

Identification of the ISWI chromatin remodeling complex of the early branching eukaryote
Trypanosoma brucei

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Background: Eukaryotes typically encode a range of ISWI chromatin remodeling complexes with different functions.

Results: We have identified and analysed three novel ISWI partners in the early branching eukaryote *Trypanosoma brucei*.

Conclusions: *T. brucei* appears to have a single major ISWI complex.

Significance: This unusually simple ISWI configuration could be a consequence of the relative lack of transcriptional regulation in this ancient eukaryote.

ABSTRACT

ISWI chromatin remodelers are highly conserved in eukaryotes and are important for the assembly and spacing of nucleosomes, thereby controlling transcription initiation

and elongation. ISWI is typically associated with different subunits, forming specialised complexes with discrete functions. In the unicellular parasite *Trypanosoma brucei* which causes African sleeping sickness, TbISWI downregulates RNA polymerase I (Pol I) transcribed Variant Surface Glycoprotein (VSG) gene expression sites (ES) which are mono-allelically expressed. Here, we use tandem affinity purification to determine the interacting partners of TbISWI. We identify three proteins which do not show significant homology with known ISWI associated partners. Surprisingly, one of these is nucleoplasmin-like protein (NLP), which we had previously shown to play a role in ES control. In addition, we identify two novel ISWI partners: Regulator of Chromosome Condensation 1-like protein

(RCCP) and phenylalanine/ tyrosine rich protein (FYRP), both containing protein motifs typically found on chromatin proteins. Knock-down of RCCP or FYRP in bloodstream form *T. brucei* results in derepression of silent VSG ESs, as had previously been shown for TbISWI and NLP. All four proteins are expressed and interact with each other in both major life-cycle stages, and show similar distributions at Pol I transcribed loci. They are also found at Pol II strand switch regions as determined with ChIP. ISWI, NLP, RCCP and FYRP therefore appear to form a single major ISWI complex in *T. brucei* (TbIC). This reduced complexity of ISWI regulation and the presence of novel ISWI partners highlights the early divergence of trypanosomes in evolution.

Eukaryotes package their genomic DNA into chromatin, whereby DNA is wrapped around octamers of histones forming nucleosomes. This allows the compaction of extensive stretches of DNA into the restricted space of the nucleus, as well as being a major factor in controlling DNA access. For example, the exact phasing or degree of compaction of nucleosomes can either block or expose promoter sequences to recognition by the transcriptional machinery (1,2). Chromatin remodeling therefore plays a major role in the regulation of gene expression, in addition to a range of other processes including chromosome segregation, DNA replication and repair (3-6).

Chromatin remodelers in the ISWI family are highly conserved among eukaryotes, and play a critical role in nucleosome assembly and spacing, as well as in the organisation of chromatin at a higher level in the cell (7-10). ISWI has a highly conserved SWI2/SNF2-family ATPase domain which provides the motor for chromatin remodeling, and characteristic HAND-SANT-SLIDE domains with DNA binding activity (3,5). Using DNA-dependent ATPase activity, ISWI remodelers change nucleosome spacing to promote chromatin

assembly, which often results in the repression of transcription (11,12). In addition to their role in remodeling existing nucleosomes, they can also facilitate the *de novo* assembly of nucleosomes in concert with core histone chaperones (13).

ISWI invariably functions as part of a complex and different eukaryotes have a diverse array of ISWI complexes, each with a discrete function (8). It is becoming increasingly clear that the ISWI partner subunits have a regulatory role, and determine ISWI complex function (8,10). In *Saccharomyces cerevisiae* there are two different ISWI variants (Isw1 and Isw2) which in combination with different subunits, form a total of four different complexes (10). ISWI (Isw1) together with the Ioc3 subunit forms the Isw1a complex which binds Pol II promoters and excludes the basal Pol II transcription machinery, thereby preventing transcription initiation (14). In contrast, Isw1 partnered with the Ioc2 and Ioc4 subunits forms the Isw1b complex, which regulates Pol II transcription elongation and termination (15-17).

In *Drosophila melanogaster* six different functional ISWI complexes have been identified (CHRAC, ACF, NURF, RSF, ToRC and NORC), each containing ISWI bound to various combinations of nine different subunits (10,18). Among these, the CHRAC and ACF complexes appear to have general roles in facilitating nucleosome sliding (19,20). NURF appears to be particularly important for the epigenetic regulation of stem cells within the testis (21). RSF has a role in assembly of chromatin through the replacement of histone variants, in addition to chromatin remodeling activities (22). ToRC is involved in the regulation of Pol II transcription (23), while NoRC is a nucleolar chromatin remodeling factor involved in silencing Pol I mediated transcription of the rDNA repeats (24).

In mammalian cells (where the ISWI equivalents are referred to as SNF2H or SNF2L/ SMARCA1), at least seven different ISWI complexes have a similar broad range of functions including facilitating DNA repair

(25,26), activating Pol III transcription (27) or playing a role in the differentiation of somatic cells (28). Similar to in *Drosophila*, a NoRC complex is also present which mediates the epigenetic regulation of rRNA genes, as well as heterochromatin formation at repetitive regions including the telomeres and centromeres (29-31).

The African trypanosome *Trypanosoma brucei* is a unicellular eukaryote and causative agent of African sleeping sickness (32). Trypanosomes are evolutionarily separated from eukaryotic model organisms, and are in a different eukaryotic supergroup (Excavata) from animals and fungi (Opisthokonta) (33). As a consequence *T. brucei* has unexpected features including the organisation of its genome. Unusually, trypanosome chromosomes consist predominantly of extensive polycistronic transcription units which are constitutively transcribed by RNA polymerase II (Pol II) (34-36). There is no evidence for regulated Pol II transcription in *T. brucei*. Levels of Pol II-derived transcripts are controlled post-transcriptionally through a variety of mechanisms, including co-transcriptional RNA degradation as well as RNA stability elements (37,38).

Another unusual feature is that RNA polymerase I (Pol I) transcribes a subset of protein coding genes in addition to the rDNA (39). These include the genes encoding the Variant Surface Glycoprotein (VSG), which forms an essential protective coat on the bloodstream form trypanosome (40,41). Although an individual trypanosome can have a repertoire of more than 2000 VSGs (42,43), only one VSG is transcribed at a time from one of about 15 telomeric VSG expression sites (ES) (44,45). The molecular mechanisms behind this mono-allelic control of VSG ESs still remain to be elucidated.

What is the role of chromatin in an organism which has little transcriptional control, and does not regulate Pol II transcription units? First of all, chromatin proteins are likely to be important for Pol II transcription in *T. brucei*. Putative Pol II transcription initiation sites have a simple

structure lacking canonical Pol II promoter elements (35). No defined motifs for Pol II promoters have yet been identified, however the H4K10ac and H3K4me3 histone modifications and H2AZ and H2BV histone variants are enriched at the probable sites of transcription initiation (35,46). It is therefore likely that these epigenetic marks play an important role in defining a functional Pol II promoter.

In addition, it is now clear that chromatin remodeling plays a key role in the control of VSG ESs. The active VSG ES is highly depleted of nucleosomes compared with the silent ESs (47,48). In addition, a steadily increasing number of chromatin proteins, chromatin remodelers, and histone modifiers have now been shown to impact on VSG ES transcriptional control (49-52).

The first chromatin remodeler discovered to play a role in VSG ES regulation is TbISWI (53). Knock-down of TbISWI results in 30-60 fold derepression of a reporter inserted immediately downstream of a silent ES promoter, as well as transcriptional read-through in the silent telomeric ESs extending to the telomeric VSGs (53,54). In addition to the role of TbISWI in silencing VSG ESs, TbISWI was also found to be enriched at transcriptional strand switch regions (SSRs) containing Pol II promoters and terminators (35,54). As ISWI is invariably part of different functional complexes in other eukaryotes, we attempted to elucidate the role of ISWI complex(es) in *T. brucei*.

Here we identify and analyse three novel ISWI partners in *T. brucei* which are expressed in both the bloodstream form (BF) and the procyclic form (PF) present in the tsetse fly insect vector. Surprisingly, these ISWI interacting proteins include the nucleoplasmin-like protein NLP, which we have previously shown to have a similar role to TbISWI in downregulating ESs (55). We also identify two previously uncharacterised proteins: RCCP and FYRP. All our experimental evidence points to the presence of a single major ISWI complex in *T. brucei*, although we cannot rule out the presence of minor sub-complexes. This relatively simple

configuration of ISWI could be a consequence of the relative lack of extensive transcriptional control in this primitive eukaryote.

EXPERIMENTAL PROCEDURES

Trypanosome strains and culturing

Procyclic form (PF) *T. brucei brucei* 427 was maintained at 27°C in SDM-79 medium supplemented with 10% heat inactivated fetal calf serum and 5 mg ml⁻¹ haemin (56). Bloodstream form (BF) *T. brucei* 427 was cultured at 37°C in HMI-9 medium supplemented with 15% fetal calf serum (57).

For tandem affinity purification (TAP), TbISWI (GeneDB: Tb927.2.1810) and NLP (GeneDB: Tb927.10.5450) were tagged at the C-terminus with a PTP epitope (Prot C-TEV protease site-Prot A) (58) in PF *T. brucei* 427. In order to ensure functionality of the TbISWI-PTP protein, PF lines were generated where the second TbISWI allele was knocked out using the pSpot5KOPhleo construct (54). Similarly, the NLP-PTP protein was shown to be functional through generation of cell lines where the second NLP allele was knocked out using the pBSphleoNLPKO construct (55).

For the co-immunoprecipitation experiments, proteins were tagged *in situ* at the endogenous locus at the C-terminus using either a triple myc-epitope or a triple HA-epitope using either the pMoTAG42M or pMoTAG4H constructs (59). These constructs were transfected into wild type PF cells or the BF 221GP1(VO2+) line (60). This line has an active *VSGVO2* expression site which is maintained using G418 selection, and the *eGFP* and puromycin resistance genes in the silent *VSG221* expression site.

For the expression site derepression and growth rate studies, the BF *T. brucei* T3-SM cell line was used (53). This cell line is a derivative of the 'single marker' cell line (61), and contains an active *VSGT3* expression site maintained with blasticidin selection, and silent *eGFP* and *VSG221* genes in the inactive *VSG221* expression site. RNAi constructs to

knock down either RCCP (GeneDB: Tb927.11.10330) or FYRP (GeneDB: Tb927.7.1060) were integrated into the *T. brucei* minichromosomes to generate either *T. brucei* T3-RCCP or *T. brucei* T3-FYRP.

The BF *T. brucei* 90-13 cell line (61), was used for the introduction of both epitope tagged proteins and RNAi constructs. RNAi was induced with tetracycline to monitor for knock-down of FYRP in the FYRP-HA epitope tagged line.

DNA constructs

TbISWI was tagged at the C-terminus with the PTP epitope for tandem affinity purification (TAP) using the pC-PTP construct as described in (58). The last 899 bp of the TbISWI C-terminus was amplified and cloned into the pC-PTP-hygro vector. This was digested with *Ava*I, and integrated via a single crossover. The final 687 bp of the NLP C-terminus and positions 68-790 of the NLP downstream sequence were amplified and cloned into the pC-PTP-hygro vector. This was digested with *Apa*I and *Sac*I, and integrated via a double crossover. TbISWI and NLP were epitope tagged with the triple myc/ and or HA epitopes as previously described (54,55). A 490 bp fragment (positions 1449-1938) of the RCCP open reading frame (ORF) was amplified and a 500 bp fragment of the 3' downstream region (positions 1-500) were inserted into the pMOTag4H or pMOTag43MB vector. A 494 bp fragment (positions 989-1173) of the FYRP ORF was amplified, and a 370 bp fragment (positions 1-370) of the 3' downstream region were inserted into the pMOTag4H or pMOTag43MB vector. The p2T7-177_hygro construct targeted to minichromosomes was used for RNA interference experiments (62). A 485 bp fragment (positions 319-803) of the RCCP ORF and a 477 bp fragment (positions 697-1173) of the FYRP ORF were inserted between the opposing T7 promoters of this construct.

