above (FDPS, POLD1, PNISR, PNKP, POLD2) were differentially bound by SRSF1, with four out five losing SRSF1 binding (Fig. 7a).

Since arginine methylation is known to regulate protein–protein interactions, we performed SRSF1 immunoprecipitation–mass-spectrometry (IP–MS) interactomics in cells with and without PRMT5. This approach identified 350 specific SRSF1 interactors (significantly enriched over an IgG control, q < 0.05). The identified interactors were enriched in proteins involved in regulating mRNA splicing, RNA transport, transcription and translation (Supplementary Table 4), consistent with previous reports on SRSF1 involvement in these processes 

Of the 350 binding partners, 162 were differentially bound to SRSF1 between the two conditions (at least a two-fold change, q < 0.05) (Fig. 7d). Most of the SRSF1 interactors showed decreased abundance in the PRMT5 KD affinity purification, suggesting that arginine methylation promotes the interaction of SRSF1 with other proteins. The decreased interactors were enriched for proteins involved in mRNA splicing, secondary structure unwinding and transport, while increased SRSF1 binding was observed for multiple ribosomal proteins (Tables 2 and 3). Such differential binding pattern indicates redistribution of SRSF1 between various cellular processes on PRMT5 KD and likely contributes to differential splicing and cell death phenotype on PRMT5 depletion (Fig. 7e).

The SRSF1 IP–MS also allowed more extensive arginine methylation analysis of the SRSF1 protein. Arginine methylation was detected on the same sites as in the global arginine methylation profiling approach (R93, R97 and R109), and all the nine identified peptides covering these sites demonstrated decreased abundance on PRMT5 KD (Extended Data Fig. 7c). While both mono- and dimethylation were detected for positions R93 and R109, only dimethylated R97 was identified.

In summary, we found that PRMT5 depletion leads to decreased arginine methylation of SRSF1 and extensive changes in its binding to mRNA and proteins.

Discussion

Here, we demonstrated the requirement of the catalytic activity of PRMT5 for the growth of the human AML cells bearing the MLL–AF9 rearrangement. This is consistent with the previous observations in a mouse model of this leukemia type, where Prmt5 knockout or chemical inhibition decreased leukemia burden and prolonged animal survival. Together, these indicated PRMT5 inhibition as a potential therapeutic approach in AML, and it would be interesting to investigate whether AML patients with mutations in spliceosome proteins are particularly sensitive to PRMT5 inhibition.

In this study, we performed large-scale identification of PRMT5 substrates. In a recent resource paper, which was published while this manuscript was prepared for submission, a number of PRMT5 substrates were identified in HeLa cells. This study employed a stable isotope labeling with amino acids in cell culture (SILAC) method (MS1-based quantitation) with two biological replicates. Many of the substrates identified by Musiani et al. were also identified and confirmed by in vitro methylation in our report (for example, WDR33, SNRPB and others). While Musiani et al. also identified SRSF1 methylated peptides, SRSF1 was not confirmed as a substrate,
since the peptides were only identified in one of the replicates, which prohibited statistical analysis. This reflects a limitation of SILAC quantitation in comparison with the TMT approach, which is more sensitive and allows for more robust statistical treatment.

We specifically focused on the identified PRMT5 substrates, which are essential for the proliferation of AML cells, since those are most likely to be downstream of PRMT5 in conferring cell survival. Our validation and rescue experiments confirmed SRSF1 as a direct PRMT5 target and demonstrated the importance of methylation for its function.

SRSF1 belongs to the family of serine/arginine-rich splicing factors. It is known to shuttle between the cytoplasm and the nuclear compartment and has a unique role in the regulation of alternative splicing. The expression of SRSF1 is altered in a variety of cancers, including AML, suggesting a potential therapeutic target.

