

Research Data Management

Increasing Openness and Collaboration



Instructors



Julie Goldman

Research Data Services Librarian
Countway Library of Medicine
Julie_Goldman@hms.harvard.edu



Meghan Kerr

Archivist and Records Manager
Center for the History of Medicine
Meghan_Kerr@hms.harvard.edu



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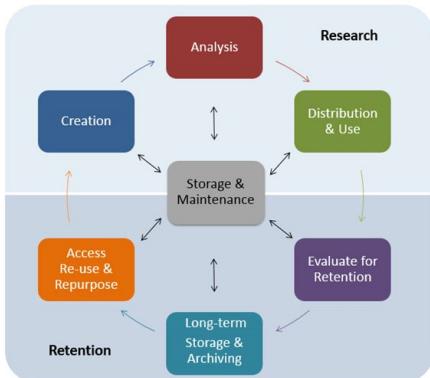
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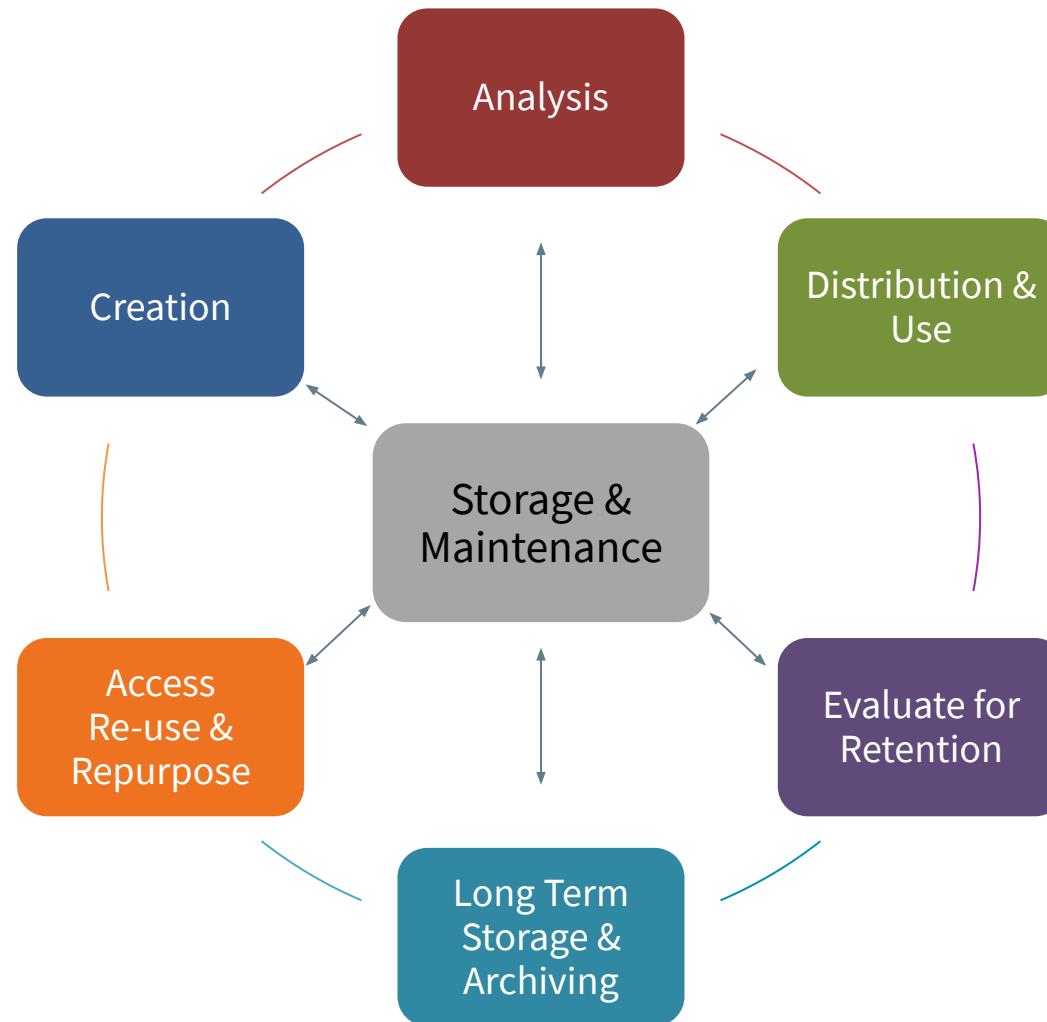
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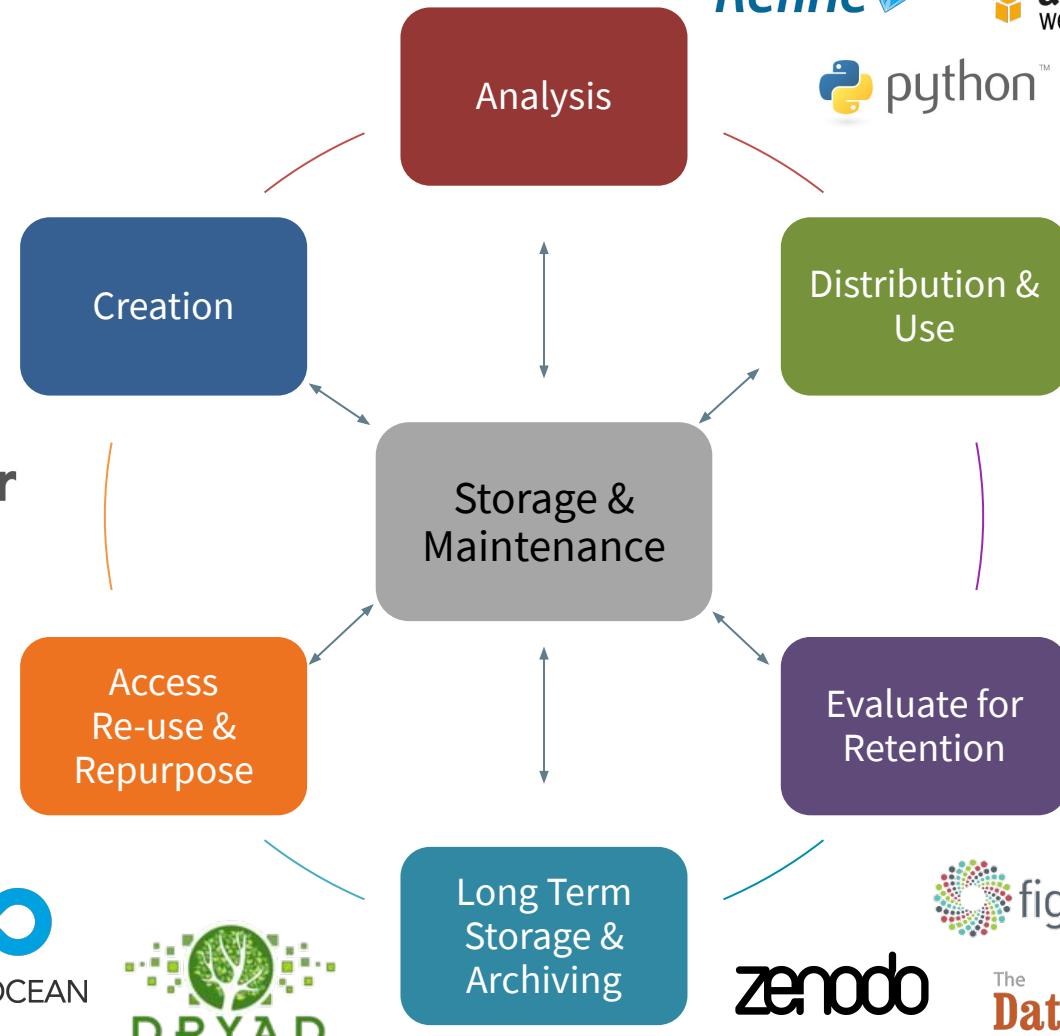


