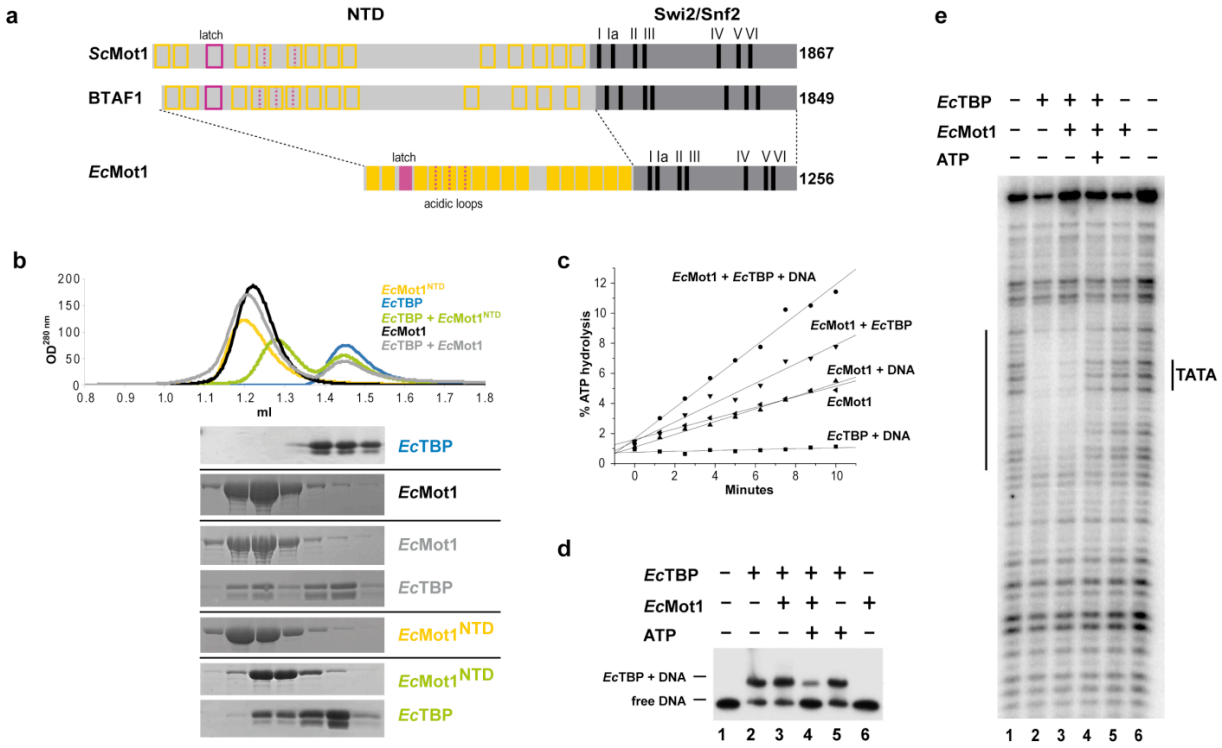
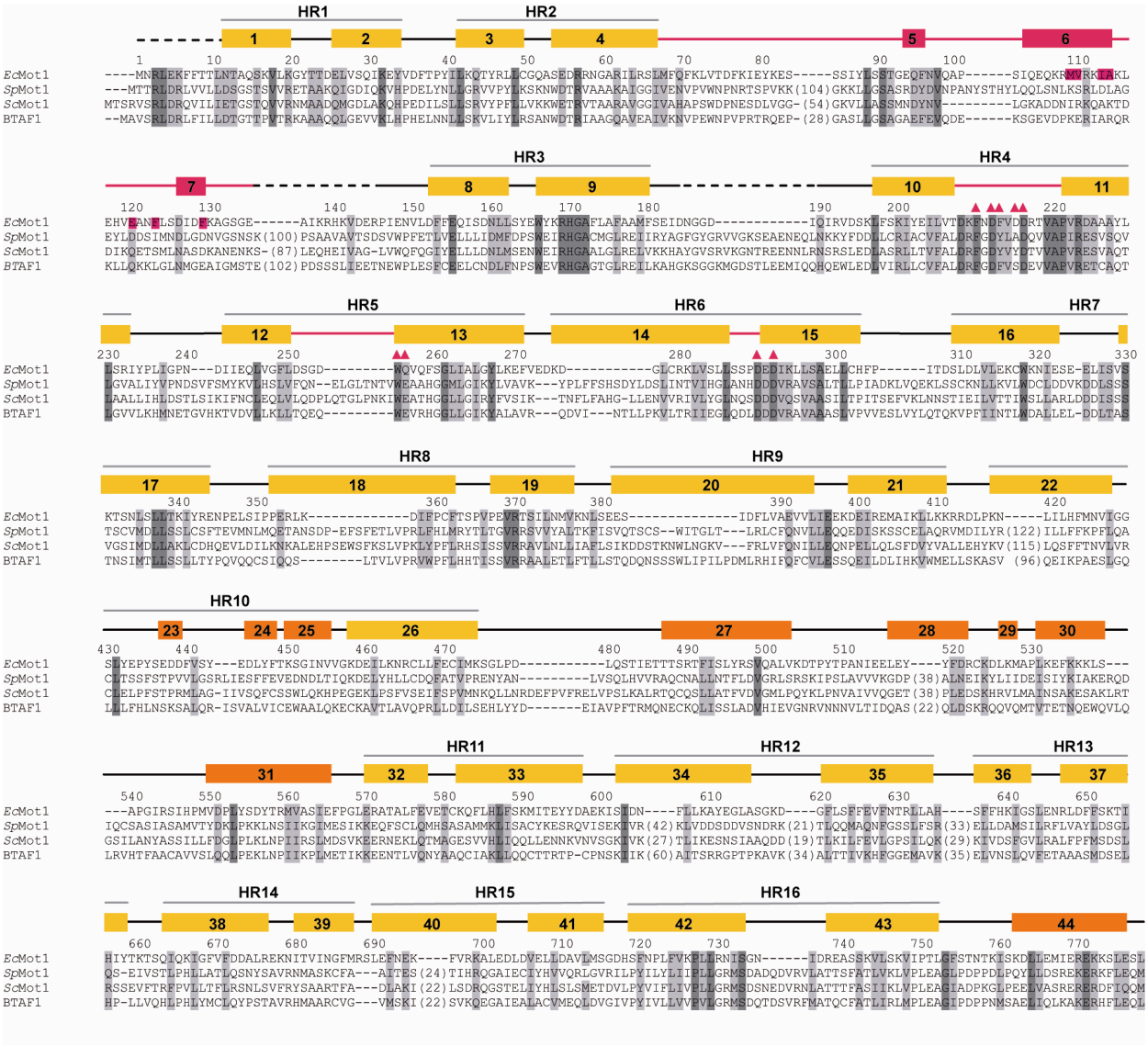
Supplementary Information

Supplementary Figures



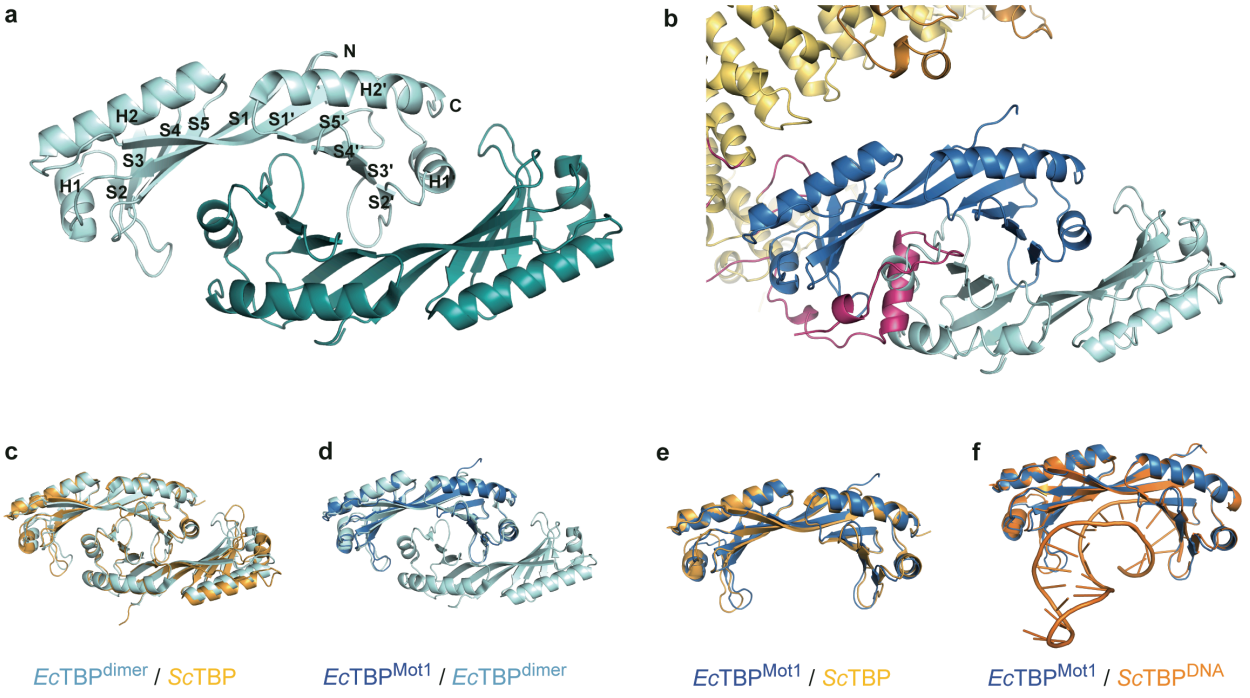
Supplementary Figure 1. ***Encephalitozoon cuniculi*** **(*Ec*) Mot1:TBP biochemistry. a,** Schematic comparison of *Ec*, *Sc* (*Saccharomyces cerevisiae*) and human Mot1 (BTAF1) with indicated sequence predicted (open yellow boxes) and structurally defined HEAT repeats (filled yellow boxes) as well as ATPase motifs I-VI. **b,** Size exclusion chromatographs of *Ec*TBP and *Ec*Mot1/*Ec*Mot1^{NTD} with samples from corresponding fractions separated by SDS-PAGE. *Ec*TBP coelutes with *Ec*Mot1/*Ec*Mot1^{NTD} indicating that they form a stable complex. *Ec*Mot1^{NTD} alone has a larger hydrodynamic radius than the *Ec*Mot1^{NTD}:*Ec*TBP complex. DLS