Our results with the KD THP-1 cells confirm the role of PRMT5 in the regulation of SRSF1 expression. This further supports the idea that PRMT5-mediated methylation is involved in the regulation of SRSF1, which in turn affects the splicing pattern of genes involved in the proliferation and survival of AML cells.
Arginine methylation of SRSF1 is functionally important for cell survival. a, Competition assays to assess the functionality of the R-to-K mutants of the essential PRMT5 substrates. Cell lines stably expressing either the wild-type or mutant versions of each substrate were transduced with an sgRNA against the substrate of interest. After the transduction, the percentage of BFP-positive (sgRNA-expressing) cells was monitored over time. b, RT-qPCR analysis of the endogenous (3′ untranslated region (UTR)) and exogenous SRSF1 expression in BFP and mCherry double positive cells co-transduced with an sgRNA targeting SRSF1 and either SRSF1 wild-type or mutant cDNA or a stuffer construct. The values are normalized to RPLP0 and shown as mean ± s.d. (n = 2 technical replicates, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not significant according to Sidak’s multiple comparisons test). The experiments in a and b were repeated twice with similar results. The source data are available online.

Fig. 6 | Arginine methylation of SRSF1 is functionally important for cell survival. a, Competition assays to assess the functionality of the R-to-K mutants of the essential PRMT5 substrates. Cell lines stably expressing either the wild-type or mutant versions of each substrate were transduced with an sgRNA against the substrate of interest. After the transduction, the percentage of BFP-positive (sgRNA-expressing) cells was monitored over time. b, RT-qPCR analysis of the endogenous (3′ untranslated region (UTR)) and exogenous SRSF1 expression in BFP and mCherry double positive cells co-transduced with an sgRNA targeting SRSF1 and either SRSF1 wild-type or mutant cDNA or a stuffer construct. The values are normalized to RPLP0 and shown as mean ± s.d. (n = 2 technical replicates, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not significant according to Sidak’s multiple comparisons test). The experiments in a and b were repeated twice with similar results. The source data are available online.

nucleus45, where it binds to exonic splicing enhancers and stimulates splicing43–45. In addition, SRSF1 is involved in other processes, including NMD, translation and mRNA transport36–38. SRSF1 is overexpressed in multiple cancers and has known oncogenic properties46. Posttranslational modifications play an important role in the function of SRSF1. Particularly, phosphorylation of SRSF1 in the arginine/serine-rich (RS) domain is required for its trans -

nucleus42, where it binds to exonic splicing enhancers and stimulates splicing43–45. In addition, SRSF1 is involved in other processes, including NMD, translation and mRNA transport36–38. SRSF1 is overexpressed in multiple cancers and has known oncogenic properties46. Posttranslational modifications play an important role in the function of SRSF1. Particularly, phosphorylation of SRSF1 in the arginine/serine-rich (RS) domain is required for its trans -

SRSF1 localization when the methylated arginines were changed to lysines or alanines or when PRMT5 was depleted. Instead, we found that PRMT5 KD induces disruption of the SRSF1 interaction network and extensive changes in the repertoire of mRNAs bound to it. Since all three PRMT5-methylated residues are located in the glycine-rich hinge connecting the two RNA recognition motifs of SRSF1, it is possible that loss of methylation in this region largely affects the protein structure. Taken together, our results suggest that SRSF1 is a key substrate for PRMT5, which can explain why PRMT5 is essential for leukemia cells and also, potentially, other types of cancer.
We did not observe extensive changes in total mRNA levels for the majority of the genes differentially spliced on PRMT5 depletion. However, decreased protein expression was observed for some of them. Alternative splicing in such cases could lead to mRNA retention in the nucleus, also known as intron detention\(^4\)\(^9\),\(^5\) inefficient translation of the alternatively spliced mRNAs\(^5\)\(^0\),\(^5\)\(^1\) or instability of the resulting protein isoforms. This agrees with the report by Braun et al. that demonstrated increased intron detection in PRMT5 inhibitor-treated glioma cells\(^2\)\(^1\). We found further similarities between the alternative splicing events induced by PRMT5 loss in human AML and glioma cells, suggesting common mechanisms of PRMT5 function. We found several essential genes that exhibit changes in splicing and a concomitant downregulation on protein level. These most likely explain the cell death phenotype on PRMT5 downregulation. It could be interesting to test whether chemical targeting of these essential proteins could synergize with PRMT5 inhibition in impeding cancer cell proliferation.