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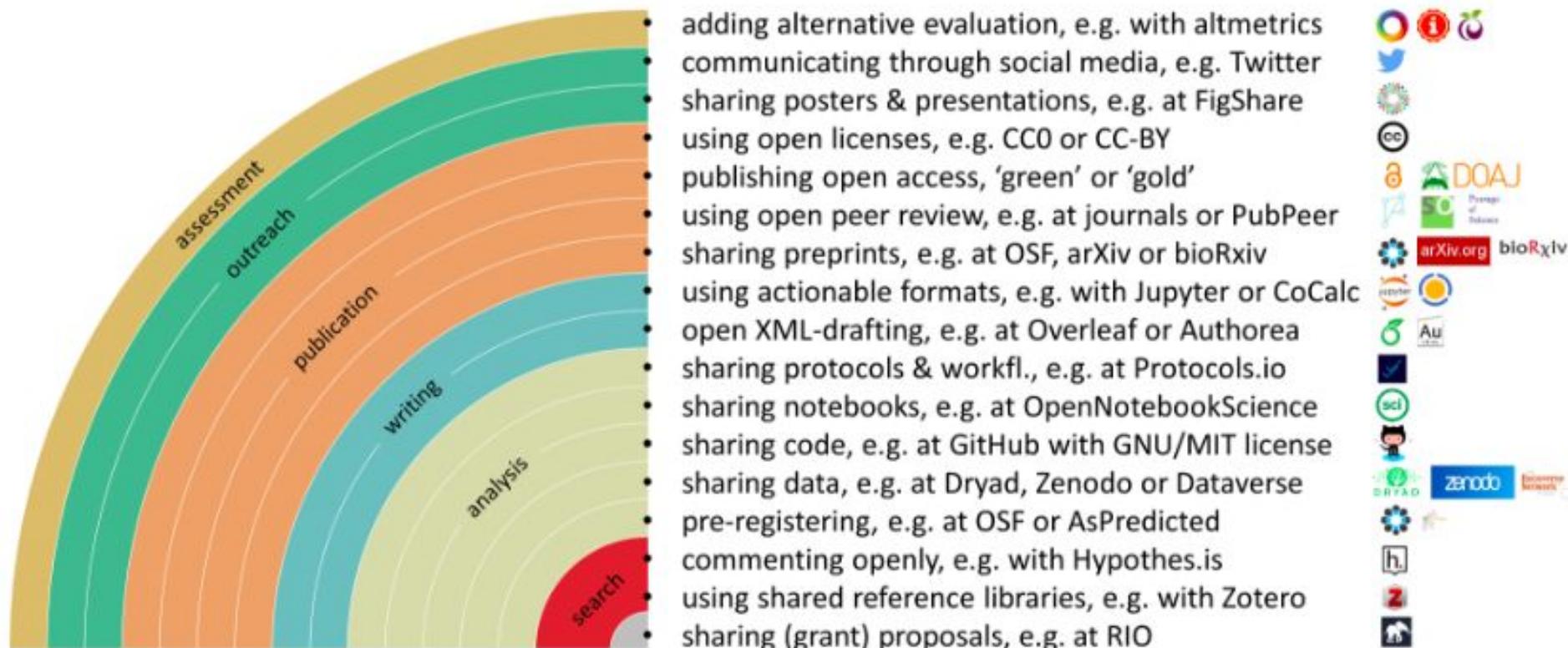
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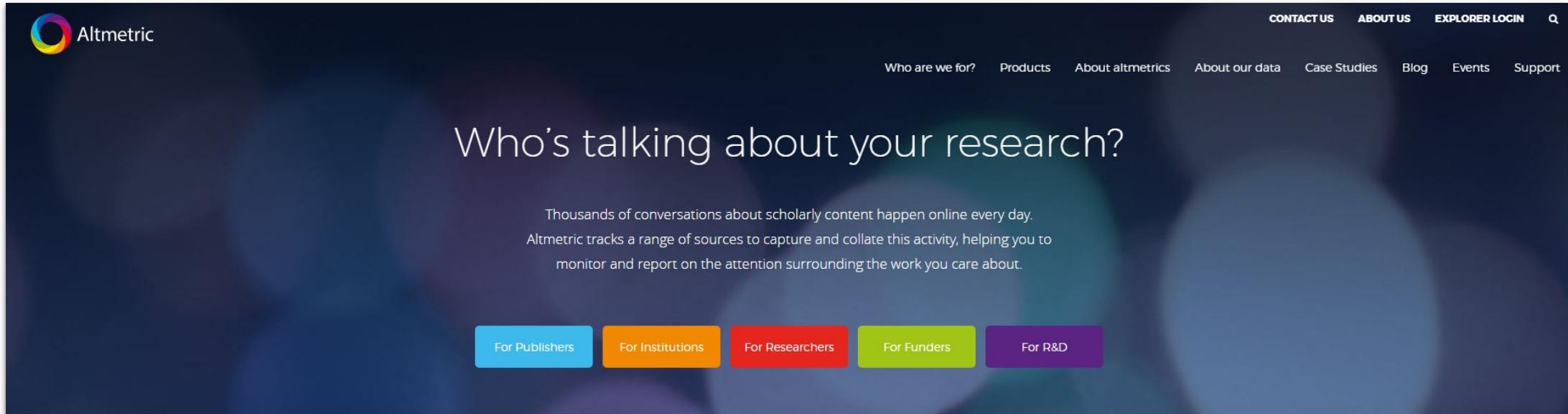
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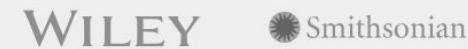
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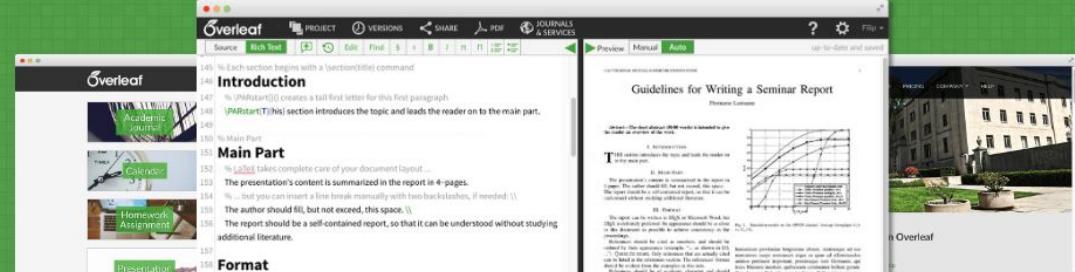