Tandem Affinity Purification

The C-termini of TbISWI and NLP were tagged with the ProtC-TEV protease site-Prot A (PTP) epitope (58), and tandem affinity purification (TAP) was performed as described in (63). Briefly, approximately two litres of PF *T. brucei* expressing PTP-epitope tagged TbISWI or NLP were lysed by douncing and shock-frozen. Proteins were extracted on ice for 20 minutes and the lysates were centrifuged twice at 20,000 g at 2°C. The supernatant was incubated at 4°C with equilibrated IgG Sepharose Fast Flow bead suspension (GE Healthcare) in a Poly-Prep chromatography column (BioRad) with protease inhibitors for 3.5 hours. The beads were washed and the Protein A portion of the tag was removed by the addition of AcTEV protease (Invitrogen). The TEV eluate was incubated with Anti-ProtC matrix (Roche) in a fresh Poly-Prep column overnight at 4°C. The Anti-ProtC matrix was washed and the final TAP purified material was eluted with EGTA. The purified product was concentrated using a vacuum concentrator and StrataClean resin (Stratagene) before separation under denaturing or non-denaturing conditions on 4-15% SDS-PAGE or PAGE gels (BioRad). Bands were visualised with Imperial Protein Stain (Thermo Scientific) or Silver Stain (Thermo Scientific), excised as specified, and subjected to analysis by liquid chromatography-tandem mass-spectrometry (Central Proteomics Facility, University of Oxford).

Co-immunoprecipitation

For co-immunoprecipitation (IP) analysis, TbISWI, NLP, RCCP and FYRP were tagged at the C-terminus with either a triple myc-epitope using the pMoTAG43M vector (59) or with a triple HA epitope using the pMoTAG4H vector (54,55,59) in PF and BF cell lines. Cell extracts were prepared as for the TAP-tagging protocol (63), except that 0.1% NP-40 was added while extracting protein. Sepharose CL-4B columns (GE Healthcare) were prepared with ice-cold IP buffer (150 mM sucrose, 20 mM L-glutamic acid, 20 mM HEPES-KOH (pH 7.7), 3 mM

MgCl₂, 1 mM DTT, 150 mM KCl, 0.1% NP-40) and incubated with either monoclonal anti-HA (Ab1424 Abcam) or anti-myc (Sigma M5546) antibodies or no antibody for 2 hours at 4°C. Crude extract (100 µl) was added to the columns with the immobilised antibodies and incubated for 2 hours at 4°C. Washes were carried out with ice-cold wash buffer (20 mM HEPES-KOH pH 7.7, 3 mM MgCl₂, 150 mM KCl, 0.1% NP40). Purified proteins were eluted into boiling SDS-PAGE loading buffer, boiled for 5 minutes and centrifuged at 1000 g for 7 minutes. The supernatant was removed and 15 µl loaded onto either 8 or 10% polyacrylamide gels.

Flow cytometry

RNAi was induced in the BF *T. brucei* T3, T3-FYRP and T3-RCCP cell lines, cells were harvested at different time points, washed once in PSG buffer, and fixed in 2% paraformaldehyde. These cell lines contain an *eGFP* reporter gene inserted behind the promoter of a silent *VSG221* expression site. Fluorescence of the cells was monitored in the FL-1 channel using a Becton Dickinson FACS Calibur (BD Biosciences). CellQuest software (BD Biosciences) was used to calculate the average of 100,000 events (BD Biosciences). The fold ES derepression was calculated by dividing the average FL-1 fluorescence of RNAi induced populations at each time point by the average FL-1 fluorescence of uninduced populations. Three independent experiments were performed with each cell line with the standard deviations shown with error bars.

Analysis of nucleic acids and proteins

The BF *T. brucei* T3-FYRP and T3-RCCP cell lines were used for quantitative reverse-transcriptase PCR (qRT-PCR). RNAi was induced against FYRP or RCCP, and total RNA was isolated at various time points using the RNeasy kit (Qiagen). RNA was treated with DNase using the TURBO DNA-free kit (Ambion) and reverse transcription carried out using the Omniscript RT kit (Qiagen) with random hexamer primers (Promega). qPCR was performed on

a 7500 Fast Real-Time PCR System (Applied Biosystems) using Brilliant II SYBR Green (Agilent Technologies). Primers were used which our bioinformatic analyses indicated would recognise single copy sequences within the *T. brucei* genome (results not shown), and the reaction conditions for each primer pair were individually optimised. Control reactions without reverse transcriptase (RT) were performed using DNase-treated RNA for each time point. Transcript levels were normalised to levels for γ -tubulin transcripts and plotted as fold increase with respect to the 0 hour time point. Three independent experiments were performed with standard deviation shown with error bars.

Whole cell protein lysates were prepared by washing cells once in PSG buffer, followed by lysis in boiling hot 1X Laemmli buffer at an end concentration of 10^5 cells/ μ l. This was incubated at 100°C for 10 minutes prior to loading on 6% or 10% SDS-polyacrylamide gels. Gels were blotted on to Hybond-P membrane (Amersham), and probed with rabbit polyclonal antibodies against protein A (Prot A), BiP (gift of Jay Bangs) (64), TbISWI (53), NLP (55), HA tag (AbCam ab9106), myc-tag (AbCam ab9110), TDP1 (gift of Klaus Ersfeld)(65) and RCCP. ECL peroxidase labelled anti-rabbit IgG antibody (GE Healthcare) was used to detect bound antibodies and the blots were visualised with Western Lightning Plus ECL (Perkin Elmer) or ECL Plus (Amersham).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described in (55). HA-tagged copies of TbISWI, NLP and FYRP in the BF *T. brucei* GP1-221(V02+) cell line (60) were immunoprecipitated using a monoclonal anti-HA antibody (Abcam ab1424) and compared with experiments performed with the parental cell line. RCCP was immunoprecipitated using polyclonal rabbit antiserum raised against RCCP, and as a control the same amount (20 μ l) of rabbit pre-immune serum was used. As a negative control, samples where no antibody was used were included for all ChIPs. The ChIP

material was analysed using quantitative PCR (qPCR), and the final values for the percentage immunoprecipitated were obtained by subtracting the relevant no antibody control from the HA or RCCP ChIP and then dividing by the total input. Three independent experiments were performed for TbISWI, FYRP and RCCP and standard deviation is shown with error bars. One representative NLP ChIP was performed and analysed by qPCR, since this has already been investigated in (55). Statistical analyses were performed using a One Way ANOVA test followed by a Tukey test to compare pairs of values.

RESULTS

Identification of TbISWI partners

In general in eukaryotes, ISWI is present in one or more functional complexes (10). We performed BLAST searches of the *T. brucei* genome with the sequences of ISWI partners in other organisms, but were unsuccessful in detecting potential homologues in *T. brucei*. In order to identify TbISWI interacting proteins, we used a Tandem Affinity purification (TAP) tagging approach with a ProtC-TEV-ProtA (PTP) epitope tag (58). We generated a procyclic form (PF) *T. brucei* cell line expressing a C-terminal PTP-tagged TbISWI protein from its endogenous locus (Fig. 1A). The second allele of TbISWI was knocked out (ISWI-sKO) without resulting in a growth defect, demonstrating that PTP-tagged TbISWI is fully functional (Fig. 1A).

This cell line was used for the TAP tagging procedure (Fig. 1B, Fig. 1C). Briefly, a crude protein extract was first purified by IgG affinity chromatography, and the Tobacco Etch Virus (TEV) protease was used to cleave off the ProtA portion of the PTP tag. Subsequently, the TEV protease eluate underwent anti-ProtC affinity purification and the final purified products were eluted with EGTA. The concentrated proteins, along with fractions obtained throughout the purification procedure were separated by SDS-PAGE under denaturing conditions (Fig. 1B, Fig. 1C).

We monitored the enrichment of TbISWI-PTP by Western blot using an anti-TbISWI antibody (Fig. 1B) to show that the purification was successful. The same samples were separated by SDS-PAGE and stained with Coomassie where a range of bands were easily detectable in the final eluate (Fig. 1C). These bands were excised and the associated proteins were identified by liquid chromatography/ tandem mass spectrometry (LC-MS/MS). As expected, TbISWI-P was recovered in the EGTA eluate (Fig. 1C) (108 unique peptide hits), as well as histones H2A, H2B and H4. As ISWI is a nucleosome remodeler, this interaction with histones is not unexpected. However, three additional potential TbISWI partners were discovered with molecular weights of approximately 110 kDa, 70 kDa and 55 kDa, with 70, 32 and 26 unique peptide hits respectively.

Unexpectedly, one of these TbISWI interacting proteins (110 kDa) was the Nucleoplasmin-like protein (NLP), which contains an AT-hook and a nucleoplasmin-like domain, and is essential in BF *T. brucei* (Fig. 2A) (55). We had previously shown that NLP plays a role in ES silencing, and knock-down of NLP results in 45-65x derepression of an *eGFP* reporter gene inserted immediately downstream of a silent ES promoter (55). Additionally, two TbISWI interacting proteins that had not been previously characterised were identified. We named the 70 KDa protein RCCP (Tb927.11.10330), as it has four Regulator of Chromosome Condensation 1 (RCC1)-like domains (Fig. 2A). The human RCC1 protein is a cell cycle regulator that binds chromatin, and acts as a guanine nucleotide exchange factor for Ran GTPase (66).

The 54 KDa TbISWI interacting protein was named FYRP (Tb927.7.1060), as it has an N-terminal phenylalanine/ tyrosine rich (FYR) domain (Fig. 2A). FYR domains are poorly characterised, but have been found in chromatin associated proteins including histone methyltransferases, such as trithorax (67,68). As NLP was one of the TbISWI interacting proteins, we subsequently also performed tandem affinity purification with

NLP tagged with the PTP epitope (results not shown). Again, we identified TbISWI, RCCP and FYRP with a significant number of unique peptide hits (95, 46 and 26 respectively).

We next investigated if TbISWI and its interacting partners were conserved in a range of different eukaryotic species (Fig. 2B). TbISWI is orthologous to ISWI in *Saccharomyces cerevisiae* and SMARCA1/ SNF2L in *Homo sapiens*. This protein is very highly conserved across eukaryotes, with orthologs present in all species analysed from a wide range of lineages. In contrast, TbISWI-interacting proteins appear to be restricted to the Kinetoplastida. NLP and FYRP homologs are only identifiable in this lineage. RCCP is an RCC1 repeat domain-containing protein, of which there are several members in trypanosomes and most other eukaryotes. However, the RCCP paralog itself is specific to the Kinetoplastida (Fig. 2B).