For the majority of the identified essential substrates R-to-K mutants were functional in our rescue assays. However, we found that PRMT5 often methylates several proteins in the same pathway or complex; for example, SNRPB and SNRPN, CCT7 and CCT4, PABPC4 and PABPN1. This could suggest functional redundancy and requirement to mutate to all of them to see an effect. Therefore,
strong proliferation defect we observe on PRMT5 depletion likely represents a combined phenotype from affecting multiple cellular processes. Despite the complexity of the PRMT5 functions, the comprehensive list of substrates we report could be used to identify additional pathways to target in cancer. One of the current clinical trials with the PRMT5 inhibitor (NCT03573310) is using SDMA plasma levels as a readout of treatment efficacy. It would be interesting to investigate if the validated essential substrates could be used as more specific biomarkers for PRMT5 activity and treatment response.

In summary, this study provides a comprehensive resource of PRMT5 substrates in human acute myeloid leukemia (AML) and directly links the alterations in splicing patterns and cell death on PRMT5 depletion to the arginine methylation of the splicing factor SRSF1.

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0313-z.

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### References

1. Larsen, S. C. et al. Proteome-wide analysis of arginine monomethylation reveals widespread occurrence in human cells. *Sci. Signal.* 9, rs9 (2016).
2. Blanc, R. S. & Richard, S. Arginine methylation: the coming of age. *Mol. Cell* 65, 8–24 (2017).
3. Gayatri, S. & Bedford, M. T. Readers of histone methylarginine marks. *Biochim. Biophys. Acta* 1839, 702–710 (2014).
4. Li, X., Wang, C., Jiang, H. & Luo, C. A patent review of arginine methyltransferase inhibitors (2010–2018). *Expert Opin. Ther. Pat.* 29, 97–114 (2019).
5. Stopa, N., Krebs, J. E. & Shcheter, D. The PRMT5 arginine methyltransferase: many roles in development, cancer and beyond. *Cell. Mol. Life Sci.* 72, 2041–2059 (2015).
6. Shalesh, H., Zakaria, H. A. & Mullikin, J. C. Detection and visualization of differential splicing in RNA-Seq data with JunctionSeq. *Nucleic Acids Res.* 44, e127 (2016).
7. Burgos, E. S. et al. Histone H2A and H4 N-terminal tails are positioned by the PRMT5/WDR77 complex regulates alternative splicing through ZNF326 in breast cancer. *Nucleic Acids Res.* 45, 11106–11120 (2017).
8. Koh, C. M. et al. MYC regulates the core pre-mRNA splicing machinery as an essential step in lymphomagenesis. *Nature* 523, 96–100 (2015).
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Author contributions
A.R. and K.H. came up with the concept. A.R., P.V.S. and V. Grinev designed the methodology. A.R., P.V.S., V. Grinev, E.L., S.K., D.S. and V. Gorshkov carried out the investigation. The original draft was written by A.R. and K.H. Review and editing of the manuscript was done by all authors. The visualization was done by A.R., P.V.S. and V. Grinev. A.R., P.V.S. O.N.J. and K.H. acquired the funding. R.C.H., O.N.J and K.H. supervised the study.

Competing interests
The authors declare no competing interests

Additional information
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**Methods**

Plasmids, sgRNA cloning and site-directed mutagenesis. phR-SFFV-KRAB-dCas9-2A-CHERY, pLentiCas9-2A-blast, pU6-sgRNA-EF1×4-puro-T2A-BFP, pLenti PGK Hgpt2 and pLEX-307 were purchased from Addgene (catalog nos. 60954, 60955, 59682, 41392 and 19066), PB-CAG-hph-dest was a gift from J. Silva. For sgRNA cloning, oligos were annealed in annealing buffer (200 mM potassium acetate, 60 mM HEKES-KOH pH 7.4, 4 mM magnesium acetate) and ligated into BstXI and BlpI digested pU6-sgRNA-EF1×4-puro-T2A-BFP. Site-directed mutagenesis was performed using QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) or Q5 Site-Directed Mutagenesis Kit (NEB).

**sgRNA design.** CRISPRko sgRNAs were designed using the sgRNA Designer: CRISPRko—Broad Institute (https://portals.broadinstitute.org/gpp/public-analysis-tools/sgrna-design). CRISPRi sgRNAs were designed as described previously52. Supplementary Table 5 contains sequences of all the sgRNAs used in the study.