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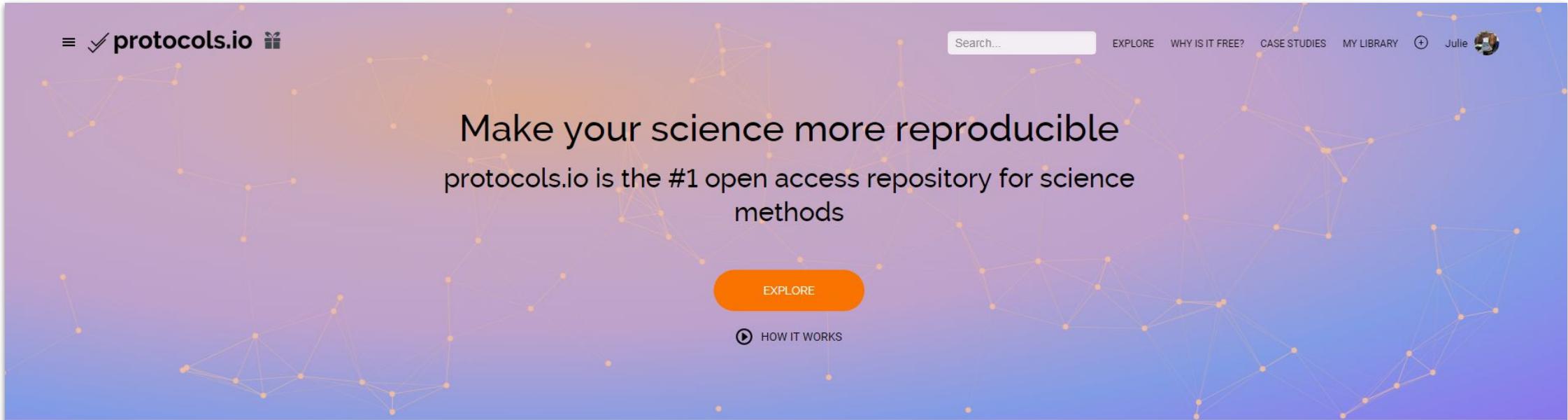
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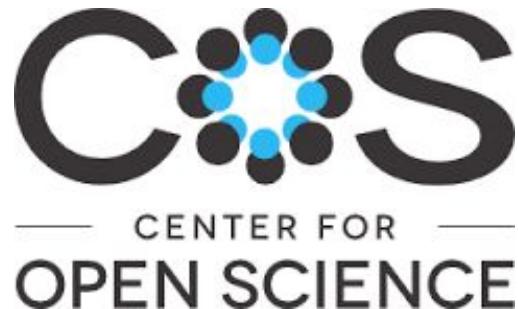
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GRIL-Seq, a method for identifying direct targets of bacterial small regulatory RNA by *in vivo* proximity ligation