TbISWI interacts with its partners forming the TbISWI complex (TbIC) in T. brucei

Is ISWI present in one or multiple complexes in *T. brucei*? Typically, in different eukaryotes ISWI is a component of a number of functional ISWI complexes, with discrete roles depending on the composition of the subunits (8,10). We investigated if the potential TbISWI partners identified through TAP affinity purification were indeed interacting with TbISWI, and with each other. We performed co-immunoprecipitation (Co-IP) experiments in PF cells which contained myc-tagged TbISWI and HA-tagged RCCP or FYRP proteins. Immunoprecipitation with either anti-myc or anti-HA monoclonal antibodies was followed by Western blot analysis to determine if other potential TbISWI complex components were co-purified. (Fig. 3). An anti-myc antibody was used to detect TbISWI-myc (138 KDa), polyclonal anti-NLP antibody to detect NLP (107 KDa) and anti-HA antibody to detect RCCP-HA and FYRP-HA (74 KDa and 57 KDa respectively). NLP, RCCP and FYRP were all co-purified when TbISWI was pulled

down. We also found that TbISWI and NLP co-purified when RCCP or FYRP were immunoprecipitated.

Further Co-IP experiments were performed in PF cells with different combinations of tagged proteins, and it was shown that when FYRP is pulled down, RCCP is co-purified and vice versa (Fig. 3). Co-IP experiments showed similar interactions between TbISWI and its proposed partners in BF cells (Fig. 4). These extensive Co-IP experiments argue that there is at least one ISWI complex containing TbISWI, NLP, RCCP and FYRP, and that all members of this complex interact with each other in both BF and PF life-cycle stages of *T. brucei*.

To elucidate whether TbISWI forms one complex or multiple subcomplexes, TbISWI-PTP and its co-purified components from the TAP affinity purification experiments were separated under nondenaturing conditions and silver stained (Fig. 5A). Similarly, the same experiment was performed with TAP affinity purified NLP-PTP (Fig. 5B). The visible bands were excised and analysed by mass spectrometry. A predominant major band was seen in both cases, corresponding to either TbISWI or NLP complexed with each other and with RCCP.

FYRP was detected in both experiments, albeit below the threshold score of 80, indicating weak association with this complex. However, based on its score in the initial TbISWI and NLP TAP tagging experiments and the extensive Co-IP experiments, we are confident that FYRP is a true member of the TbISWI complex. Additional minor bands observed below the main band contain different stoichiometries of complex partners indicating possible different degradation states of a single complex. These data therefore indicate that there is a single major *Trypanosoma brucei* ISWI complex (TbIC) (Fig. 5C). However, we cannot exclude the presence of additional minor subcomplexes composed of just some of the TbISWI complex subunits.

Depletion of FYRP or RCCP results in derepression of silent VSG expression sites

We have previously established that both TbISWI and NLP play a role in ES silencing (53,55). We investigated the role of RCCP and FYRP on ES control using a BF *T. brucei* VSGT3 expressing reporter cell line where eGFP had been inserted immediately downstream of the promoter of the inactive VSG221 ES (53). RNAi was induced against RCCP, resulting in a reduction in transcript levels to about 60% normal levels, with a simultaneous reduction in levels of protein (Fig. 6). Only a minor reduction in cell growth was observed. However, there was an observed 17-37 fold derepression of eGFP in the silent VSG221 ES after 72 hours as monitored in the FL-1 channel using flow cytometry (Fig. 6C).

We performed a similar analysis of the role of FYRP (Fig. 7). FYRP transcript was reduced to 50% normal levels after 24 hours. FYRP protein knock-down was investigated using a cell line with an HA tagged copy of FYRP, which was knocked down to undetectable levels after 96 hours induction of RNAi (Fig. 7E). Here too, although the induction of RNAi resulted in only a minor reduction in cell growth (Fig. 7B), there was 26-61 fold derepression of the silent VSG221 ES.

Genomic localisation of the TbISWI complex

The native gels and the co-IP experiments suggested that there is a single predominant TbISWI complex (TbIC) in *T. brucei*. However, to investigate this further we determined the genomic localisation of the four potential components using chromatin immunoprecipitation (ChIP) experiments. ChIP was performed in different BF cell lines expressing either HA-tagged TbISWI, HA-NLP or HA-FYRP using a monoclonal anti-HA antibody. Multiple attempts of ChIP using HA epitope tagged RCCP proved unsuccessful, indicating possible lack of accessibility of the HA epitope to antibodies when the ISWI complex is in association with DNA. We therefore used a rabbit polyclonal

antibody against RCCP in the RCCP ChIP experiments.

We first investigated the localisation of the TbISWI complex components at the RNA polymerase I (Pol I) transcribed rDNA loci (Fig. 8A). TbISWI and NLP are relatively depleted within Pol I transcription units but enriched at non-transcribed regions (53,55). This pattern of localisation was also observed for RCCP and FYRP (Fig. 8B). In the case of FYRP, the statistical significance of this differential localisation was extremely significant ($p < 0.001$) (primers a vs primers b or d, or primers e vs primers b, c or d). In the case of RCCP, although there was a trend, this was not statistically significant. Similarly, at the Pol I transcribed procyclin loci (Fig. 8C), TbISWI and NLP are relatively enriched upstream compared with within the transcription units (53,55). This was also the case for both RCCP and FYRP with a statistical significance of ($p = 0.01-0.05$) (primers a vs primers b or c) in both cases (Fig. 8D).

Pol II transcription units in *T. brucei* are polycistronic. Pol II transcription initiates in strand switch regions (SSRs) where two opposing transcription units diverge, and terminates where they converge. TbISWI was proposed to be enriched at these SSRs, and particularly in the regions around divergent SSRs containing promoters (54). ChIP experiments with ISWI are very difficult to perform, presumably as a consequence of the relatively low affinity of this chromatin remodeler for DNA.

TbISWI, NLP, RCCP and FYRP appeared to bind regions around different Pol II SSRs (Fig. 9). In parallel, ChIP experiments were also performed with histone H3, serving as a positive control for the ChIP procedure (result not shown). There was possible colocalisation of ISWI subunits at the SSR divergent regions D1 and D2, however these results were not statistically significant. All members of the TbISWI complex associate with chromatin, and show a trend of localising to similar genomic regions, which is statistically significant at Pol I loci. All of these different experimental

approaches which show all TbIC components interacting and present at a variety of genomic loci argue that there is a single predominant ISWI complex in *T. brucei*.

DISCUSSION

In eukaryotes, the ISWI chromatin remodeler is typically present in a variety of different complexes with distinct functions depending on exactly which subunits ISWI is partnered up with. Here, we have characterised TbISWI and its interacting partners in *T. brucei* and provide evidence for a single major ISWI complex (TbIC) in both BF and PF *T. brucei*. Using a number of different experimental methods we show that all of the TbIC subunits are expressed and interact with each other in both trypanosome life-cycle stages. The previously characterised nucleoplasmin-like protein NLP was found to be a member of this TbIC complex. This unexpected discovery explains the observation that knock-down of either TbISWI or NLP both lead to similar phenotypes including the derepression of VSG ESs. In addition, using TAP affinity purification with either TbISWI or NLP, we identify two novel and previously uncharacterised TbIC components: RCCP and FYRP. Neither of these ISWI partners is a homologue of known ISWI partners in other eukaryotes. However, both proteins contain amino acid sequence motifs indicating a possible interaction with chromatin.

The TbISWI interacting RCCP protein contains four RCC1 (Regulator of Chromosome Condensation 1) protein motifs which characterise the RCC1 superfamily of proteins (66). The RCC1 family is a diverse group of proteins which contain variable numbers of the RCC1-like domain, with a tertiary structure resembling a seven-bladed propeller (69). RCC1 is the best characterised member of this family and is a DNA binding protein which regulates the onset of chromosome condensation (70). RCC1 is localised to chromatin throughout the cell cycle, and is a guanine nucleotide exchange factor for Ran (71-73). RCC1 binds nucleosomes, recruits Ran to the chromatin

and activates Ran's nucleotide exchange activity (72,74). It therefore plays a central role in establishing the RanGTP concentration gradient around the chromosome, which is key for a number of processes to occur including mitosis (75-77). In this regard, it is interesting that it has been reported that in *Xenopus*, ISWI is a RanGTP dependent microtubule associated protein required for chromosome segregation (78). Although in *T. brucei* knock-down of TbISWI and its subunits leads to derepression of VSG ESs, we have not seen obvious disruption of chromosome segregation.

In contrast, the TbISWI interacting protein FYRP is characterised by a FYRC domain. FYRC protein motifs contain a phenylalanine and tyrosine-rich region which is poorly characterised and is found in an assortment of chromatin associated proteins (68). FYRC domains are typically found in association with protein modules that recognise histone modifications (79). FYRC motifs have been identified in the *Drosophila* trithorax protein, involved in the epigenetic regulation of gene expression during fly development, and X-chromosome interacting proteins (67).

One possibility that could explain our data is that in *T. brucei*, FYRP is the most prone to disassociate from the TbIC ISWI complex compared with the other three subunits. Although we repeatedly identified FYRP by mass spectrometry using TAP affinity purification with either ISWI or NLP as bait, the score was consistently the lowest of the four TbIC components. In addition, FYRP was not identified in the TbIC complex using native gel analysis. However, Co-IP experiments showed clear interaction of FYRP with every other TbIC subunit (TbISWI, NLP and RCCP). In addition, ChIP experiments showed a trend for localisation of FYRP with other TbIC members on similar regions of genomic DNA. Similarly, knock-down of FYRP also lead to comparable derepression of silent VSG ESs as observed after knock-down of the other TbIC subunits. Our data therefore indicate that FYRP could have a weak or transient interaction with other

complex members, making it prone to disassociation during protein affinity purification.

Is there indeed only one ISWI complex in *T. brucei*? Both the TbISWI and NLP affinity purification experiments identified each other, as well as the RCCP and FYRP subunits. In addition as mentioned above, co-IP experiments in both life-cycle stages show that all four components interact with each other, and ChIP experiments indicate that all four proteins associate with similar regions of genomic DNA. Therefore, all of the available evidence using a variety of different experimental approaches would argue that a single predominant TbISWI complex is present in the early branching eukaryote *T. brucei*. As expected for subunits participating in the same complex, knock-down of each of these TbIC subunits leads to VSG ES derepression. However, these experiments do not rule out the presence of minor TbISWI complexes containing a subset of the subunits.

Chromatin remodelers including ISWI complexes are extremely difficult to analyse using ChIP (80). This may be indicative of the transient nature of the interactions between these remodeling complexes and specific DNA sequences as they move along the genome changing nucleosome spacing (81). Despite these technical hurdles, co-localisation of ISWI with different interacting subunits using ChIP can indicate the presence of discrete functional ISWI complexes at different genomic locations (80). Previous ChIP analyses of TbISWI have argued that there is a possible enrichment of TbISWI at the Pol II SSRs which contain transcriptional boundaries including Pol II promoters and terminators (35,54). This is comparable with what has been found in other organisms including *S. cerevisiae*.

In *S. cerevisiae* ISWI is important for regulation of Pol II transcription, and ISWI variants are found both within Pol II gene bodies as well as at both promoters and terminators. The Isw1 variant has different functions depending on which Ioc subunits it

is partnered up with (14,17). Isw1 in complex with Ioc3 forms the Isw1a complex which represses initiation of transcription at Pol II promoters (15). In contrast Isw1 partnered up with the Ioc2 and Ioc3 subunits forms the Isw1b complex, which either acts within Pol II coding regions to control elongation of transcription, or alternatively facilitates transcription termination (15). The Isw2 ISWI variant is particularly enriched at the nucleosome depleted region around Pol II promoters, where it appears to play a role in maintaining a high density of nucleosomes within the Pol II transcribed gene bodies (81). This reduces the amount of inappropriate Pol II transcription initiation from gene internal cryptic sites and suppresses anti-sense transcription.

