SUPPLEMENTARY DATA

Insights into the evolutionary conserved regulation of the Rio ATPase activity

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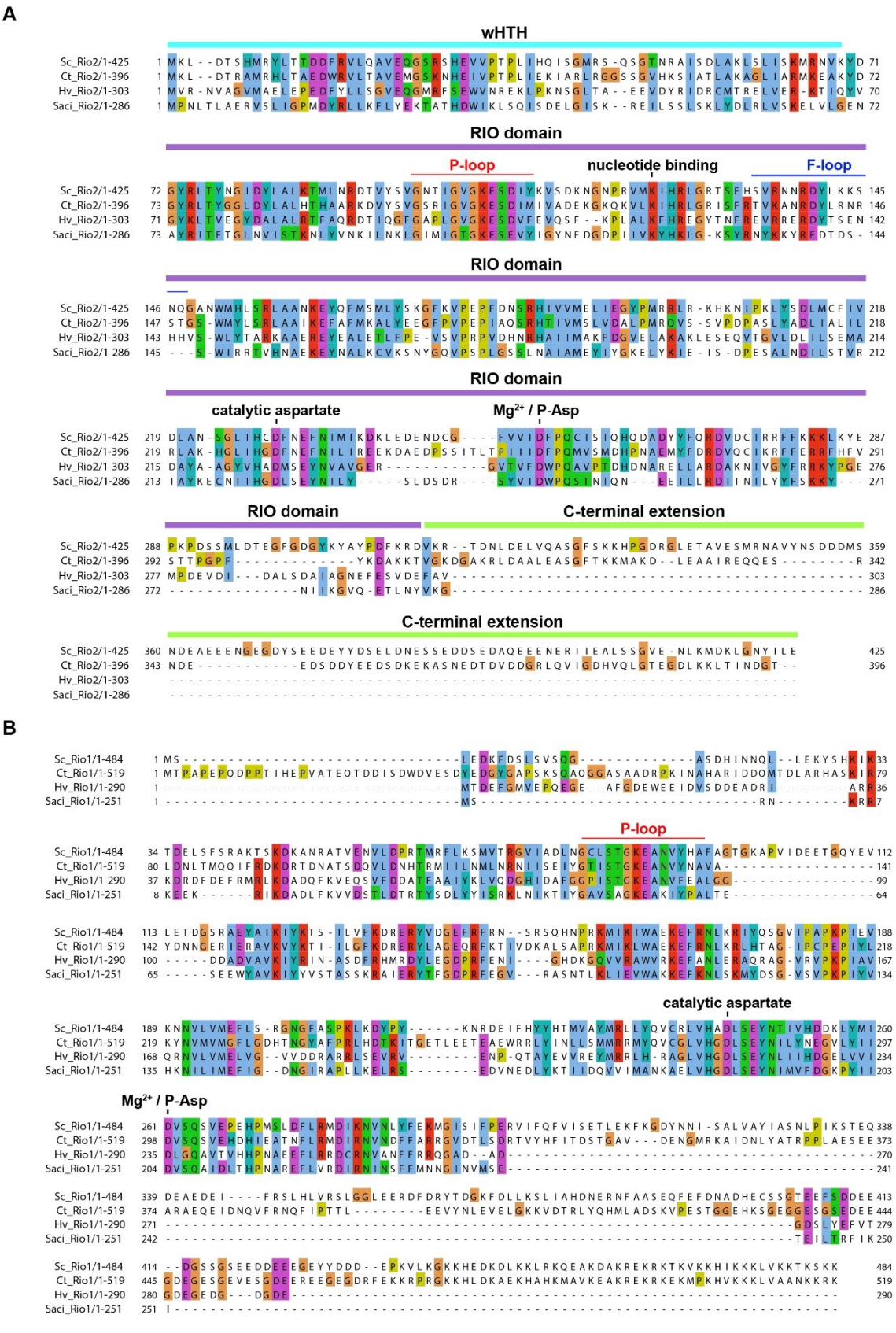
Supplementary Figures and Tables