Kook Han,¹ Brian Tjaden,² and Stephen Lory^{1,*}

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The first step in the post-transcriptional regulatory function of most bacterial small non-coding RNAs (sRNAs) is base-pairing with partially complementary sequences of targeted

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Bacterial small RNA regulators: versatile roles and rapidly

GRIL-seq provides a method for identifying direct targets of bacterial small regulatory RNA by *in vivo* proximity ligation

Kook Han¹, Brian Tjaden² and Stephen Lory^{1*}

The first step in the post-transcriptional regulatory function of most bacterial small non-coding RNAs (sRNAs) is base pairing with partially complementary sequences of targeted transcripts. We present a simple method for identifying sRNA targets *in vivo* and defining processing sites of the regulated transcripts. The technique, referred to as global small non-coding RNA target identification by ligation and sequencing (GRIL-seq), is based on preferential ligation of sRNAs to the ends of base-paired targets in bacteria co-expressing T4 RNA ligase, followed by sequencing to identify the chimaeras. In addition to the RNA chaperone Hfq, the GRIL-seq method depends on the activity of the pyrophosphorylase RppH. Using Prrf1, an iron-regulated sRNA in *Pseudomonas aeruginosa*, we demonstrated that direct regulatory targets of this sRNA can readily be identified. Therefore, GRIL-seq represents a powerful tool not only for identifying direct targets of sRNAs in a variety of environments, but also for uncovering novel roles for sRNAs and their targets in complex regulatory networks.

Post-transcriptional regulation mechanisms, primarily through the activities of regulatory small RNAs (sRNAs), play an important role in the bacterial stress response, metabolism, quorum sensing and virulence^{1–6}. The sharp increase in the description of this abundant class of RNA regulators during the past decade is the direct result of transcriptome analyses using next-generation RNA sequencing (RNA-seq) and the development of *in silico* methods to identify non-protein-coding transcripts⁷. In bacteria, regulation of gene expression by sRNAs can be divided into two mechanistically distinct categories. One class of sRNAs functions by modifying the activities of regulatory proteins⁸. Other sRNAs regulate gene expression by base pairing with messenger RNAs (mRNAs); this process is accelerated by the RNA chaperone Hfq⁹. The targets of base-pairing sRNAs can range from a few to as many as 1% of total cellular transcripts¹⁰. Transcripts positively controlled by these sRNAs form secondary structures near their ribosome binding sites and can be disrupted by alternative base pairing with sRNAs, allowing translational initiation^{11,12}. Negative regulation depends on base pairing of sRNAs near the translation initiation regions, and on downstream protein coding regions, which can lead to degradation of the transcripts¹³.

In spite of the relative ease of identifying regulatory RNAs, their targets are less well defined. The main difficulty in predicting sRNA targets is the limited and non-contiguous base-pairing regions with frequent internal secondary structures and the existence of multiple targets with different base-pairing configurations. Consequently, although several different computational algorithms have been developed, their performance in predicting direct regulatory targets of sRNAs is highly variable¹⁴. A number of experimental approaches have also been developed to facilitate the identification of direct targets of regulatory RNAs. Several methods (such as cross-linking ligation and sequencing of hybrids (CLASH)¹⁵ and *in vivo* photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)¹⁶) have been used to identify targets of eukaryotic microRNAs (miRNAs) based on immunoprecipitation of transcripts crosslinked to Argonaute proteins.

Crosslinking followed by *in vitro* ligation and sequencing has been used to define the targets of non-coding RNAs in human cells¹⁷. In bacteria, transcriptome analysis following brief expression of sRNAs can be used to predict likely targets using translational reporters or ribosome profiling^{18,19}. However, these methods are often unable to distinguish between direct and indirect effects of sRNA regulation. A variation of these methods was applied to the identification of Hfq-bound mRNAs and sRNAs in *Escherichia coli* and *Salmonella*; however, the assignment of a direct regulatory relationship could not be made^{18,19}. Another approach relies on fusing the MS2 coat protein-binding hairpin sequence to the sRNA and capturing the sRNA/mRNA complex with the MS2 bacteriophage coat protein²⁰.

To facilitate the analysis of global effects of bacterial sRNA-target mRNA interactions, we have developed a robust yet simple method for identifying targets of sRNAs. We have named this method global sRNA target identification by ligation and sequencing (GRIL-seq). The method takes advantage of the proximity of the sRNA and mRNA target sites in a complex that is likely to be stabilized by the Hfq protein. This arrangement facilitates a preferential ligation of the 3' and 5' ends by bacteriophage T4 RNA ligase, coexpressed in the same cell, and detection of the chimaeric RNAs by sequencing. The GRIL-seq method is an easy, readily accessible approach towards defining post-transcriptional regulatory networks controlled by sRNAs. Conceivably, this method could also be applicable to the analyses of miRNA-directed silencing of eukaryotic mRNAs.

Results

sRNA-target RNA ligation by T4 RNA ligase in the cell. We exploited the ability of bacteriophage T4 RNA ligase to link two base-paired RNA molecules expressed in the same cell. To ligate two RNAs, the 5' terminal donor sequence must be monophosphorylated. While most bacterial sRNAs are primary transcripts with 5' triphosphoryl termini, a recent study demonstrated that a fraction of *Salmonella* sRNAs carries 5' monophosphate, and their enrichment

The eluted RNA (in 47 µl) was collected, and nuclease-free water (153 µl) was added to reach 200 µl. The enriched RNA was precipitated overnight at -80 °C with 500 µl 100% ethanol (2.5 volumes), 20 µl 0.1 M sodium acetate (0.1 volume) and 4 µl glycogen (5 µg µl⁻¹).