In *T. brucei*, we found a trend for TbISWI and the NLP, RCCP and FYRP subunits binding at both divergent and convergent Pol II strand switch regions, however this data supporting four proteins being relatively enriched in these regions was not statistically significant. This relative simplicity of ISWI complex architecture could be a consequence of the lack of control of Pol II expression in *T. brucei* either at the level of transcription initiation or elongation (82).

In most eukaryotes Pol I exclusively transcribes the rDNA arrays, of which typically about half are transcriptionally silent (83). ISWI variants also play a role in this regulation of Pol I, which in mammals is mediated by the ISWI containing NoRC complex consisting of ISWI (SNF2H) in complex with the TIP5 subunit (84). This NoRC complex mediates the formation of heterochromatin at both the silent rDNA repeats as well as at the centromeres (31). In *T. brucei* all of the TbIC components are located at the rDNA, particularly in the non-transcribed spacers. This is also the case at the Pol I transcribed procyclin loci and the ESs (85), although no particular enrichment

was observed at either active or silent ESs (54). As knock-down of all of the TbIC components leads to de-repression of silent ESs, it is clear that ISWI plays a role in regulation of Pol I transcription in *T. brucei*.

All of our experimental evidence therefore points to a single ISWI containing complex in *T. brucei*, which is a very early branching eukaryote, although we cannot rule out the presence of relatively minor sub-complexes. The apparent presence of all TbIC components at a range of different genomic loci including Pol II SSRs, as well as at different Pol I loci argues that the predominant TbIC complex could be multifunctional. Chromatin remodeling enzymes appear to have arisen soon after the origin of the eukaryotic lineage, and as eukaryotic genomes expanded in size and complexity there was an increasing need for a larger array of specialised chromatin remodeling factors (1). In common with other parasites, *T. brucei* appears to have a relatively reduced set of these chromatin remodelers, coupled with a greatly reduced set of Pol II transcription factors (1,86). Possibly as *T. brucei* evolved, large amounts of gene loss occurred as a consequence of the lack of the need for intricate control systems as the organism relied on constitutive transcription by Pol II. We show that the major *T. brucei* TbIC complex contains novel subunits compared with other non Kinetoplastid eukaryotes.

The challenge for us now is to understand the role of these unique chromatin remodelers in the maintenance of genome architecture in these ancient eukaryotes. In addition, hopefully increased knowledge of the role that these divergent chromatin remodelers play in transcriptional control, including that of the VSG expression sites, will allow us to disrupt this process and thereby leading to new forms of antiparasitic therapies.

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Author contributions: TS, MN, AL and GR designed the study. GR and SR wrote the paper. BW performed bioinformatic analyses. TS, MN, SR, AL, KW, MK, SW and JW performed experiments. All authors analysed the results and approved the final version of the manuscript.

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FOOTNOTES

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FIGURE 1.

Identification of ISWI partners in *T. brucei*.

A. Tagging TbISWI with the PTP epitope. Western blot analysis of whole cell protein lysates from procyclic form *T. brucei* lines probed with an anti-TbISWI antibody. Extracts from wild type cells (WT) are compared with those from cells where a single TbISWI allele was either C-terminally tagged with the PTP epitope (ISWI-PTP), or knocked out (ISWI-sKO), or in two clones (1.1 and 1.2) where one TbISWI allele was knocked out and the other allele was PTP tagged (ISWI-sKO/ ISWI-PTP). Relevant bands are indicated, including TbISWI tagged with PTP (ISWI-PTP), untagged ISWI, or a cross-reactive band (CRB) which functions as a loading control. The signal for ISWI-PTP is particularly strong, presumably as the tagged protein binds to both the primary and the secondary antibodies. An equivalent of 1×10^7 cells were analysed on a 6% gel. Size markers in kiloDaltons (kDa) are indicated on the left.

B. Enrichment of PTP-tagged TbISWI using the Tandem Affinity Purification (TAP) procedure. Samples isolated during the procedure were monitored using Western blot analysis of a 4-15% SDS-polyacrylamide gel. Samples from the input (1x), the IgG column flow-through (FT-IgG), the TEV protease eluate (TEV elu) (5x), flow-through from the anti-Prot C column (FT α -ProtC), or from the final eluate (EGTA elu), (20x) were compared. The blot was probed with an anti-TbISWI antibody. The location of TbISWI-PTP following TEV cleavage (ISWI-P) is indicated with an arrowhead.

C. Monitoring of TbISWI complex purification using Coomassie blue staining of an SDS-PAGE gel. A sample of the input (0.002% total) was compared with a sample of the IgG column flow-through (FT-IgG, 0.002%), the TEV eluate (TEV elu, 5% total), flow-through anti-Prot C column (FT α -Prot C, 0.08%) or the total concentrated EGTA eluate (EGTA elu, 100%). Bands were excised for mass spectrometry analysis, and the main hit for each band is indicated beside the arrowhead. In addition to TbISWI, three TbISWI interacting partners were identified: the previously identified Nucleoplasmin-like protein NLP, and two novel proteins which we called RCCP (~70 kDa) and FYRP (~54 kDa). Histones H2A, H2B and H4 (which ISWI interacts with) were also purified from the final eluate. Additional minor bands which are not labelled contained peptides corresponding to TbISWI, NLP, RCCP and FYRP. Size markers in kiloDaltons (kDa) are indicated on the left.

FIGURE 2.

T. brucei ISWI and its partners.

A. Schematics of TbISWI and its partners with relevant protein motifs indicated with coloured boxes. TbISWI has a SNF2_N domain, a helicase C domain, a HAND and a SLIDE domain. NLP contains an AT hook motif and a nucleoplasmin-like domain. RCCP contains four RCC1 (regulator of chromosome condensation) domains. FYRP contains a FYRC (F/Y rich C-terminus domain). The domains (with e-values indicated below) were identified using Pfam 27, with the exception of NLP where MyHits (ISB-SIB) and ISWI where Superfamily v1.75 were also used.

B. Conservation of TbISWI components across eukaryotes. Distribution of homologs was investigated by analysis of BLAST hits in the predicted proteomes of model species from a range of eukaryotic lineages. Spot size represents the strength of BLAST hit (e-value). Red shows reciprocal best-BLAST hits between proteomes (likely orthologs) and grey shows non-reciprocating hits (likely paralogs).

FIGURE 3.

Co-immunoprecipitation reactions show interaction between *T. brucei* ISWI and each of its partners in insect form trypanosomes. *T. brucei* cell lines were generated using procyclic form

trypanosomes (PF WT) containing a myc-tagged TbISWI and either an HA-tagged RCCP or HA-tagged FYRP (respectively ISWI-myc/ RCCP-HA or ISWI-myc/ FYRP-HA). Alternatively, cell lines contained HA-tagged NLP, and either myc-tagged RCCP or myc-tagged FYRP. Last, cell lines containing HA-tagged RCCP and myc-tagged FYRP were analysed. Using protein lysates from these cell lines, immunoprecipitation reactions were performed using either anti-myc (α -myc) or anti-HA (α -HA) monoclonal antibodies or a no antibody control (No Ab). These immunoprecipitated samples were separated on SDS-PAGE gels together with samples from the input (0.4% amount used for immunoprecipitation). Each blot was probed with antibodies against myc (α -myc), NLP (α -NLP), HA (α -HA), TbISWI (α -ISWI) or the chromatin protein TDP1 (α -TDP), which served as a negative control. Relevant proteins are indicated on the right with arrowheads. Protein size markers in kiloDaltons (kDa) are indicated on the left.

FIGURE 4.

Coimmunoprecipitation experiments show that TbISWI partners show similar interactions in bloodstream form as well as procyclic form *T. brucei*. Lysates from bloodstream form *T. brucei* GPI (VO2+) (BF WT) containing RCCP tagged with the HA epitope and FYRP tagged with the myc epitope (RCCP-HA/ FYRP-myc) were compared with a line containing TbISWI tagged with the myc epitope and FYRP tagged with the HA epitope (ISWI-myc/ FYRP-HA). Immunoprecipitation experiments were carried out with antibodies against the myc (α -myc) or HA epitope (α -HA) or a no antibody control (no Ab). These immunoprecipitated samples were separated on SDS-PAGE gels together with samples from the input (0.4% amount used for immunoprecipitation). Blots were probed with antibodies against TbISWI, NLP, HA, myc or the chromatin protein TDP as a negative control. Relevant proteins are indicated on the right with arrowheads. Sizes of protein markers are indicated on the left in kiloDaltons (kDa).

FIGURE 5.

Native gels show the presence of a single predominant TbISWI complex in *T. brucei*

A. TAP affinity purification was performed with lysates from procyclic form *T. brucei* containing TbISWI tagged with the PTP epitope. The purified material was separated on a 4-15% non-denaturing gel and silver stained. The bands which were excised and sent for mass spectrometry are indicated with numbers on the right. The proteins subsequently identified in each band are also indicated. The sizes of the native gel protein marker are indicated in kiloDaltons (kDa) on the left.

B. As in Panel A), only lysates were used from procyclic form cells where the TbISWI partner NLP was tagged with the PTP epitope.

C. A schematic of the *T. brucei* TbISWI complex (TbIC) with TbISWI and its different partners indicated with coloured balls.

FIGURE 6.

Knockdown of RCCP in bloodstream form *T. brucei* leads to *VSG* expression site derepression.

A. Schematic of the *T. brucei* T3-RCCP cell line containing a silent GFP gene in the *VSG221* expression site, and an active *VSGT3* expression site containing a blasticidin resistance gene (blast). RNAi against RCCP can be expressed from opposing tetracycline inducible T7 promoters. Relevant genes are indicated with coloured boxes, transcription is indicated with arrows, with expression site promoters shown with white flags and T7 promoters with black flags.

B. There is no significant reduction in growth after knockdown of RCCP. The parental T3 cell line and the T3-RCCP1 and T3-RCCP2 clones were incubated in the presence (+) or

absence (-) of tetracycline. The cumulative growth was plotted over time, with error bars indicating the standard deviation from three replicate experiments.

C. Depletion of RCCP leads to 17-37 fold derepression of the silent *VSG221* expression site as monitored using GFP. The upper panel shows representative flow cytometry traces in the FL-1 channel either before or after induction of RCCP RNAi with tetracycline (Tet) for three days. The bottom panel shows the mean fold derepression in the T3-RCCP1 or T3-RCCP2 clones compared with the parental cell line (T3) after the induction of RCCP1 RNAi for the time indicated in days. Error bars indicate the standard deviation from three independent experiments.

D. Knockdown of RCCP transcript after the induction of RCCP RNAi for the time indicated in days. Transcript levels were determined using qRT-PCR, normalised using γ -tubulin and are shown relative to the 0 hours timepoint. The results shown are the average of three independent experiments with error bars showing the standard deviation.

E. Reduction in levels of RCCP protein after the induction of RCCP RNAi with tetracycline (Tet) for the time indicated in days. Protein lysates from the T3-RCCP cell line in the presence of RCCP RNAi were analysed by Western blot. Blots were probed with a rabbit polyclonal antibody against RCCP. A cross reactive band (CRB) is indicated as a loading control. The size of a marker protein in kiloDaltons (kDa) is indicated on the left.

FIGURE 7.

Knockdown of FYRP in bloodstream form *T. brucei* leads to derepression of the silent *VSG221* expression site.