**Cell lines and culture.** THP-1 were obtained from ATCC, MOLL-13 and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycoplasma negative, no cell authentication was performed. Human embryonic kidney (HEK)293FT packaging and MONOMAC-6 from DSMZ. HeLa cells were a kind gift from the X. Jiang laboratory. All the cells used in the study were mycopyla...
In vitro methyltransferase assay. The assay was performed in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, 5 mM DTT and 0.2 mM S-adenosylmethionine. Each reaction was performed in 25 µl for 12 h at 37 °C with 300 ng of the corresponding peptide and 0.2 µl of active human recombinant PRMT5–MEP50 complex (Sigma, SRP0145) or water (negative control). The product of the reaction was desalted on a stageTip with 100 µl of 0.1% TFA and eluted in 30 µl of 50% ACN 0.1% FA. Then, 2 µl of the desalted product was directly infused into LTQ Orbitrap XL using nanoAcquity ultra-performance liquid chromatography (Waters) and analyzed at 100,000 resolution. The data were deconvoluted with Xtract with signal-to-noise threshold of ten, spectra extracted from the .xml outputs and plotted with the ggplot2 R package.

Defining PRMT5 methylation motif. PRMT5 methylation motif was predicted using the iceLogo online tool (https://iomics.ugent.be/icelogoserver/). Next, 20 amino acid sequences with the arginine modification site in the center were compared to the Swiss-Prot human proteome reference set.

SRSF1 RNA immunoprecipitation and sequencing. The cells were transduced with either a negative control sgRNA or sgRNAs inducing KD of PRMT5 or SRSF1. For each condition three biological replicates of 20 million cells were collected 6 d after transduction and subjected to RNA immunoprecipitation protocol using Magna RIP Kit (Millipore) according to the manufacturer's instructions. The immunoprecipitation was performed with 5 µg of anti-SRSF1 antibody 96 (Santa Cruz). The obtained RNA was used for the preparation of sequencing libraries using the TruSeq RNA Library Prep Kit v2, starting at the Elute, Prime, Fragment step. The details of the RIP-sequencing data analysis can be found in the Supplementary Information.

SRSF1 interactomics analysis. The cells were lysed in lysis buffer (50 mM EPPS, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors), briefly sonicated, cleared from cell debris, pre-cleared with protein A Sepharose beads and immunoprecipitated with anti-SRSF1 antibody 96 (Santa Cruz) and protein A Sepharose beads. After immunoprecipitation, the beads were washed five times in wash buffer (50 mM EPPS, pH 7.4, 150 mM NaCl) and subjected to trypsin digestion overnight at 37 °C (20 mM EPPS pH 8.5, 5 mM TCEP, 20 mM chloroacetamide, 10 ng µl⁻¹ LysC and 20 ng µl⁻¹ Trypsin). The digest was labeled with 20 g l⁻¹ TMT tags, as recommended by the manufacturer, and half of the material was fractionated by Pierce High pH Reversed-Phase Peptide Fractionation Kit concatenating two fractions into a superfraction (for example, 1 and 5). After desalting the samples were evaporated using vacuum centrifuge, resuspended in 0.1% TFA and analyzed by LC–MS/MS.

Emetine treatment. The cells were transduced with either a negative control or a PRMT5 sgRNA and cultured for 6 d. After that, either water or emetine (at a final concentration of 100 µg ml⁻¹) were added for 3 h. The cells were harvested and analyzed by RT–qPCR.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Next-generation sequencing has been submitted to the Gene Expression Omnibus (accession number GSE129652). Proteomics data has been submitted to ProteomeXchange (accession number PXD013611). Source data for all the main Figures and Extended Data Figs. 1, 2, 4, 6, 7 are available with the paper online either as Source Data or in Supplementary Tables. All other data will be made available on request.

Code availability
GitHub project with the RNA-sequencing analysis code is available at: https://github.com/VGrinev/transcriptome-analysis/blob/master/TranscriptsFeatures. Any additional code will be provided upon request from the authors.