and SLS experiments (dynamic and static light scattering, see Supplementary Table 3) revealed that *Ec*Mot1^{NTD} forms a dimer, possibly to saturate exposed hydrophobic surfaces at the

truncated C-terminus. **c,** ATPase activity of *Ec*Mot1 in the presence of different substrates. We used 10 nM *Ec*Mot1 and 150 nM *Ec*TBP with or without 150 nM TATA-containing DNA. *Ec*Mot1 alone had an ATP hydrolysis rate of 8.9 $\pm$ 0.4 moles ATP hydrolyzed per min per mol *Ec*Mot1. For comparison, the rates were 10.3 $\pm$ 0.3 for *Ec*Mot1 plus DNA and 16.0 $\pm$ 1.0 for *Ec*Mot1 plus TBP. The ATPase was most active when incubated with pre-formed TBP:DNA complexes, yielding a rate of 25.6 $\pm$ 1.1. **d,** *Ec*Mot1 dissociates the *Ec*TBP:DNA complex in an ATP-dependent reaction. Electrophoretic mobility shift assays were performed using radiolabeled TATA-containing DNA, proteins and ATP as indicated. A stable complex containing *Ec*Mot1 was not detectable in this assay. Note that the addition of *Ec*Mot1 and ATP resulted in a marked decrease in the abundance of the *Ec*TBP:DNA complex (lane 4 versus 2, 3 and 6). **e,** Analysis of *Ec*Mot1 activity by DNase I footprinting. Reactions contained radiolabeled TATA-containing DNA and *Ec*TBP, *Ec*Mot1 and/or ATP as indicated. Incubation of *Ec*TBP with the DNA resulted in protection of a ~20 bp region bracketing the TATA sequence (denoted by the vertical bar, compare lanes 1 and 2)[1]. There was no detectable change in the *Ec*TBP footprint when *Ec*Mot1 was added (lane 3 versus 2). However, the addition of *Ec*Mot1 and ATP caused *Ec*TBP to be stripped from the probe (lane 4).