A. The schematic shows the *T. brucei* T3-FYRP reporter cell line, which has an active *VSGT3* expression site containing a blasticidin resistance gene (blast) and an inactive *VSG221* expression site with a silent GFP gene. RNAi against FYRP can be expressed from a construct containing two opposing tetracycline inducible promoters. Relevant genes are shown with coloured boxes, transcription is indicated with arrows, expression site promoters with white flags and T7 promoters with black flags.

B. Only minor growth reduction is observed after the induction of FYRP knockdown. The parental T3 cell line, as well as the T3-FYRP1 and T3-FYRP2 clones were incubated in the presence (+) or absence (-) of tetracycline for the time indicated in hours. The cumulative growth was plotted against time, with error bars indicating the standard deviation of three replicate experiments.

C. FYRP knockdown leads to 26-61 fold derepression of the silent *VSG221* expression site as monitored using GFP. The upper panel shows representative flow cytometry histograms in the FL-1 channel either before or after induction of FYRP RNAi for 72 hours (h) using tetracycline (Tet). The bottom panel shows mean derepression in the *T. brucei* T3-FYRP1 or T3-FYRP2 clones compared with the parental cell line (T3) after induction of FYRP RNAi for the time indicated in hours. Error bars indicate the standard deviation from three independent experiments.

D. Decrease in levels of FYRP transcript in *T. brucei* T3-FYRP1 following the induction of FYRP RNAi. Transcript levels were determined using qRT-PCR, normalised to γ -tubulin and shown relative to values at the 0 hours timepoint. Results are the average of three independent experiments, with error bars showing the standard deviation.

E. Efficient knockdown of FYRP protein after the induction of FYRP RNAi. Protein lysates were analysed after the induction of FYRP RNAi with tetracycline (Tet) for the time indicated in hours (h) in a cell line containing a copy of FYRP tagged with the HA epitope. Western blot analysis was performed using an anti-HA antibody (α HA). A cross reacting band (CRB) is indicated below as a loading control. The sizes of protein markers in kilodaltons (kDa) are indicated on the left.

FIGURE 8.

T. brucei ISWI and its partners colocalise at the Pol I transcribed rDNA and procyclin loci in bloodstream form *T. brucei*.

A. Schematic of a typical ribosomal DNA (rDNA) transcription unit, with genes indicated with black boxes, and the rDNA promoter with a black flag. Regions analysed by qPCR are indicated with letters. Primers are described in the supplementary material.

B. Colocalisation of TbISWI and its partners at the rDNA locus. Chromatin from *T. brucei* ISWI-HA, NLP-HA, FYRP-HA or wild type (WT) cells was immunoprecipitated with an anti-HA antibody. Chromatin from parental cells was immunoprecipitated with an anti-RCCP antibody, and rabbit pre-immune serum (PI) was used as a negative control. The genomic regions analysed are indicated in the schematic and listed above the graphs. Results are presented as the amount immunoprecipitated (percentage of input or % IP) after subtraction of the no antibody control. Results shown are the mean of three independent experiments with the standard deviation indicated with error bars, apart from NLP. Here the results are from one representative ChIP experiment, as similar data has been published previously in Narayanan et al., 2011.

C. A diagram of the EP procyclin locus transcribed by multi-functional Pol I. A black flag depicts the procyclin promoter, and letters indicate the regions that were analysed using qPCR.

D. Different TbISWI partners colocalise at the procyclin locus. Immunoprecipitated chromatin at the procyclin genomic loci was analysed as indicated in the legend for panel B. Regions analysed are shown above the graphs.

FIGURE 9.

Location of different TbISWI partners at two different Pol II convergent and divergent strand-switch regions.

A. A schematic of different Pol II strand switch regions (SSRs) from Chromosome 10 are shown (Convergent regions C2 and C3 and Divergent regions D1 and D2). These regions were initially described by Siegel *et al* (2009), and also analysed in Stanne *et al* 2011. Convergent SSRs contain putative Pol II termination sites, and divergent SSRs putative Pol II promoters. Genes are indicated with black boxes, with arrows showing the direction of transcription. Genomic regions analysed by qPCR are indicated with letters. Primer pairs a, f and l are located approximately in the middle of the polycistronic transcription units. Primers are described in the supplementary material.

B. Distribution of the TbISWI partners at different Pol II SSRs. ChIP was performed using an anti-HA antibody on either wild type cells (WT) or cells containing an HA epitope tagged ISWI, HA-NLP or HA-FYRP. An anti-RCCP antibody was used to immunoprecipitate RCCP, and is compared with ChIP performed with rabbit pre-immune (PI) serum. The results are expressed as percentage of total input (% IP), followed by subtraction of the no antibody control. Results are shown as the average of three independent experiments with error bars showing the standard deviation with the exception of some of the ISWI results as these confirm previously published data (54).

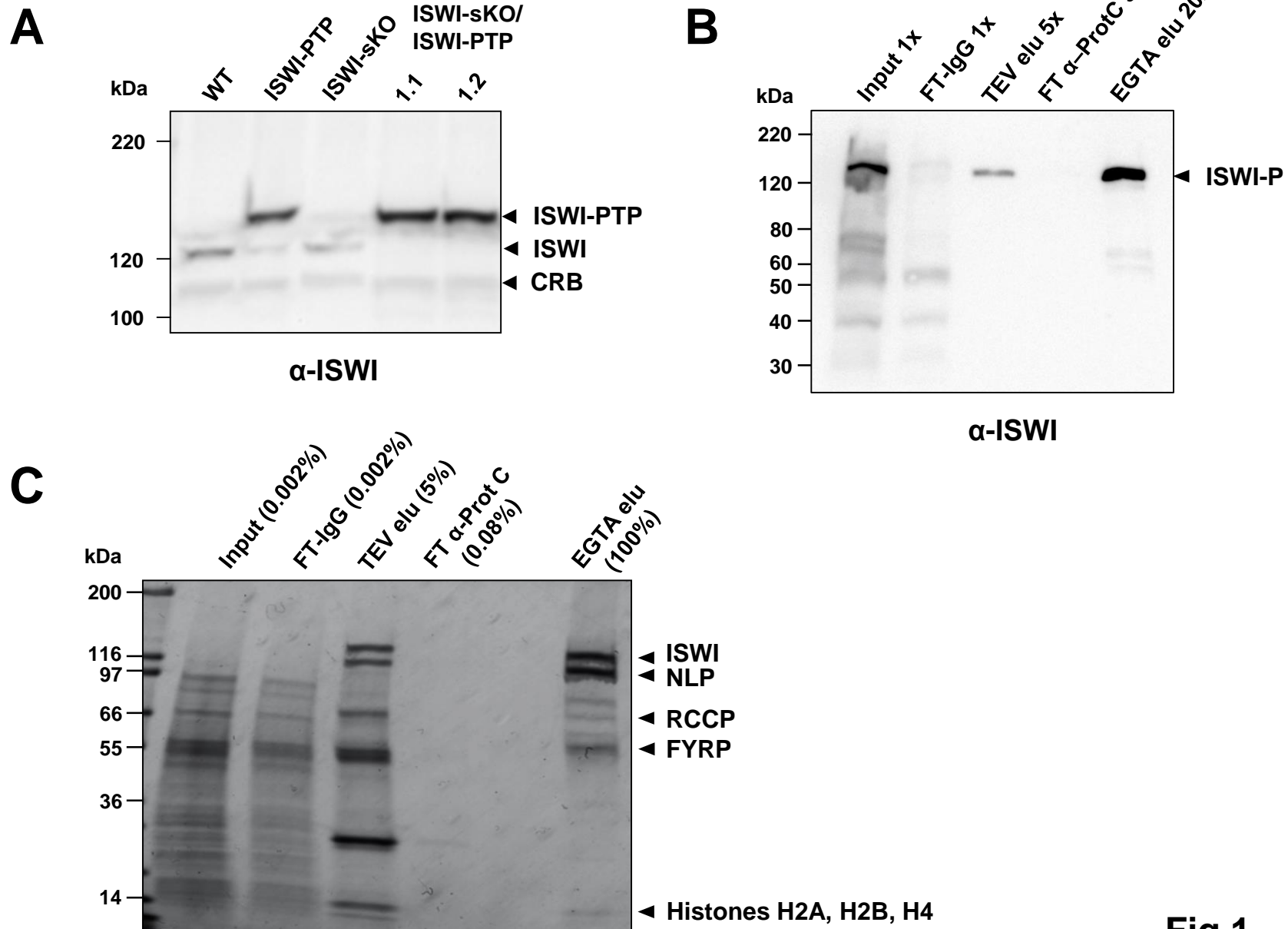
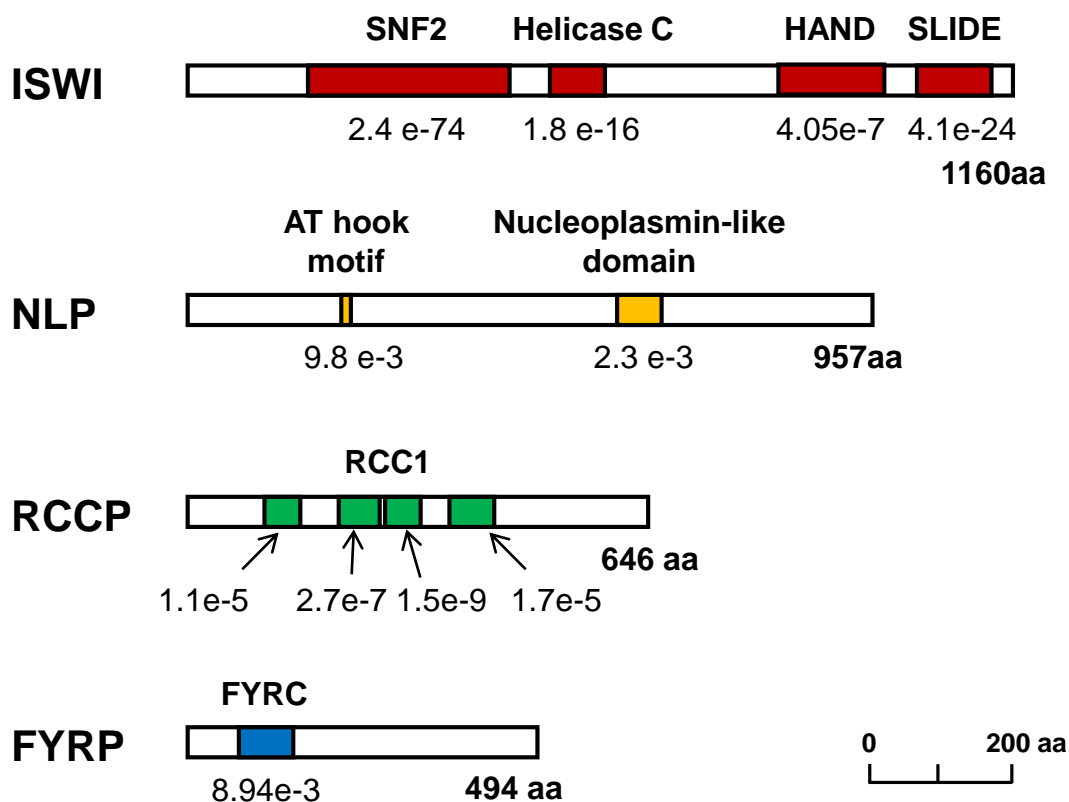