Supplementary Figures 1-3

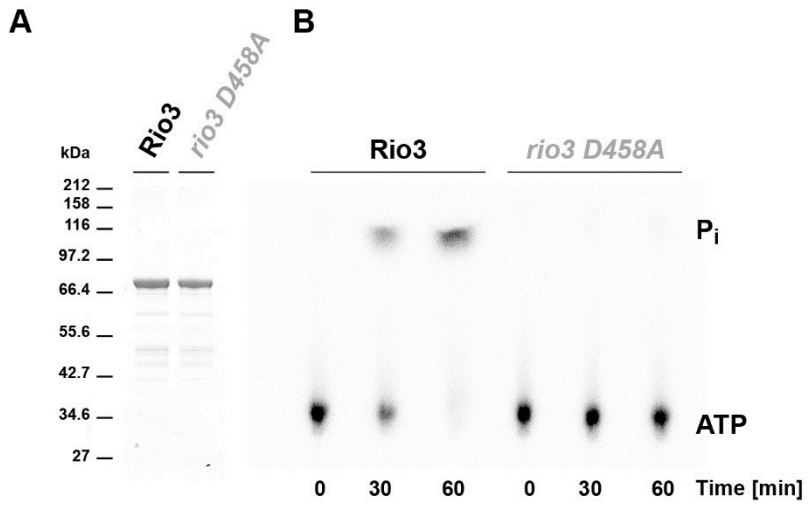
Supplementary Tables 1-3

Supplementary Figures



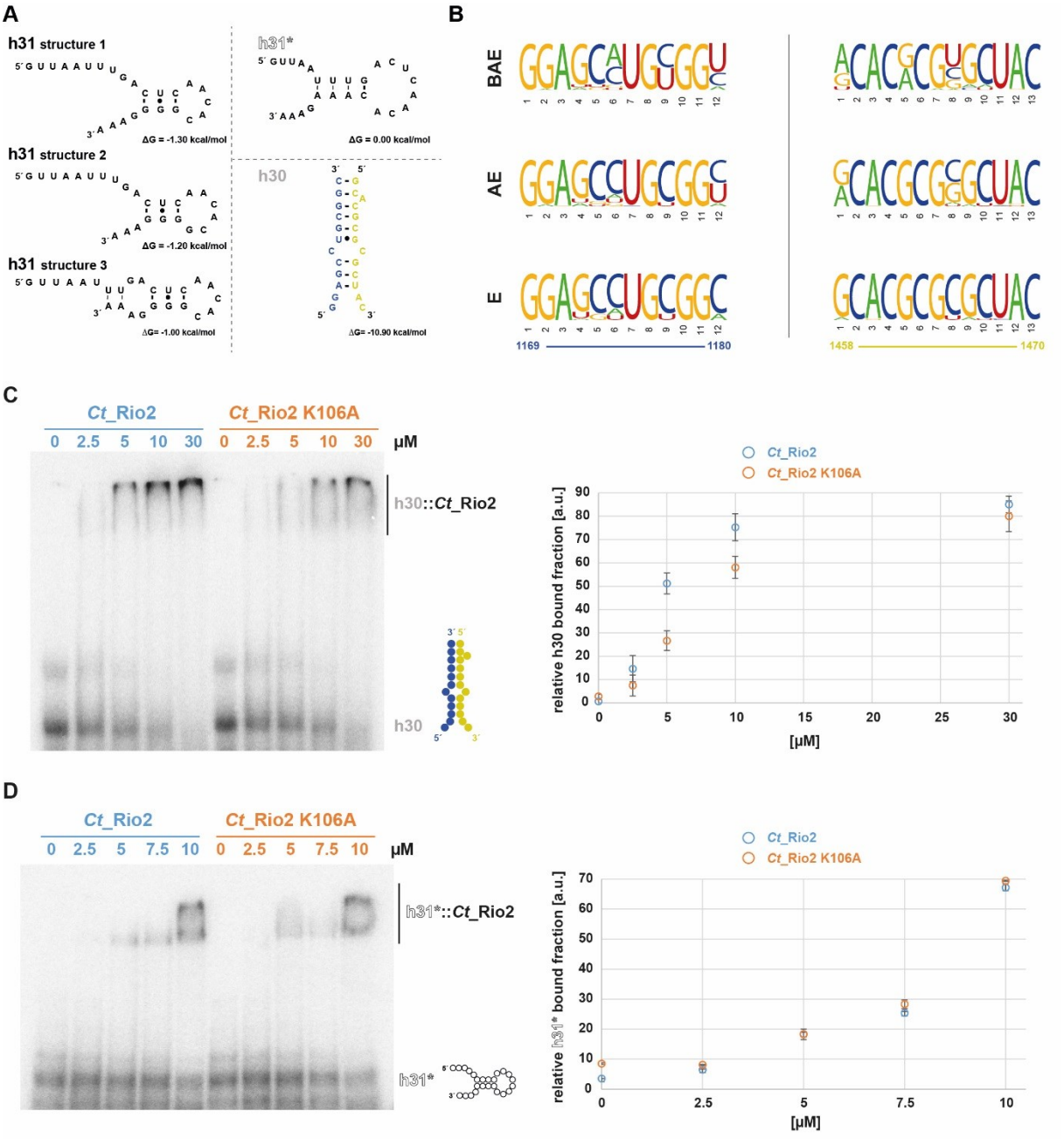
Supplementary Figure 1: Sequence alignments of representative Rio proteins