Preparation of the cDNA library for RNA-seq. The precipitated enriched chimaeric sRNA was recovered in 15 µl nuclease-free water. The enriched RNAs (100 ng) were used for cDNA library preparation. Library construction for Illumina sequencing was carried out using an NEB Next Ultra Directional RNA Library Prep kit for Illumina (New England Biolab) following the manufacturer's instructions (Supplementary Notes 5). The index primers used in this study are listed in Supplementary Table 5. To enrich the libraries, 16 cycles of PCR were carried out at the last enrichment step. To obtain high purity of the library from the adaptor dimer, bead purification was carried out twice at the last step.

Detection of sRNA-target chimeras by RT-PCR. To remove residual DNA from total RNAs, 10 µg RNA sample in a 50 µl reaction volume was treated with TURBO DNase (1 U) for 25 min, followed by its inactivation for 5 min using a TURBO DNA-free kit (Thermo Fisher Scientific). Total RNA was recovered (45 µl) by centrifugation at 10,000 × g for 2 min, and total RNA (1 µg) was converted to cDNA using a SuperScript III First-Strand Synthesis system (Invitrogen) with random hexamers. When required, 5 pmol gene-specific primer was used for the gene-specific cDNA synthesis. Reverse transcription was carried out at 30 °C for 1 h and terminated at 85 °C for 10 min. The residual RNA was removed using 2 µl of an enzyme mixture containing RNase H (1 µg; New England Biolab) and Ribonuclease (0.5 µl; EpiCentre) for each reaction. Approximately 10% of the reaction was used as the template for PCR amplification using a GoTaq Green Master Mix (Promega) and the primer pairs listed in Supplementary Table 5. Cycling conditions were: 95 °C for 3 min; 30 cycles of 94 °C for 25 s, 58 °C for 25 s and 72 °C for 60 s; and a final cycle of 72 °C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis; the DNA bands were eluted and cloned into pET1L.2 vector (Thermo Fisher Scientific) following the manufacturer's instructions, and the inserts were sequenced using the pET1L.2 reverse primer.

RNA-seq data analysis. Sequencing reads were aligned with the PAO1 genome using the Rockhopper software²¹. The two biological replicate RNA-seq experiments corresponding to the PAO1 ApfF1ApfF2 strain resulted in 7,520,964 sequencing reads and 8,655,351 sequencing reads, of which 87% mapped to the reference genome. The two biological replicate RNA-seq experiments corresponding to PerFI overexpression resulted in 5,687,213 sequencing reads and 9,884,869 sequencing reads, of which 88% mapped to the reference genome. Sequencing read data were normalized using upper quartile normalization²². Differential expression in the two conditions was tested using the approach of DESeq²³, where *P* values were computed to indicate the probability of observing each gene's two expression levels, in the two conditions, by chance. Because multiple tests were performed across the set of genes, *P* values were corrected to *q* values to control the false discovery rate at less than 10%²⁴.

Data availability. The sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession codes SAMN05933141–SAMN05933146.

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*Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts 02115, USA. †Computer Science Department, Wellesley College, Wellesley, Massachusetts 02481, USA. *e-mail: stephen.lory@hms.harvard.edu

GRIL-seq provides a method for identifying direct targets of bacterial small regulatory RNA by *in vivo* proximity ligation

Kook Han¹, Brian Tjaden² and Stephen Lory^{1*}

The first step in the post-transcriptional regulatory function of most bacterial small non-coding RNAs (sRNAs) is base pairing with partially complementary sequences of targeted transcripts. We present a simple method for identifying sRNA targets *in vivo* and defining processing sites of the regulated transcripts. The technique, referred to as global small non-coding RNA target identification by ligation and sequencing (GRIL-seq), is based on preferential ligation of sRNAs to the ends of base-paired targets in bacteria co-expressing T4 RNA ligase, followed by sequencing to identify the chimaeras. In addition to the RNA chaperone Hfq, the GRIL-seq method depends on the activity of the pyrophosphorylase RppH. Using Prrf1, an iron-regulated sRNA in *Pseudomonas aeruginosa*, we demonstrated that direct regulatory targets of this sRNA can readily be identified. Therefore, GRIL-seq represents a powerful tool not only for identifying direct targets of sRNAs in a variety of environments, but also for uncovering novel roles for sRNAs and their targets in complex regulatory networks.

Post-transcriptional regulation mechanisms, primarily through the activities of regulatory small RNAs (sRNAs), play an important role in the bacterial stress response, metabolism, quorum sensing and virulence^{1–6}. The sharp increase in the description of this abundant class of RNA regulators during the past decade is the direct result of transcriptome analyses using next-generation RNA sequencing (RNA-seq) and the development of *in silico* methods to identify non-protein-coding transcripts⁷. In bacteria, regulation of gene expression by sRNAs can be divided into two mechanistically distinct categories. One class of sRNAs functions by modifying the activities of regulatory proteins⁸. Other sRNAs regulate gene expression by base pairing with messenger RNAs (mRNAs); this process is accelerated by the RNA chaperone Hfq⁹. The targets of base-pairing sRNAs can range from a few to as many as 1% of total cellular transcripts¹⁰. Transcripts positively controlled by these sRNAs form secondary structures near their ribosome binding sites and can be disrupted by alternative base pairing with sRNAs, allowing translational initiation^{11,12}. Negative regulation depends on base pairing of sRNAs near the translation initiation regions, and on downstream protein coding regions, which can lead to degradation of the transcripts¹³.