Fig 1

A



B

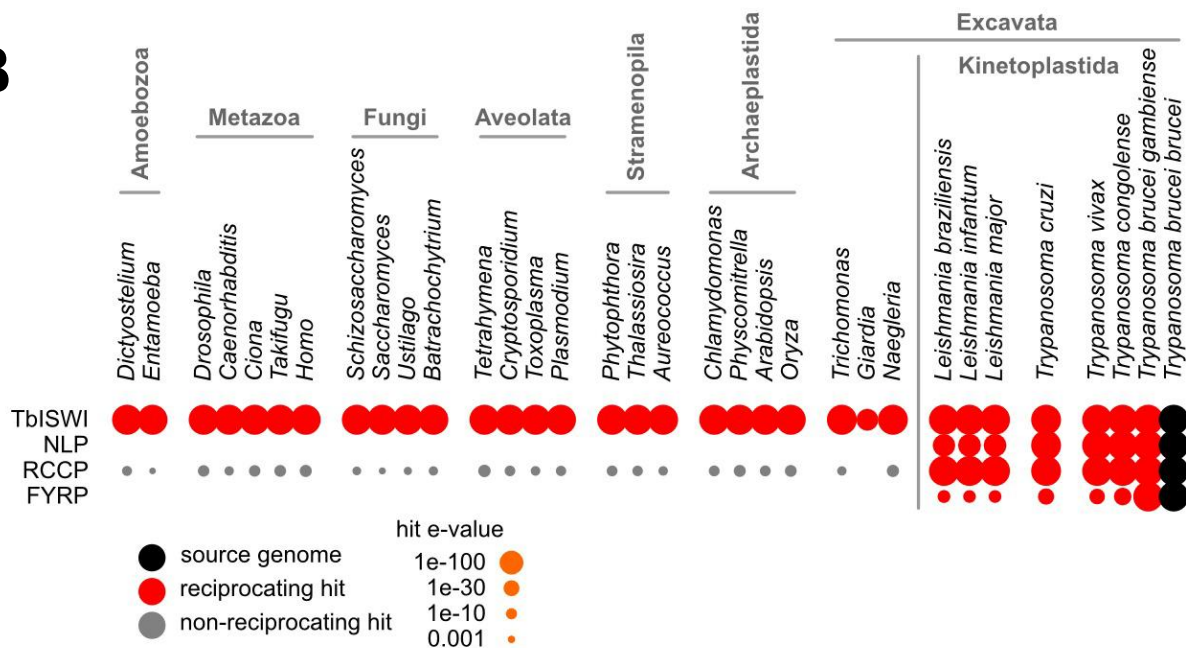


Fig 2

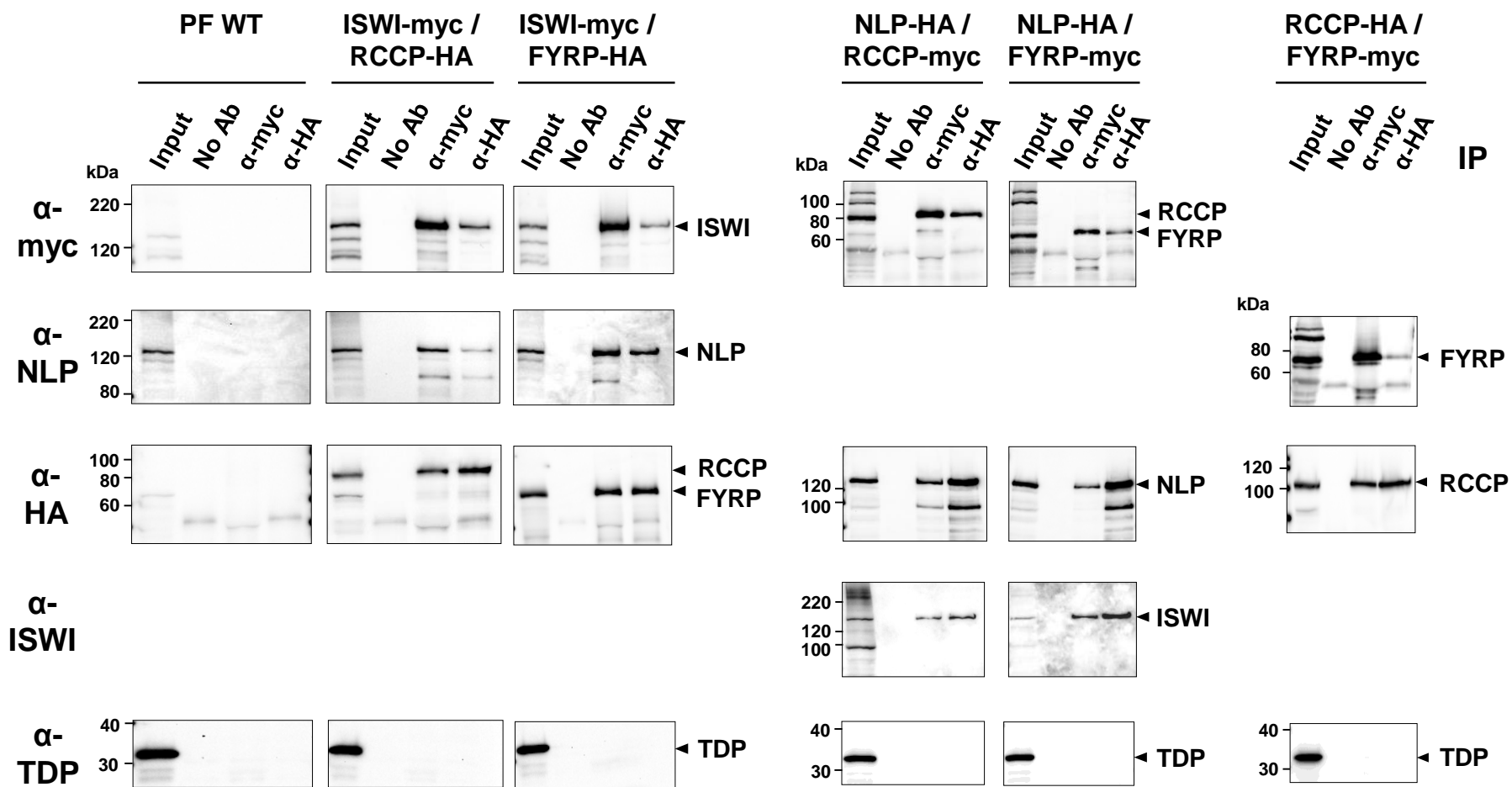


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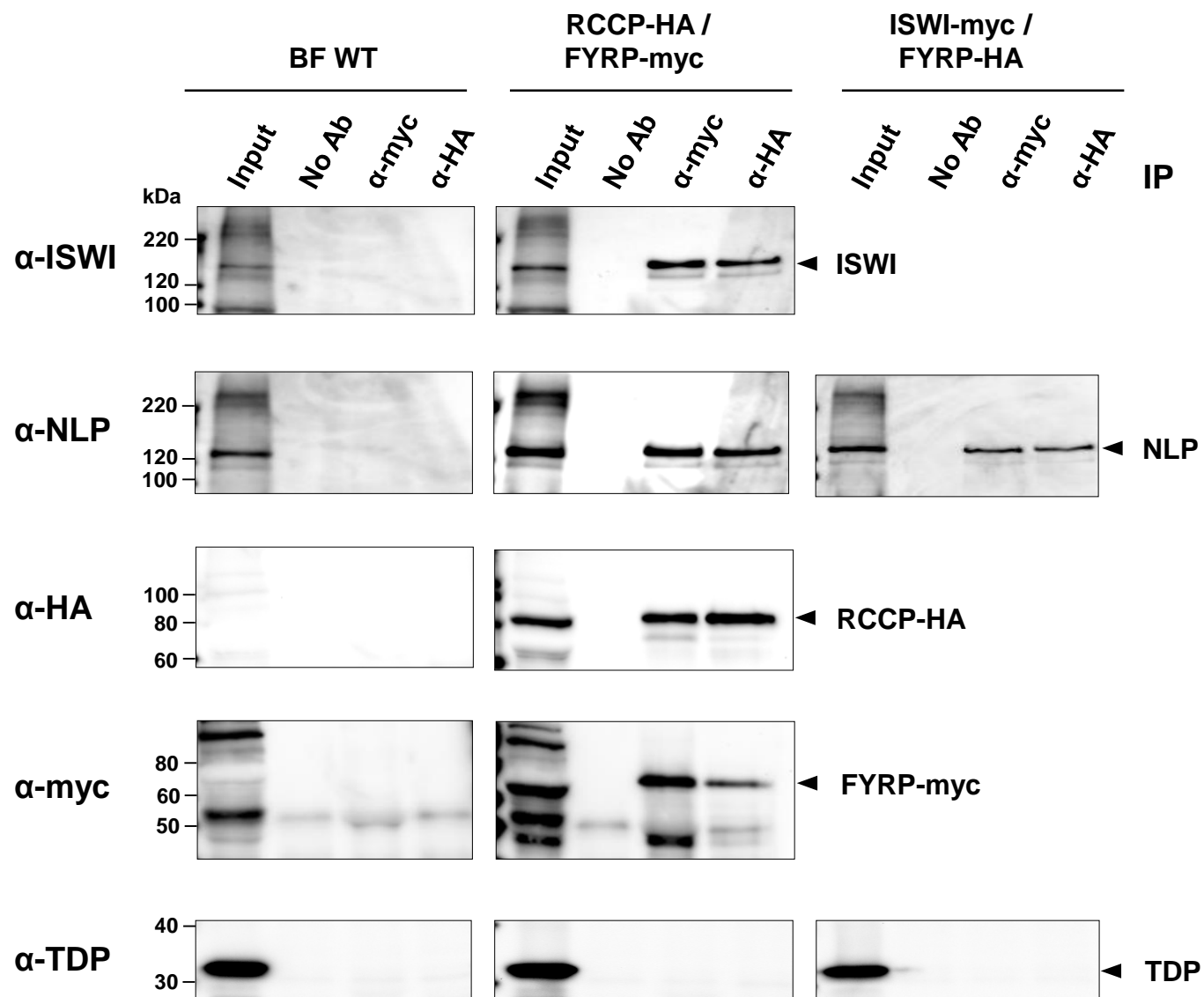