References
52. Radzisheuskaya, A., Shlyueva, D., Müller, I. & Helin, K. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucleic Acids Res. 44, e141 (2016).
Extended Data Fig. 1 | see figure caption on next page.
Extended Data Fig. 1 | PRMT5 and WDR77 are required for the survival of mouse and human AML cells. a, Overview of the CRISPR interference and knockout approaches. b, RT-qPCR analysis of PRMT5 expression in THP-1-cdCas9-KRAB cells transduced with a non-targeting (NegCtrl) sgRNA or two sgRNAs targeting PRMT5 (3 and 6 days post-transduction). The values are normalized to RPLP0 and shown as mean ± SD (n = 3, **** is p-value < 0.0001 using Sidak’s multiple comparisons test). c-d, Western blot analysis of PRMT5 and GAPDH (c) and symmetrical arginine dimethylation (SDMA) (d) levels in THP-1-cdCas9-KRAB cells transduced with a non-targeting sgRNA or two sgRNAs targeting PRMT5 (3 and 6 days post-transduction). Bar charts show quantification of protein levels relative to a loading control. e, Growth curves of THP-1-cdCas9-KRAB cells transduced with a non-targeting sgRNA or two sgRNAs targeting PRMT5 (3 and 6 days post-transduction). The experiments were repeated at least twice with similar results. The uncropped western blots are presented in the Source Data.
Extended Data Fig. 2 | Chemical inhibition of PRMT5 leads to growth defects in AML cells.

a. Western blot analysis of symmetrical arginine dimethylation (SDMA) levels and Vinculin in THP-1 cells treated with DMSO or different doses of PRMT5 inhibitor EPZ015666 at 6 days after the addition of a compound. Bar chart shows quantification of protein levels relative to a loading control.

b. Growth curves of THP-1-cdCas9-KRAB-stuffer cells treated with DMSO or different doses of PRMT5 inhibitor EPZ015666. X-axis indicates number of days after addition of the compound.