(A) Multiple sequence alignment of eukaryotic Rio2 orthologues in comparison to archaeal Rio2. Sequence alignment of eukaryotic *Sc*_Rio2 (*Saccharomyces cerevisiae*; P40160*)* and *Ct*_Rio2 (*Chaetomium thermophilum*; G0S5R3) in comparison to archaeal *Hv*_Rio2 (*Haloferax volcanii*; D4SG4) and *Saci*_Rio2 (*Sulfolobus acidocaldarius*; Q4JAL3) using Cobalt multiple sequence alignment server (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web) and Jalview. Indicated above the alignment are the different Rio2 structural domains including wHTH, RIO domain and C-terminal extension. Moreover, the catalytic residues, the flexible- and phosphate-loops (F-loop and P-loop, respectively) are indicated. (B) Multiple sequence alignment of eukaryotic Rio1 orthologues in comparison to archaeal Rio1. Sequence alignment of eukaryotic *Sc*_Rio1 (*Saccharomyces cerevisiae;* Q12196) and *Ct*_Rio1 (*Chaetomium thermophilum;* G0S3J5) in comparison to archaeal *Hv*_Rio1 (*Haloferax volcanii;* D4GYY1) and *Saci*_Rio1 (*Sulfolobus acidocaldarius;* Q4JA53) using Cobalt multiple sequence alignment server (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Web) and Jalview. Indicated above the alignment are the different Rio2 structural domains including wHTH, RIO domain and C-terminal extension. Moreover, the catalytic residues, the flexible- and phosphate-loops (F-loop and P-loop, respectively) are indicated.



Supplementary Figure 2: ATPase activity of *Drosophila melanogaster Dm*_Rio3

ATPase activity of purified *Dm*_Rio3 (Q9VR42) and catalytic mutant *Dm_rio3* D458A (DFG motif) (A) was analyzed by single-turnover experiment (B).



Supplementary Figure 3: Interaction of *Ct*_Rio2 with the conserved h30.

(A) 2D structure prediction of RNA mimic used in this study. Single strand RNA 2D structure prediction where performed with mfold using default parameters (http://unafold.rna.albany.edu/?q=mfold/) (Zuker, 2003). Since, the h31 RNA mimic (18S rRNA 1181-1204 *Sc* numbering) was predicted to fold in an ensemble of RNAs (top hits – left panel) which were not folding into a "native" h31-like structure, we designed h31* (upper right panel) allowing stabilization into an h31-like structure. For double RNA structure prediction (h30 lower right panel) the RNAcofold server was used (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi) (Gruber et al., 2008). Region 18S rRNA 1169-80 and 1458-70 (*Sc* numbering) forming h30 are indicated in blue and yellow, respectively (B) Sequence conservation of h30. Representative SSU rRNA sequences (133 rRNA sequences) (Bernier et

al., 2014) from bacteria (B – 67 sequences), archaea (A – 36 sequences) and eukarya (E - 30 sequences) were aligned and visualized in Jalview (Waterhouse et al., 2009). The corresponding alignments obtained for region 1169-80 (*Sc* numbering) and 1458-70 of the 18S rRNA building h30 were used to generate sequence logo (Crooks et al., 2004) depicting position-dependent nucleotide frequency. (C-D) Interaction of *Ct*_Rio2 and *Ct*_Rio2 K106A with h31* and h30 mimic RNA. Interaction of the indicated recombinant proteins with RNA mimic (depicted as cartoon) were analyzed by electro mobility shift assay (left panels). Quantitation and standard deviation were derived from 3 independent experiments (right panels). Note that the apparent weak affinity (in the µM range) of *Ct*_Rio2 and *Ct*_Rio2 K106A towards h31* is similar, whereas the apparent binding affinity, also in the µM range, of h30 towards *Ct*_Rio2 K106A is slightly reduced in comparison to wild-type (Apparent Kd *Ct*_Rio2= 4.02 ± 1.04 µM and *Ct*_Rio2 K106A= 8.12 ± 1.57 µM).