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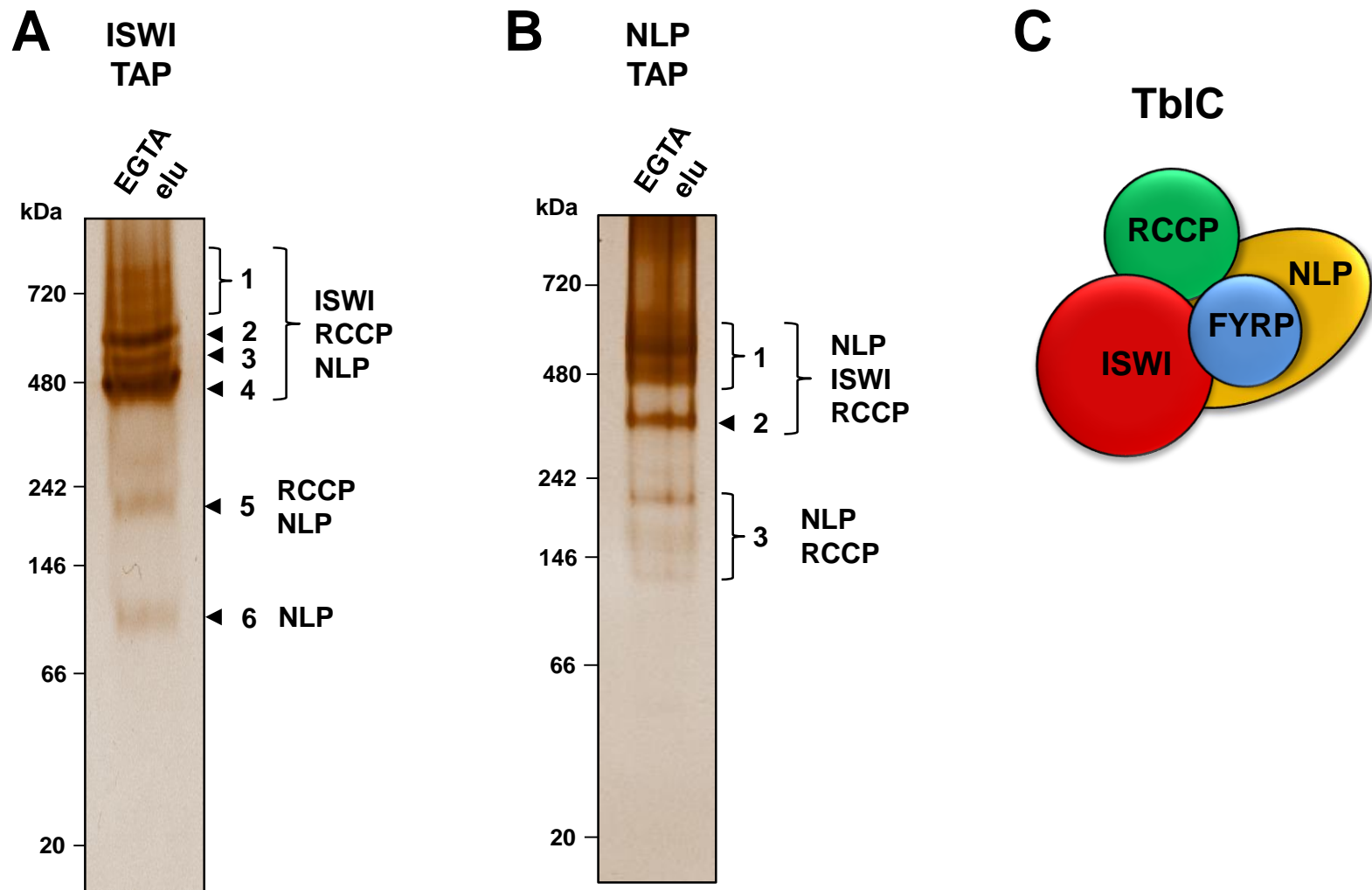
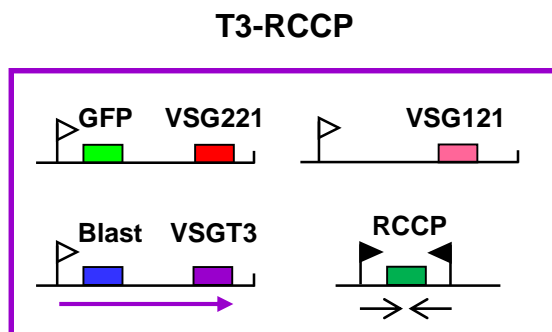
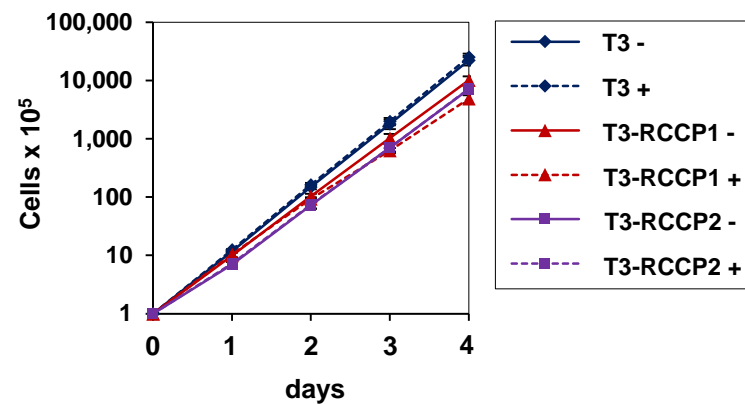
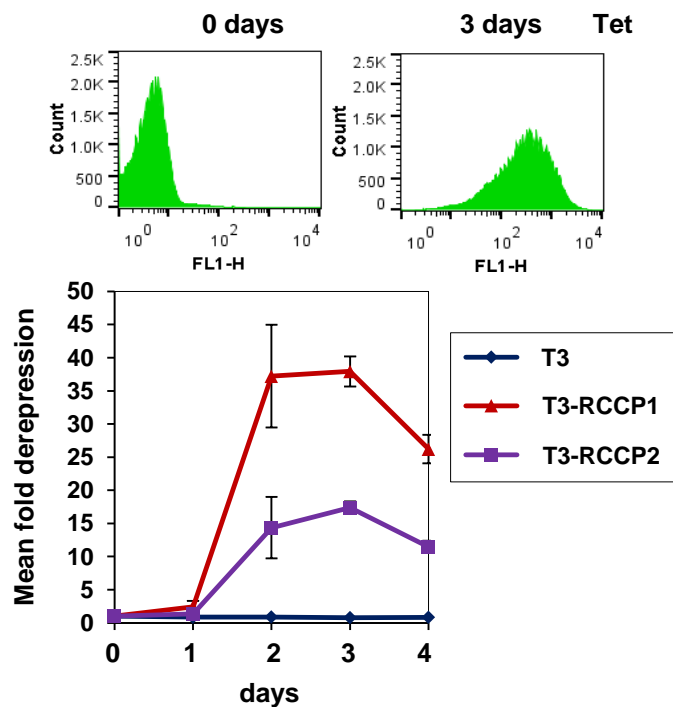
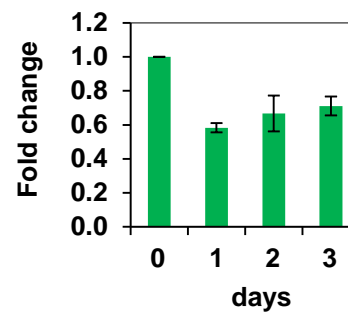
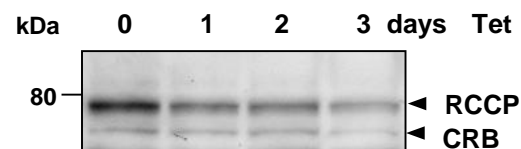
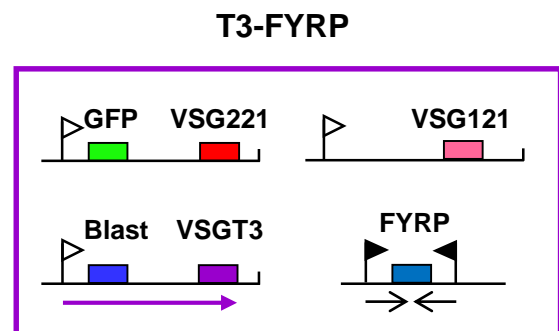
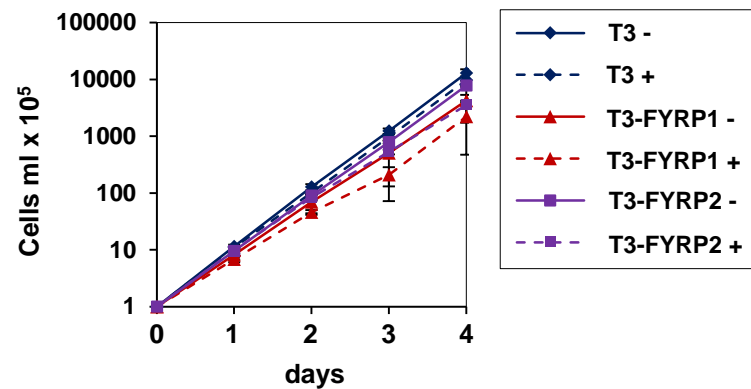
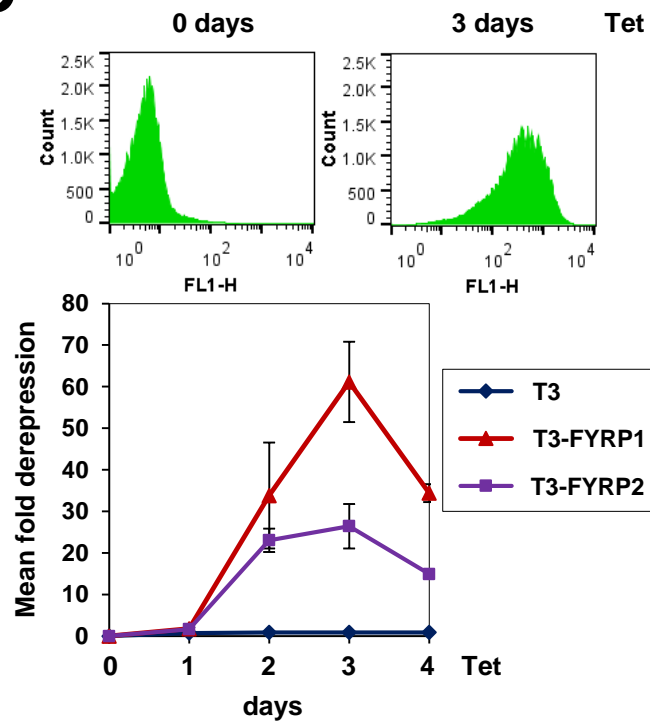
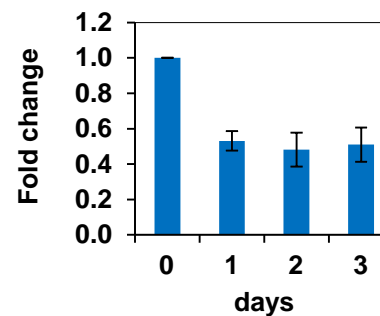
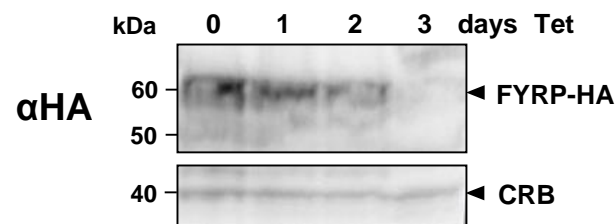