c. Growth curves of THP-1-cdCas9-KRAB-wtPRMT5 cells treated with DMSO or different doses of PRMT5 inhibitor EPZ015666. X-axis indicates number of days after addition of the compound. The experiments were repeated twice with similar results. The uncropped western blots are available in the Source Data.
Extended Data Fig. 3 | see figure caption on next page.
Extended Data Fig. 3 | Validation of the essential PRMT5 substrates. a, 17 out of 62 potential PRMT5 substrates were chosen as potentially essential according to a previously published CRISPRko screen in THP-1 cells. Y axis represents log2FC of the relative abundance of sgRNA in the screen and -1.5 was chosen as a cut-off. b, Distributions of relative abundances of unmethylated and methylated peptide forms after the incubation with or without recombinant PRMT5-WDR77 complex. Only the peptides belonging to the unconfirmed PRMT5 substrates are shown here. c, RT-qPCR analysis of CCT4, CC7, PNN, SFPO, SNRPB, SRSF1, SUPT5H, TAF15, CPSF6 and RPS10 expression demonstrates efficient knockdown of the genes upon CRISPRi sgRNA transduction (n = 3, * is p-value < 0.033, *** is p-value < 0.001, **** is p-value < 0.0001 according to the unpaired t test). The experiments were repeated twice with similar results.
Extended Data Fig. 4 | see figure caption on next page.
Extended Data Fig. 4 | Knockdown of PRMT5 leads to differential splicing in the transcriptome of THP-1 AML cells. a, Two independent algorithms (DESeq2 and edgeR-limma) identified 2974 RIs in the transcriptome of THP-1 cells. b, In total 2923 of 45450 Cufflinks-assembled transcripts of the THP-1 cells contain DESeq2- or edgeR-limma-detected RIs. Of these, 2668 transcripts are common between the two algorithms. c, Density plot of the transcript abundance demonstrating that the transcripts with RIs (+ RIs) are highly expressed in the transcriptome of THP-1 cells comparing to RI-free (−RIs) ones. d, The knockdown of PRMT5 leads to differential usage of a subset of EEJs in the transcriptome of THP-1 cells. The differentially used EEJs were determined using two independent algorithms (limma-diffsplice and JunctionSeq) with moderate overlap between the results. e-g, SRSF1 (e), SRSF2 (f) and SRSF3 (g) motifs are significantly enriched both at the 5′ and 3′ splice sites of the differential EEJs (dynamic thresholding). h, SFPQ motif is not significantly enriched at the 5′ or 3′ splice sites of the differential EEJs (dynamic thresholding). i-j, Density diagrams of SRSF1 motif frequency at the 5′ and 3′ splice sites of the differential and non-differential EEJs in U-87 MG cells. Stars indicate statistically significant differences (p < 0.01) (dynamic thresholding). k-l, Median absolute numbers of SRSF1 motifs in differential and non-differential splicing events in U-87 MG cells (fixed thresholding). Boxplot summary (e–h, k, l): outliers (diamonds), minimum (lower whisker), first quartile (lower bound of box), median (horizontal line inside box), third quartile (upper bound of box), interquartile range (box), and maximum (upper whisker).
Extended Data Fig. 5 | SRSF1 motif number is increased around the differential splicing sites of the selected essential candidate genes. a, Median absolute numbers of SRSF1 motifs near all the splicing sites that do not change upon PRMT5 depletion and near the splicing sites that change upon PRMT5 KD in the selected essential candidate genes (FDPS, PDCD2, PNISR, PNKP, POLD1, POLD2, PPIR7) (fixed thresholding). Boxplot summary: outliers (diamonds), minimum (lower whisker), first quartile (lower bound of box), median (horizontal line inside box), third quartile (upper bound of box), interquartile range (box), and maximum (upper whisker). b, Table summary of the identified SRSF1 binding sites in all the splicing events that change upon PRMT5 KD in the FDPS, PDCD2, PNISR, PNKP, POLD1, POLD2, PPIR7 genes.
Extended Data Fig. 6 | see figure caption on next page.
Extended Data Fig. 6 | PRMT5 depletion doesn’t demonstrate detectable effects on SRSF1 cellular localization. a, Western blot validation of SRSF1 antibody. Significant decrease in the signal observed after the SRSF1 knockdown, demonstrating antibody specificity. Bar chart shows quantification of protein levels relative to a loading control. b, Western blotting for SRSF1, Lamin B1 and GAPDH after cell transduction with either a negative control or a PRMT5 sgRNA and subsequent nuclear-cytoplasm fractionation. Lamin B1 and GAPDH were used as controls for successful fractionation into nuclear and cytoplasmic (cyto) fractions, respectively. Bar chart shows quantification of protein levels. c, Representative immunofluorescence images of HeLa cells transiently transfected with either triple-FLAG-tagged wild type, triple R-to-K or triple R-to-A mutant SRSF1 cDNAs driven by the CAG promoter. Scale bar = 10 μm. d, Representative immunofluorescence images of HeLa cells transiently transfected with either triple-FLAG-tagged wild type, triple R-to-K or triple R-to-A mutant SRSF1 cDNAs driven by the EF1a promoter. Scale bar = 10 μm. The experiments in the figure were repeated at least twice with similar results. The uncropped western blots are available in the Source Data.
Extended Data Fig. 7 | PRMT5 depletion affects the binding of SRSF1 to mRNA and proteins. **a**, Western blotting for SRSF1 and PRMT5 in the input and immunoprecipitation samples (either SRSF1 or IgG). Bar chart shows quantification of protein levels. **b**, RNA yield after RNA-immunoprecipitation and purification in three biological replicates of each sample. **c**, Heatmap of the methylated peptides identified for SRSF1 in the negative control and PRMT5 KD SRSF1 IP-MS samples. “aa” stands for amino acid. Each IP was performed in three biological replicates. The uncropped western blots are available in the Source Data.
Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

RNA-sequencing and RIP-sequencing data were collected using NextSeq System Suite. Proteomics data was collected using Orbitrap Fusion Lumos platform.

Data analysis

GitHub project with the RNA-sequencing analysis code is available here: https://github.com/VGrinev/transcriptome-analysis/blob/master/TranscriptsFeatures. Proteomics data was analyzed by Proteome Discoverer 2.3 with Mascot 2.3.2 and DabeR package.

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Next-generation sequencing data have been submitted to GEO (GSE129652). Proteomics data have been submitted to ProteomeXchange (PXD013611).