Supplementary Tables

Table 1: Strains

S. cerevisiae strains	Name	Description	Source
	RIO2 shuffle strain	Mat a; his3Δ; leu2Δ; lys2Δ; met15Δ; ura3Δ; YNL207w::kanMX4: pURA3-RIO2-GFP	(Ferreira-Cerca et al., 2012)
	RIO2 shuffle strain Δ*ltv1*	his3Δ; leu2Δ; ura3Δ; YNL207w::kanMX4; YKL143W::kanMX4; pURA3-RIO2-GFP	(Ferreira-Cerca et al., 2012)
	Tsr1-TAP	Mat a; his3Δ; leu2Δ; lys2Δ; met15Δ; ura3Δ; Tsr1-TAP::His3MX6	This study
H. volcanii strains	Name	Description	Source
	DS70		(Allers et al., 2004)
	H26	Δ*pyrE2* (DS70)	(Allers et al., 2004)
	H53	Δ*trpA* Δ*pyrE2* (DS70)	(Allers et al., 2004)
	H98	Δ*hdrB* Δ*pyrE2* (DS70)	(Allers et al., 2004)
	H99	Δ*trpA* Δ*hdrB* Δ*pyrE2* (DS70)	(Allers et al., 2004)
	SFC - *rio1::trpA*	*rio1::trpA* Δ*trpA* Δ*pyrE2* (H53)	This study - Ferreira-Cerca Lab
	SFC - *rio2::trpA*	*rio2::trpA* Δ*trpA* Δ*pyrE2* (H53)	This study - Ferreira-Cerca Lab
	SMH666/ SMH677 - *rio1::trpA*	*rio1::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab
	SMH668/ SMH669 - *rio2::trpA*	*rio2::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab
	SMH670/ SMH671 - *rio1::hdrB*	*rio1::hdrB* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab
	SMH672/ SMH673 - *rio2::hdrB*	*rio2::hdrB* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab
	SMH674/ SMH 675 - *rio1::trpA rio2::hdrB*	*rio1::trpA rio2::hdrB* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab
	SMH676/ SMH 677 - *rio1::hdrB rio2::trpA*	*rio1::hdrB rio2::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2* (H99)	This study - MacNeill Lab

Table 2: Plasmids

Yeast vectors		
Name	Description	Source
pRS315-*RIO2*	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	(Ferreira-Cerca et al., 2012)
pRS315-*rio2* D253A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	(Ferreira-Cerca et al., 2012)
pRS315-*rio2* K105E	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	(Ferreira-Cerca et al., 2012)
pRS315-*rio2* K105A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G99A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G102A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G104A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G106E	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G106S	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
pRS315-rio2 G107A	CEN; LEU2; RIO2 ORF including 500nt before ATG and 140nt after stop codon	This study
YEP352-*GAL::RIO2*	2µ; URA3; *RIO2* ORF under *GAL* promoter	(Ferreira-Cerca et al., 2012)
YEP352-*GAL*::*rio2* D253A	2µ; URA3; *RIO2* ORF under *GAL* promoter	(Ferreira-Cerca et al., 2012)
YEP352-*GAL*::*rio2* K105A	2µ; URA3; *RIO2* ORF under *GAL* promoter	This study
YEP352-*GAL*::*rio2* K105A D253A	2µ; URA3; *RIO2* ORF under *GAL* promoter	This study
Ycplac111-Rio2-TAP	CEN; LEU2; RIO2 ORF fused to TAP-tag including 500nt before ATG	(Ferreira-Cerca et al., 2012)
Ycplac111-*rio2* D253A-TAP	CEN; LEU2; RIO2 ORF fused to TAP-tag including 500nt before ATG	(Ferreira-Cerca et al., 2012)
Ycplac111-*rio2* K105A-TAP	CEN; LEU2; RIO2 ORF fused to TAP-tag including 500nt before ATG	This study