Fig 5

A**B****C****D****E****Fig 6**

A**B****C****D****E****Fig 7**

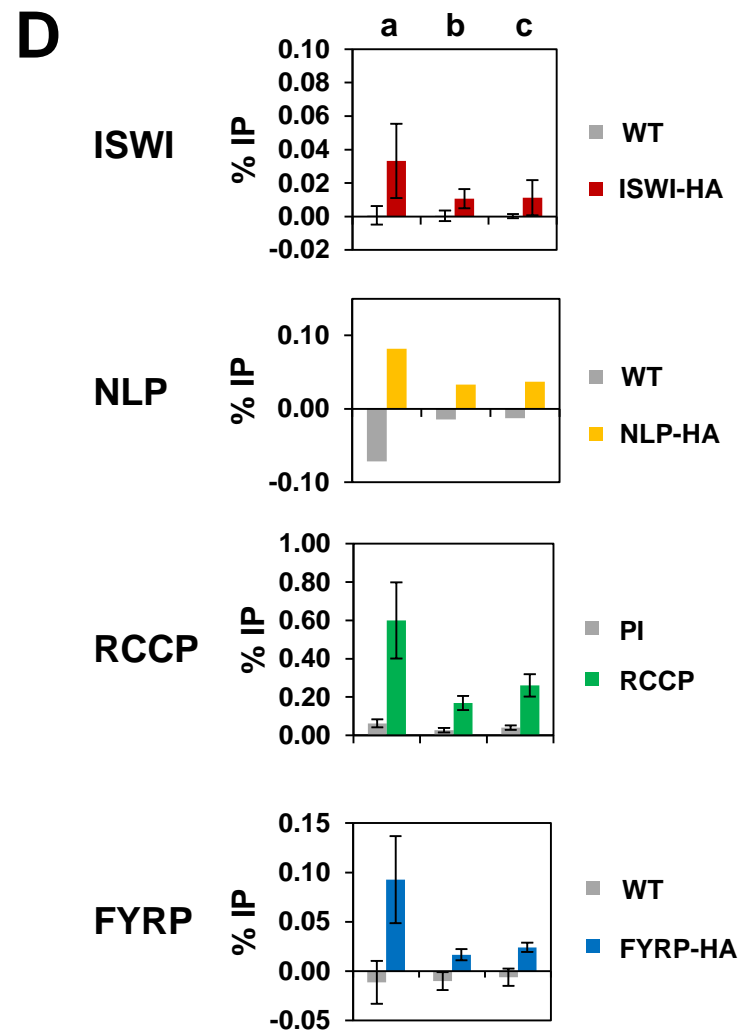
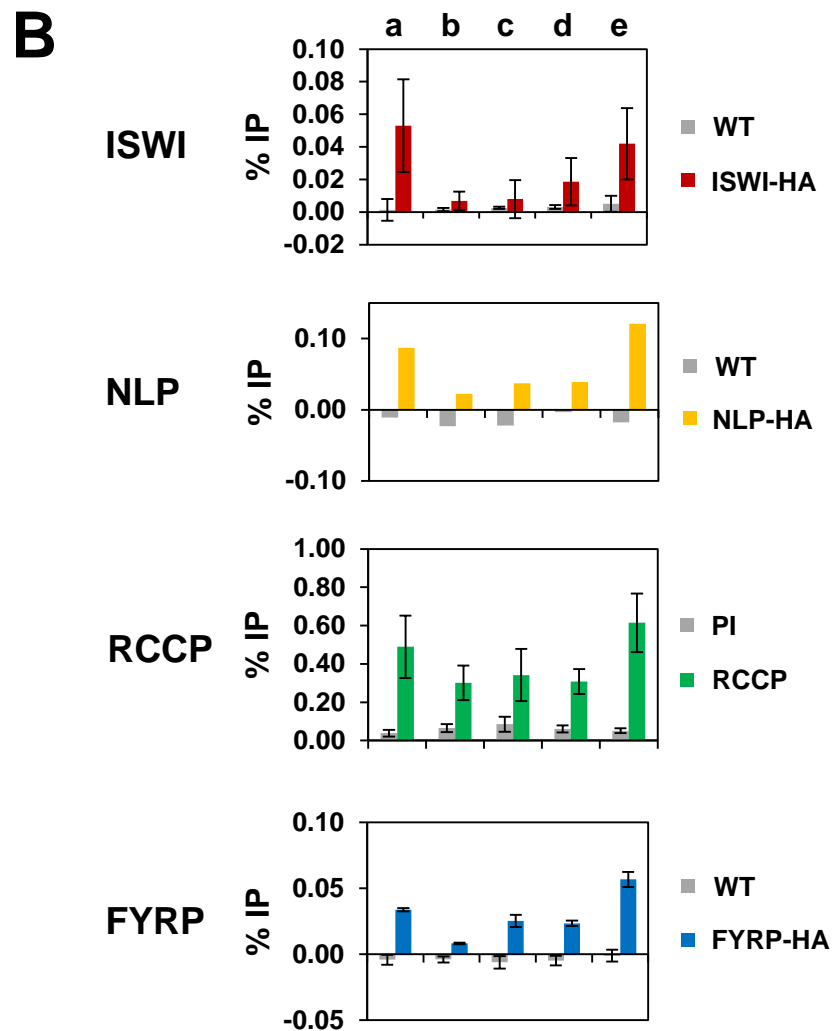
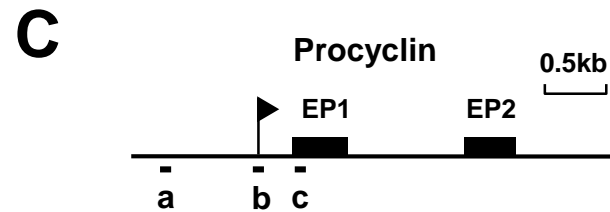
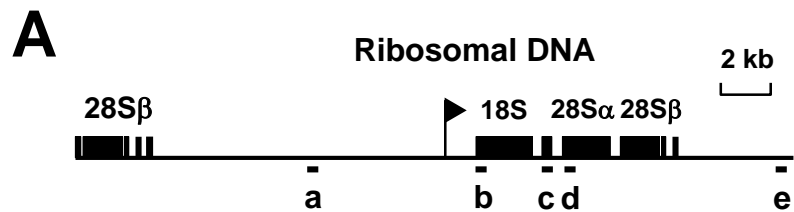
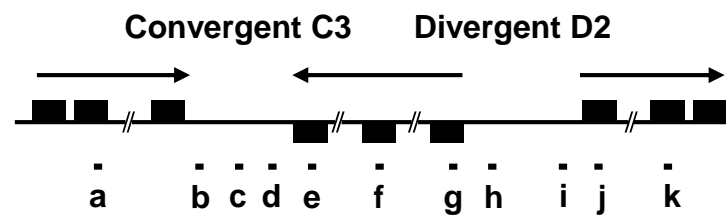
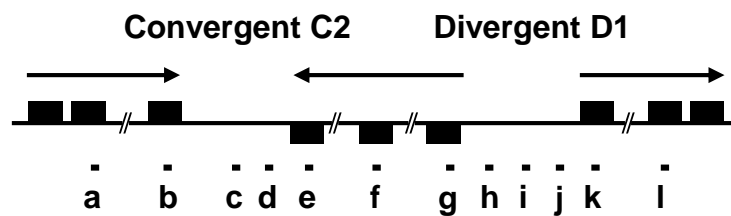
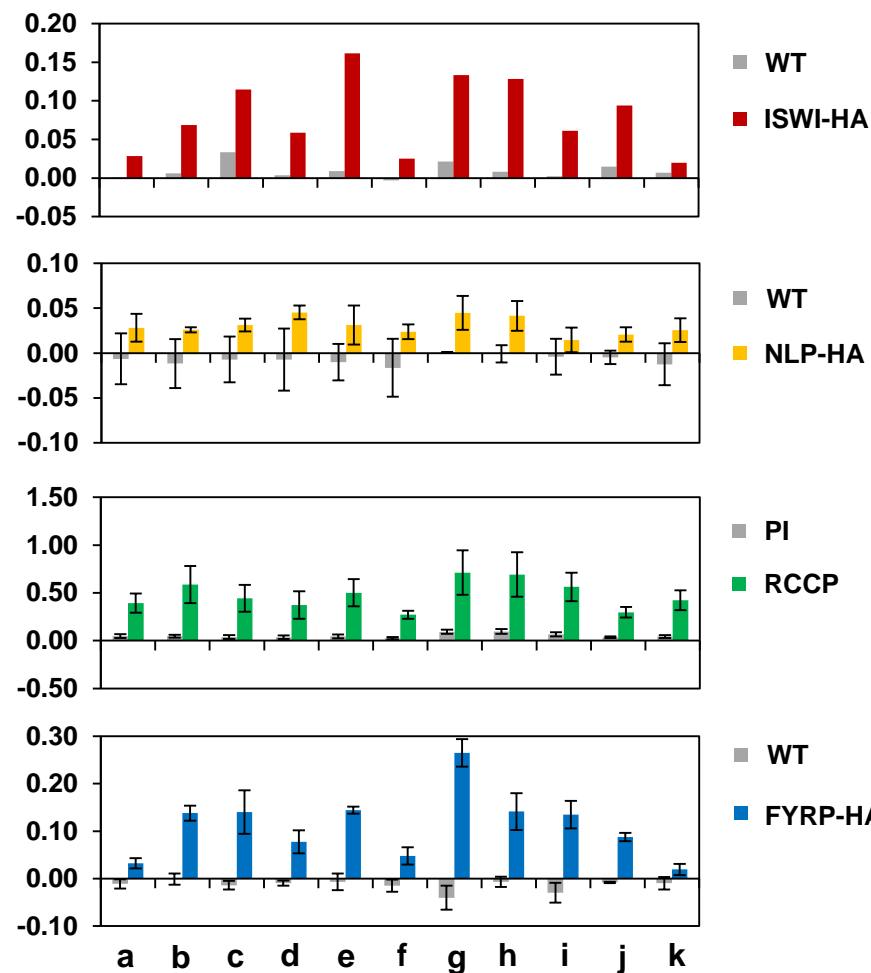
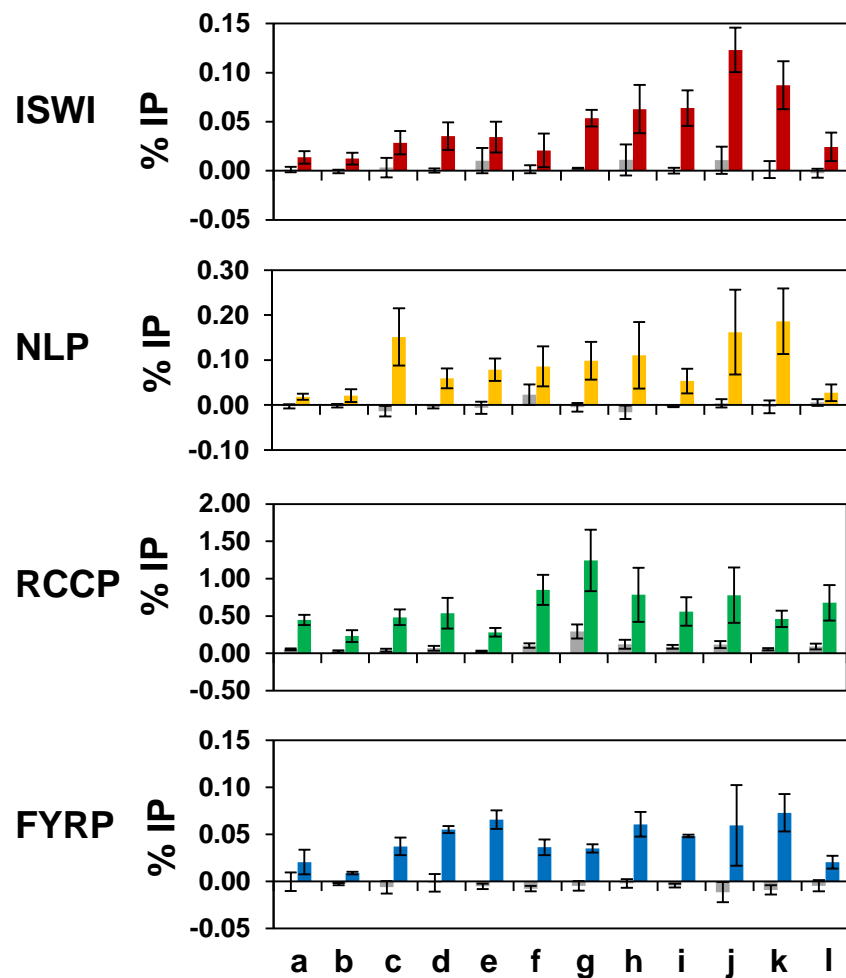


Fig 8

A**B****Fig 9**