In spite of the relative ease of identifying regulatory RNAs, their targets are less well defined. The main difficulty in predicting sRNA targets is the limited and non-contiguous base-pairing regions with frequent internal secondary structures and the existence of multiple targets with different base-pairing configurations. Consequently, although several different computational algorithms have been developed, their performance in predicting direct regulatory targets of sRNAs is highly variable¹⁴. A number of experimental approaches have also been developed to facilitate the identification of direct targets of regulatory RNAs. Several methods (such as cross-linking ligation and sequencing of hybrids (CLASH)¹⁵ and *in vivo* photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)¹⁶) have been used to identify targets of eukaryotic microRNAs (miRNAs) based on immunoprecipitation of transcripts crosslinked to Argonaute proteins.

Crosslinking followed by *in vitro* ligation and sequencing has been used to define the targets of non-coding RNAs in human cells¹⁷. In bacteria, transcriptome analysis following brief expression of sRNAs can be used to predict likely targets using translational reporters or ribosome profiling^{18,19}. However, these methods are often unable to distinguish between direct and indirect effects of sRNA regulation. A variation of these methods was applied to the identification of Hfq-bound mRNAs and sRNAs in *Escherichia coli* and *Salmonella*; however, the assignment of a direct regulatory relationship could not be made^{18,19}. Another approach relies on fusing the MS2 coat protein-binding hairpin sequence to the sRNA and capturing the sRNA/mRNA complex with the MS2 bacteriophage coat protein²⁰.

To facilitate the analysis of global effects of bacterial sRNA-target mRNA interactions, we have developed a robust yet simple method for identifying targets of sRNAs. We have named this method global sRNA target identification by ligation and sequencing (GRIL-seq). The method takes advantage of the proximity of the sRNA and mRNA target sites in a complex that is likely to be stabilized by the Hfq protein. This arrangement facilitates a preferential ligation of the 3' and 5' ends by bacteriophage T4 RNA ligase, coexpressed in the same cell, and detection of the chimaeric RNAs by sequencing. The GRIL-seq method is an easy, readily accessible approach towards defining post-transcriptional regulatory networks controlled by sRNAs. Conceivably, this method could also be applicable to the analyses of miRNA-directed silencing of eukaryotic mRNAs.

Results

sRNA-target RNA ligation by T4 RNA ligase in the cell. We exploited the ability of bacteriophage T4 RNA ligase to link two base-paired RNA molecules expressed in the same cell. To ligate two RNAs, the 5' terminal donor sequence must be monophosphorylated. While most bacterial sRNAs are primary transcripts with 5' triphosphoryl termini, a recent study demonstrated that a fraction of *Salmonella* sRNAs carries 5' monophosphate, and their enrichment

The eluted RNA (in 47 µl) was collected, and nuclease-free water (153 µl) was added to reach 200 µl. The enriched RNA was precipitated overnight at -80 °C with 500 µl 100% ethanol (2.5 volumes), 20 µl 0.1 M sodium acetate (0.1 volume) and 4 µl glycogen (5 µg µl⁻¹).

Preparation of the cDNA library for RNA-seq. The precipitated enriched chimaeric sRNA was recovered in 15 µl nuclease-free water. The enriched RNAs (100 ng) were used for cDNA library preparation. Library construction for Illumina sequencing was carried out using an NEB Next Ultra Directional RNA Library Prep kit for Illumina (New England Biolab) following the manufacturer's instructions (Supplementary Notes 5). The index primers used in this study are listed in Supplementary Table 5. To enrich the libraries, 16 cycles of PCR were carried out at the last enrichment step. To obtain high purity of the library from the adaptor dimer, bead purification was carried out twice at the last step.

Detection of sRNA-target chimeras by RT-PCR. To remove residual DNA from total RNAs, 10 µg RNA sample in a 50 µl reaction volume was treated with TURBO DNase (1 U) for 25 min, followed by its inactivation for 5 min using a TURBO DNA-free kit (Thermo Fisher Scientific). Total RNA was recovered (45 µl) by centrifugation at 10,000 × g for 2 min, and total RNA (1 µg) was converted to cDNA using a SuperScript III First-Strand Synthesis system (Invitrogen) with random hexamers. When required, 5 pmol gene-specific primers was used for the gene-specific cDNA synthesis. Reverse transcription was carried out at 30 °C for 1 h and terminated at 85 °C for 10 min. The residual RNA was removed using 2 µl of an enzyme mixture containing RNase H (1 µg; New England Biolab) and Ribonuclease (0.5 µl; Epientre) for each reaction. Approximately 10% of the reaction was used as the template for PCR amplification using a GoTaq Green Master Mix (Promega) and the primer pairs listed in Supplementary Table 5. Cycling conditions were: 95 °C for 3 min; 30 cycles of 94 °C for 25 s, 58 °C for 25 s and 72 °C for 60 s; and a final cycle of 72 °C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis; the DNA bands were eluted and cloned into pET1L.2 vector (Thermo Fisher Scientific) following the manufacturer's instructions, and the inserts were sequenced using the pET1L.2 reverse primer.

RNA-seq data analysis. Sequencing reads were aligned with the PAO1 genome using the Rockhopper software²¹. The two biological replicate RNA-seq experiments corresponding to the PAO1 ApfF1ApfF2 strain resulted in 7,520,964 sequencing reads and 8,655,351 sequencing reads, of which 87% mapped to the reference genome. The two biological replicate RNA-seq experiments corresponding to PefF1 overexpression resulted in 5,687,213 sequencing reads and 9,884,869 sequencing reads, of which 88% mapped to the reference genome. Sequencing read data were normalized using upper quartile normalization²². Differential expression in the two conditions was tested using the approach of DESeq²³, where *P* values were computed to indicate the probability of observing each gene's two expression levels, in two conditions, under the assumption that both genes belong to the same set of genes. *P* values were corrected to *q* values to control the false discovery rate at least three times.

Data availability. The sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession codes SAMN05933141-SAMN05933146.