E. coli expression vectors		
Name	Description	Source
pT7::His6-TEV-*ct RIO2*	*E.coli* expression vector; Amp	(Ferreira-Cerca et al., 2012)
pT7::His6-TEV-*ct rio2* D257A	*E.coli* expression vector; Amp	(Ferreira-Cerca et al., 2012)
pT7::His6-TEV-*ct rio2 K106E*	*E.coli* expression vector; Amp	(Ferreira-Cerca et al., 2012)
pT7::His6-TEV-*ct rio2 K106A*	*E.coli* expression vector; Amp	This study
pET24a-Hv_Rio1	*E.coli* expression vector; Kan	This study
pET24a-Hv_Rio1 D235A	*E.coli* expression vector; Kan	This study
pET24a-Hv_Rio2	*E.coli* expression vector; Kan	This study
pET24a-Hv_Rio2 D242A	*E.coli* expression vector; Kan	This study
pET15b-Saci_Rio1	*E.coli* expression vector; Amp	This study
pET15b-Saci_Rio1 D204A	*E.coli* expression vector; Amp	This study

E. coli expression vectors (continued)		
Name	Description	Source
pET15b-Saci_Rio2	*E.coli* expression vector; Amp	This study
pET15b-Saci_Rio2 D243A	*E.coli* expression vector; Amp	This study
pET24a-Dm_Rio3	*E.coli* expression vector; Kan	This study
pET24a-Dm_*rio3* D458A	*E.coli* expression vector; Kan	This study

H.volcanii vectors		
Name	Description	Source
pTA131	Amp; *pyrE2*	(Allers et al., 2004)
pTA131-Rio1usds	pTA131 derivative	This study - Ferreira-Cerca Lab
pTA131-Rio2usds	pTA131 derivative	This study - Ferreira-Cerca Lab
pTA131-*rio1::trpA*	pTA131 derivative	This study - Ferreira-Cerca Lab
pTA131-*rio2::trpA*	pTA131 derivative	This study - Ferreira-Cerca Lab
pTA131-HfxRio1-EBSX	pTA131 derivative	This study - MacNeill Lab
pTA131-HfxRio2-EBSX	pTA131 derivative	This study - MacNeill Lab
pTA131-HfxRio1Δ-trpA	pTA131 derivative	This study - MacNeill Lab
pTA131-HfxRio1Δ-hdrB	pTA131 derivative	This study - MacNeill Lab
pTA131-HfxRio2Δ-trpA	pTA131 derivative	This study - MacNeill Lab
pTA131-HfxRio2Δ-hdrB	pTA131 derivative	This study - MacNeill Lab