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Sample size  Preliminary experiments demonstrated tight correlation of the degree of PRMT5 knockdown using the sgRNAs in the study. Thus, we chose three biological replicates as a sample size and performed necessary statistics on those.

Data exclusions  No data exclusion was performed in the study

Replication  We performed each experiment at least two times to ensure data reproducibility. The exact number of times each experiment was performed is stated in the corresponding figure legend. For RNA-sequencing and RIP-sequencing experiments three biological replicates were used, for proteomics experiments 3-4 biological replicates were used.

Randomization  No randomization was performed in the study

Blinding  No blinding was performed in the study

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### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|☐ | Antibodies |
|☒ | Eukaryotic cell lines |
|☐ | Palaeontology |
|☐ | Animals and other organisms |
|☐ | Human research participants |
|☐ | Clinical data |

### Antibodies

**Antibodies used**

PRMT5 (Abcam, ab109451), Vinculin (Sigma, SA94200080), SDMA (Cell Signalling, 13222), GAPDH (Abcam, ab181602), SRSF1 (Abcam, ab538017), SRSF1 (Santa Cruz, sc-33652), Lamin B1 (Abcam, ab16048), b-Actin (Abcam, ab6276).

**Validation**

PRMT5 and SRSF1 antibodies were validated by the knockdown of the gene of interest. SDMA antibody was validated by the knockdown of the main enzyme for the mark. Validation of Vinculin, GAPDH, Lamin B1 and b-Actin antibodies for western blotting can be found on the manufacturer’s website using the provided catalog number.

### Eukaryotic cell lines

**Policy information about** cell lines

**Cell line source(s)**

THP-1 from ATCC, MOLM-13 and MONOMAC6 from DSMZ, HeLa cells were a kind gift from the Xuejun Jiang Lab.

**Authentication**

No cell authentication was performed

**Mycoplasma contamination**

All the cells used in the study were mycoplasma negative

**Commonly misidentified lines (See IGAC register)**

No commonly misidentified cell lines were used in the study

### Palaeontology

**Specimen provenance**

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Indicate where the specimens have been deposited to permit free access by other researchers.

**Dating methods**

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement).
Dating methods

where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

### Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals. |
|--------------------|------------------------------------------------------------------------------------------------------------------|
| Wild animals       | Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals. |
| Field-collected samples | For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field. |
| Ethics oversight   | Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Human research participants

Policy information about studies involving human research participants

| Population characteristics | Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you outlined the behavioural & social sciences study design questions and have nothing to add here, write "See above." |
| Recruitment              | Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results. |
| Ethics oversight         | Identify the organization(s) that approved the study protocol. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

| Clinical trial registration | Provide the trial registration number from ClinicalTrials.gov or an equivalent agency. |
| Study protocol             | Note where the full trial protocol can be accessed OR if not available, explain why. |
| Data collection            | Describe the settings and locales of data collection, noting the time periods of recruitment and data collection. |
| Outcomes                   | Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures. |

### ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

| Data access links | For "initial submission" or "revised version" documents, provide reviewer access links. For your "final submission" document, provide a link to the deposited data. |
|-------------------|-------------------------------------------------------------------------------------------------|
| Files in database submission | Provide a list of all files available in the database submission. |
| Genome browser session (e.g. UCSC) | Provide a link to an anonymized genome browser session for "initial submission" and "revised version" documents only, to enable peer review. Write "no longer applicable" for "final submission" documents. |

Methodology

| Replicates | Describe the experimental replicates, specifying number, type and replicate agreement. |
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

- Sample preparation: No special sample preparation was necessary
- Instrument: BD LSRII, CytoFLEX
- Software: BD FACSDIVA, CytExpert, FlowJo
- Cell population abundance: The starting abundance of the populations of interest was 50-70%
- Gating strategy: Relevant gating strategy is presented in the Supplementary Information

Magnetic resonance imaging

Experimental design

- Design type: Indicate task or resting state; event-related or block design.
- Design specifications: Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.
- Behavioral performance measures: State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

- Imaging type(s): Specify: functional, structural, diffusion, perfusion.
- Field strength: Specify in Tesla
- Sequence & imaging parameters: Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.
- Area of acquisition: State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.
- Diffusion MRI: Used
### Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
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