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*Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts 02115, USA. †Computer Science Department, Wellesley College, Wellesley, Massachusetts 02481, USA. *e-mail: stephen.lory@hms.harvard.edu

GRIL-seq provides targets of bacterial proximity ligation

Kook Han¹, Brian Tjaden² and St

The first step in the post-transcriptional pairing with partially complementary sequences in vivo and defining processing pathways for non-coding RNA target identification by the ends of base-paired targets in bacteria. In addition to the RNA chaperone Hfq, PrrF1, an iron-regulated sRNA in *Pseudomonas aeruginosa*, can readily be identified. Therefore, GRIL-seq can be used not only for identifying targets in a variety of environments, but also for understanding the regulatory mechanism of sRNAs.

D post-transcriptional regulation mechanism of sRNAs. The activities of regulatory small RNAs (sRNAs) in *Pseudomonas aeruginosa* can be studied by using the GRIL-seq method. This method can be used to identify targets in a variety of environments, but also for understanding the regulatory mechanism of sRNAs.

RNA-seq data analysis

Sequencing reads were aligned with the PAO1 genome using the Rockhopper system⁴⁶. The two biological replicate RNA-seq experiments corresponding to the PAO1ΔprrF1ΔprrF2 strain resulted in 7,520,964 sequencing reads and 8,655,351 sequencing reads, of which 87% mapped to the reference genome. The two biological replicate RNA-seq experiments corresponding to PrrF1 overexpression resulted in 5,687,213 sequencing reads and 9,884,669 sequencing reads, of which 88% mapped to the reference genome. Sequencing read data were normalized using upper quartile normalization⁴⁷. Differential expression in the two conditions was tested using the approach of DESeq2⁴⁸, where P values were computed to indicate the probability of observing each gene's two expression levels, in the two conditions, by chance. Because multiple tests were performed across the set of genes, P values were corrected to q values to control the false discovery rate at less than 1%⁴⁹.

Data availability

The sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under accession codes SAMN05933141–SAMN05933146.

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The first step in the post-transcriptional regulatory function of most bacterial small non-coding RNAs (sRNAs) is base-pairing with partially complementary sequences of targeted transcripts. We present a simple, yet robust method for identifying sRNA targets in vivo and defining processing sites of the regulated transcripts. The technique (referred to as GRIL-Seq) is based on preferential ligation of sRNAs to the ends of base-paired targets in bacteria expressing the T4 RNA ligase followed by sequencing to identify the ligated chimeras. In addition to the RNA chaperone Hfq, the GRIL-Seq method depends on the activity of the pyrophosphorylase RppH. We used PrrF1, an iron-regulated sRNA in *Pseudomonas aeruginosa*, to demonstrate that direct regulatory targets of this sRNA can be readily identified. Therefore, GRIL-Seq represents a powerful tool not only for identifying direct targets of sRNAs in a variety of environments, but also for better understanding the mechanistic details of their activity during post-transcriptional regulation of gene expression.

Sample:
[SAMN05933141](#) • [SRS1755301](#) • All experiments • All runs
Organism: *Pseudomonas aeruginosa PAO1*

Library:
Name: GS_R1
Instrument: Illumina HiSeq 2500
Strategy: OTHER
Source: TRANSCRIPTOMIC
Selection: RANDOM
Layout: PAIRED

Runs: 1 run, 47.2M spots, 14.3G bases, [8.6Gb](#)

Run	# of Spots	# of Bases	Size	Published
SRR4436850	47,247,376	14.3G	8.6Gb	2016-10-26

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Data Type	Raw sequence reads
Scope	Multispecies
Grants	<ul style="list-style-type: none">"System and Methods for Analysis of Bacterial Transcriptomes" (Grant ID R15 GM102755, National Institute of General Medical Sciences)"P. aeruginosa Virulence Determinants" (Grant ID R37 AI021451, National Institute of Allergy and Infectious Diseases Extramural Activities)
Submission	Registration date: 21-Oct-2016 Wellesley College

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SRR4436848	5.7M	859.6Mbp	425.7M	59.8%	2016-10-26	public

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This run has 2 reads per spot:

L=76, 100% L=76, 100% [Legend](#)

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SRX2255951 to BLAST	RS_PrrF1_R1	Illumina	RNA-Seq	TRANSCRIPTOMIC	RANDOM	PAIRED

Biosample	Sample Description	Organism	Links
SAMN05933145 (SRS1755300)		Pseudomonas aeruginosa PAO1	PRJNA349811 [undefined]

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GRIL-seq provides a method for identifying direct targets of bacterial small regulatory RNA by *in vivo* proximity ligation

Kook Han, Brian Tjaden & Stephen Lory ✉

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Figure 1: T4 RNA ligase-catalysed *in vivo* linking of sRNA to mRNAs.

a Schematic diagram of the GRIL-seq workflow. A bacterial cell contains mRNA, sRNA, and T4 RNA ligase. T4 RNA ligase catalyzes the ligation of sRNA to mRNA at the 3' end of mRNA. The linked RNA is isolated and analyzed by RT-PCR. The PCR product is then analyzed by gel electrophoresis.

b Growth curve of PAO1 (□) and PAO1ΔPAU (○) strains. Cells were grown in minimal medium with or without IPTG. Cell density was measured at 600 nm over time (0–8 h).

c RT-PCR analysis of total RNA isolated from PAO1 (left) and PAO1ΔPAU (right) cells. Lanes are grouped by target (top) or non-target (bottom). Lanes are labeled with IPTG concentration (0, 5, 10, 20 μM) and time (0, 5, 10, 20 min). qRT-PCR analysis of *PAU* mRNA levels is shown below each RT-PCR gel.

d Schematic of the *PAU* gene structure. The *PAU* gene is transcribed from left to right. The *PAU* mRNA is indicated by a red line. The *PAU* sRNA is indicated by a green line. The T4 RNA ligase cleavage sites are marked with asterisks (*). The T4 RNA ligase ligation sites are marked with plus signs (+). The *PAU* mRNA 3' ends are marked with arrows. The *PAU* sRNA 5' ends are marked with arrows. The *PAU* gene promoter is marked with a triangle.