Table 3: Oligonucleotides

Primers for yeast Rio2 mutants	
scRio2 G98A Fw	5´-GAGACACTGTCTATTCTGTCgccAACACTATTggtGTTgg-3´
scRio2 G98A Rv	5´-ccAACaccAATAGTGTTggcGACAGAATAGACAGTGTCTC-3´
scRio2 G102A Fw	5´-CTGTCggtAACACTATTgccGTTggtAAGGAATCTGACATC-3´
scRio2 G102A Rv	5´-GATGTCAGATTCCTTaccAACggcAATAGTGTTaccGACAG-3´
scRio2 G104A Fw	5´-GTCggtAACACTATTgccGTTggtAAGGAATCTGACATCTATAAAG-3´
scRio2 G104A Rv	5´-CTTTATAGATGTCAGATTCCTTaccAACggcAATAGTGTTaccGAC-3´
scRio2 K105A Fw	5´-GGTAACACTATTGGTGTTGGTgccGAATCTGACATCTATAAAGT-3´
scRio2 K105A Rv	5´-ACTTTATAGATGTCAGATTCggcACCAACACCAATAGTGTTACC-3´
scRio2 E106A Fw	5´-GTAACACTATTGGTGTTGGTAAGgccTCTGACATCTATAAAGTAAG-3´
scRio2 E106A Rv	5´-CTTACTTTATAGATGTCAGAggcCTTACCAACACCAATAGTGTTAC-3´
scRio2 E106S Fw	5´-GTAACACTATTGGTGTTGGTAAGtctTCTGACATCTATAAAGTAAG-3´
scRio2 E106S Rv	5´-CTTACTTTATAGATGTCAGAagaCTTACCAACACCAATAGTGTTAC-3´
scRio2 S107A Fw	5´-CACTATTGGTGTTGGTAAGGAAgccGACATCTATAAAGTAAGTGAC-3´
scRio2 S107A Rv	5´-GTCACTTACTTTATAGATGTCggcTTCCTTACCAACACCAATAGTG-3´
Primers for cloning of recombinant Rio proteins	
oHv011-HvRio2-Nde-Fw	5´-gaggcgccatATGGTACGGAACGTCGCCGGC-3´
oHv012-HvRio2-His6-Bam-Rv	5´-tattatggatccTCAgtggtggtggtggtggtggccGACGGCGAACTCGTCGACGCTC-3´
oHv122-HvRio2 D242A Fw	5´-GCGCGGCGTCACGGTGTTCGcCTGGCCGCAGGCCGTCCCG-3´
oHv123-HvRio2 D242A Rv	5´-CGGGACGGCCTGCGGCCAGgCGAACACCGTGACGCCGCGC-3´
Saci_0796_Rio2 Fw NdeI	5´-AAACATATGATGCCTAATCTCACTTTAGCGC-3´
Saci_0796_Rio2 Rv BamHI	5´-AAAGGATCCCTACCCCTTAACATAGTTTAAGGTTTC-3´
Saci_001_Rio2 Nde Fw	5´-ggcagcCATATGCCTAATCTCACTTTAGCG-3´
Saci_002_Rio2 XhoI Rv	5´-cggatcCTCGAGCTACCCCTTAACATAGTTTAAG-3´
Saci_003_Rio2 D243A Fw	5´-CGATAGATCTTACGTTATCGcTTGGCCTCAAAGTACAAATATAC-3´
Saci_004_Rio2 D243A Rv	5´-GTATATTTGTACTTTGAGGCCAAgCGATAACGTAAGATCTATCG-3´
Saci_0965_Rio1 Fw NdeI	5´-AAACATATGTTGTCACGAAACAAAAGGAGAAA-3´
Saci_0965_Rio1 Rv BamHI	5´-AAAGGATCCTTAAATTTTAATAAAACGAGTTAATATTTCCGTC-3´
Saci_005_Rio1 Nde Fw	5´-ggcagcCATATGttgtcacgaaacaaaaggag-3´
Saci_006_Rio1 Bam Rv	5´-gcagccGGATCCttaaattttaataaaacgag-3´
Saci_007_Rio1 D204A Fw	5´-GATGGTAAACCGTATATAATAGcTGTTAGCCAAGCCATAGATC-3´
Saci_008_Rio1 D204A Rv	5´-GATCTATGGCTTGGCTAACAgCTATTATATACGGTTTACCATC-3´
CtRio2 K106A	5´-GGTAGCCGCATCGGCGTTGGAgctGAGAGTGATATCATGATTGTG-3´
CtRio2 K106A	5´-CACAATCATGATATCACTCTCagcTCCAACGCCGATGCGGCTACC-3´
dmRioK3 Nde Fw	5´-gcgctacatATGTCGTCGCCATGGGTTAAG-3´
dmRioK3 XhoI Rv	5´-cacgtgctcgagATGGTTGGCAGTTTTGTTAATC-3´
Primers for *H. volcanii* deletion constructs (Ferreira-Cerca Lab)	
oHv025-KOus-HvRio2-KpnI-Fw	5´-GCATCGAGGGTACCTCGTCGTGCGAATCGCGGACG-3´
oHv026-KOus-HvRio2-Bam-Rv	5´-AGCGGAGGGGTCTCGACGGCGGATCCGCACGTCCGTCCGTCGGCGA-3´
oHv027-KOds-HvRio2-Bam-Fw	5´-TCGCCGACGGACGGACGTGCGGATCCGCCGTCGAGACCCCTCCGCT-3´
oHv028-KOds-HvRio2-XbaI-Rv	5´-CTAGCCACTCTAGAGACCTCGCGCTCGACGGCGTGGC-3´
oHv029-KOus-HvRio1-KpnI-Fw	5´-GCATCGAGGGTACCCCCGCGGTGCGAAAACGGGTGT-3´
oHv030-KOus-HvRio1-Bam-Rv	5´-TCTCGCGGCGGGTCTGGGTTGGGATCCTGGACTAGGGCTACGAGAGG-3´
oHv031-KOds-HvRio1-Bam-Fw	5´-CCTCTCGTAGCCCTAGTCCAGGATCCCAACCCAGACCCGCCGCGAGA-3´
oHv032-KOds-HvRio1-XbaI-Rv	5´-CTAGCCACTCTAGAGGTTTATCTGTGGCTTTCGCC-3´´

Note: Corresponding restriction sites are underlined.