Altmetric Score in Action

Altmetric

GRIL-seq provides a method for identifying direct targets of bacterial small regulatory RNA by in vivo proximity ligation

Overview of attention for article published in Nature Microbiology, December 2016

SUMMARY Twitter

Title GRIL-seq provides a method for identifying direct targets of bacterial small regulatory RNA by in vivo proximity ligation

Published in Nature Microbiology, December 2016

DOI 10.1038/nmicrobiol.2016.239

Pubmed ID 28005055

Authors Kook Han, Brian Tjaden, Stephen Lory

Abstract The first step in the post-transcriptional regulatory function of most bacterial small non-coding... [show]

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OUTPUTS FROM NATURE MICROBIOLOGY #**340** of 600 outputs

OUTPUTS OF SIMILAR AGE #**24,517** of 317,171 outputs

OUTPUTS OF SIMILAR AGE FROM NATURE MICROBIOLOGY #**34** of 67 outputs

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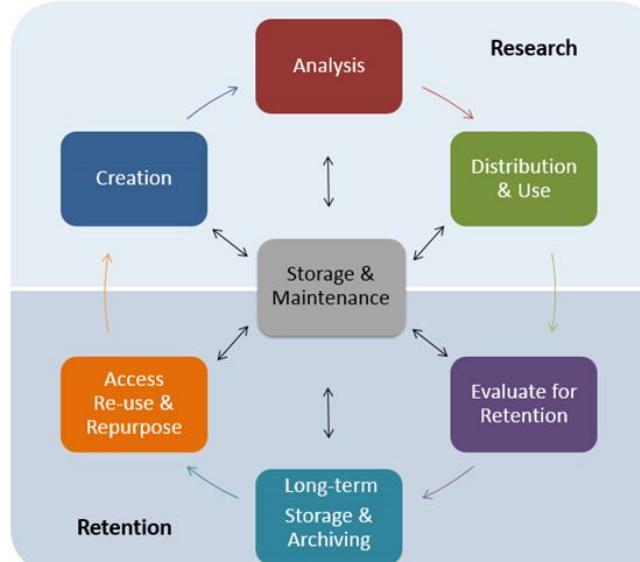
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Harvard Biomedical Data Management
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← December 2018 →

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9	10	11	12	13	14	15
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23	24	25	26	27	28	29
30	31					

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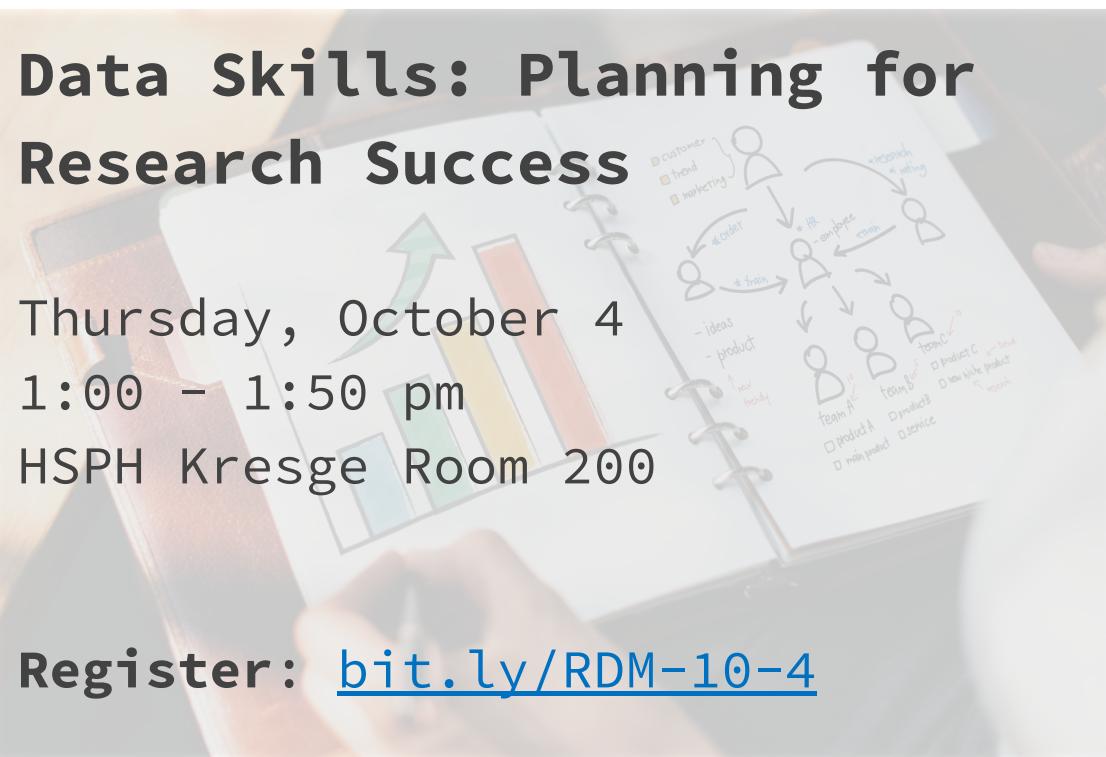
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Data Skills: Planning for Research Success

Thursday, October 4
1:00 – 1:50 pm
HSPH Kresge Room 200

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Digital Tools for the Lab

Friday, November 16
1:00 – 1:50 pm
HMS TMEC 227

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