Primers for *H. volcanii* deletion constructs (MacNeill Lab)	
HfxRio1-U5E	5′-GTGGTGGTGTGAATTCCATACCACGACGGCGTTT-3′
HfxRio1-U3B	5′-GTGGTGGTGTGGATCCTGGACTAGGGCTACGAGAG-3′
HfxRio1-D5B	5′-GTGGTGGTGTGGATCCCAACCCAGACCCGCCGCGA-3′
HfxRio1-D3S	5′-GTGGTGGTGTACTAGTTGGCCGATGATGCCGAGC-3′
HfxRio2-U5E	5′-GTGGTGGTGTGAATTCGTGGGTTGATTCACGCCCG-3′
HfxRio2-U3B	5′-GTGGTGGTGTGAATTCGTGGGTTGATTCACGCCCG-3′
HfxRio2-D5B	5′-GTGGTGGTGTGGATCCGCCGTCGAGACCCCTCCGC-3′
HfxRio2-D3S	5′-GTGGTGGTGTACTAGTTGTACCACAACTTCCGCC-3′
Note: Indicated restriction sites are underlined (E for EcoRI, B for BamHI, S for SpeI).	

Primers used for diagnostic PCR (shown in Figure 1A)	
HfxRio1-P1	5′-AGAGGCGACGATACCGATAC-3′
HfxRio1-P2	5′-GGGCACCGTCGAGAATCATC-3′
HfxRio2-P1	5′-CGACGGCGAGGGCGCGGTCG-3′
HfxRio2-P2	5′-AACTACTGGCCGATGGCCGC-3′
HfxTrpA-F	5′-TCGGCGTGGTCGCCGTCGCT-3′
HfxTrpA-R	5′-CTTCCGTTCTCGGAGCCCAT-3′
HfxHdrB-F	5′-CGTGGCACCTCCCCGAGGAC-3′
HfxHdrB-R	5′-GTCGCCTTCGTGTGCCGCGT-3′
HfxMCM-NTD-5Bsa	5′-TGTGTGGTCTCTGCGCCGCGCAGGCCCCCCAGAACCGTGACCT-3′
HfxMCM-NTD-3Bsa	5′-GTTGTTGTGTGGTCTCGTATCAGACCATCTTCTCGTAGAT-3′

Primers used for Northern blot analysis (shown in Figure 2A)	
oHv189-Hv-16S-003	5′-CTCCTCCGGTTGTAGTGCTC-3′
oHv190-Hv-S2-004	5′-cgtcggggtggtctcggtcg-3′
oHv194-Hv-23S-008	5′-CCTCGGCTGATTGACAGTGCC-3′

Primers used for *in vitro* transcription	
T7+h31 Fw	5′-TAATACGACTCACTATAGGGTTAATTTGACTCAACACGGGGAAA-3′
T7+h31 Rv	5′-TTTCCCCGTGTTGAGTCAAATTAACCCTATAGTGAGTCGTATTA-3′
T7+h31-Stab Fw	5′-TAATACGACTCACTATAGGGTTAATTTGACTCAACACAAAGAAA-3′
T7+h31-Stab Rv	5′-TTTCTTTGTGTTGAGTCAAATTAACCCTATAGTGAGTCGTATTA-3′
T7-18S-1169-80 Fw	5′-TAATACGACTCACTATAGGGgagcctgcggc-3′
T7-18S-1169-80 Rv	5′-gccgcaggctcCCCTATAGTGAGTCGTATTA-3′
T7-18S-1458-70 Fw	5′-TAATACGACTCACTATAGGGcacgcgcgctac-3′
T7-18S-1458-70 Rv	5′-gtagcgcgcgtgCCCTATAGTGAGTCGTATTA-3′
Note: Synthesized RNA is indicated in bold	

Synthetic RNA	
18S rRNA 1169-1180	5′-ggagccugcggc-3′
18S rRNA 1458-1470	5′-gcacgcgcgcuac-3′
18S rRNA h31-Stab	5′-uuaauuugacucaacacaaagaaa-3′
Note: All RNA were synthesized at Biomers and HPLC purified	

Supplementary References

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