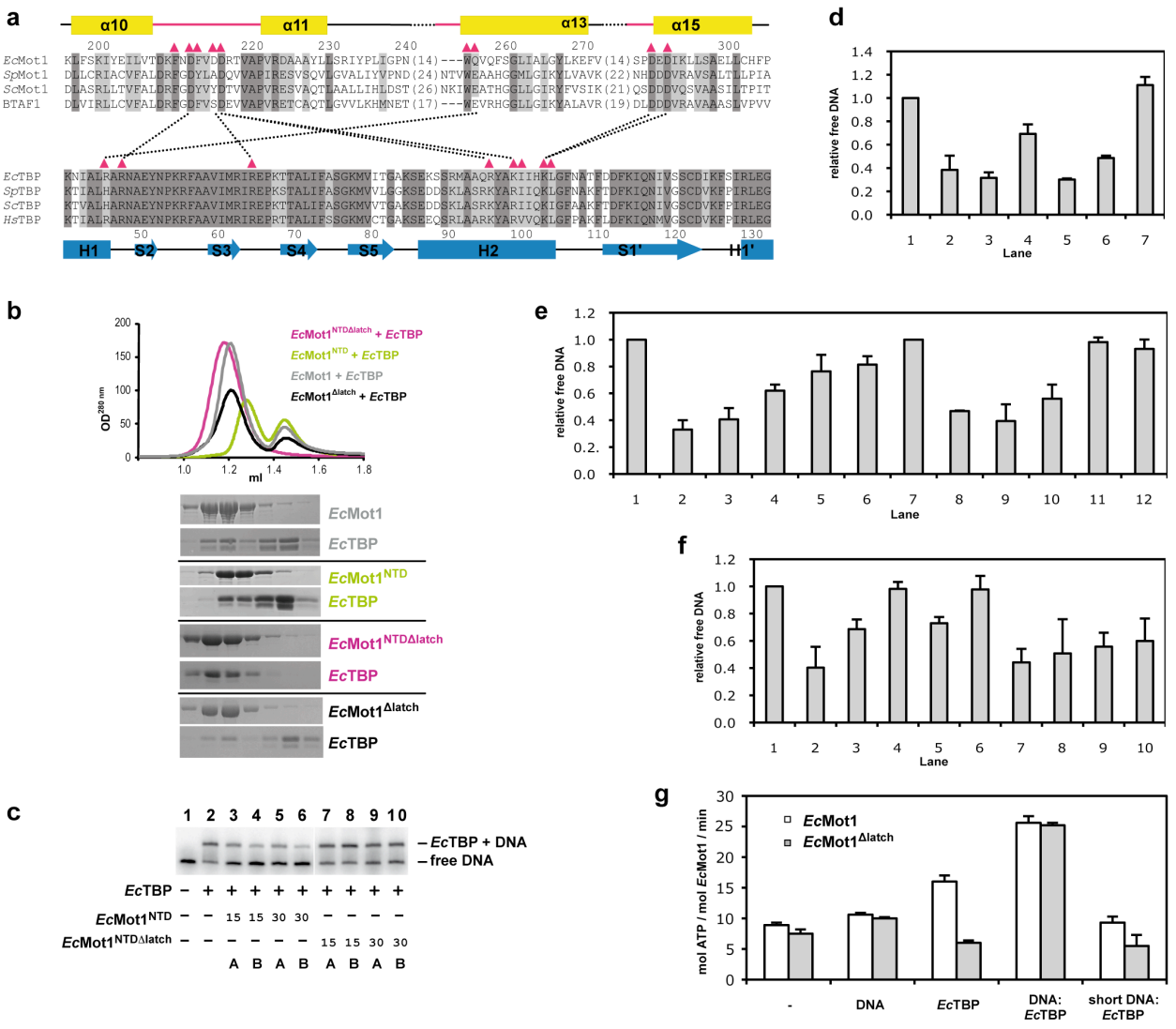


Supplementary Figure 2. Sequence alignment of the N-terminal domain of Mot1 from different species. Identical residues and highly conserved residues are colored in dark and light grey, respectively. Numbers on top of the alignment specify the sequence of *Ec*Mot1. The annotated secondary structure of *Ec*Mot1^{NTD} is depicted on top of the alignment with numbers corresponding to the specified helices. Some helices are grouped to HR (HEAT repeats) by grey lines. Residues mentioned in the text are highlighted in pink or marked with a pink triangle.

Dashed lines correspond to regions without structural information. The latch (residues 101-130) and the loops of HR 4 to HR 6 are highlighted in magenta. Abbreviations: *Ec*Mot1, *Encephalitozoon cuniculi* Mot1; *Sp*Mot1, *Schizosaccharomyces pombe* Mot1; *Sc*Mot1, *Saccharomyces cerevisiae* Mot1; BTAF1 human Mot1 homologue.

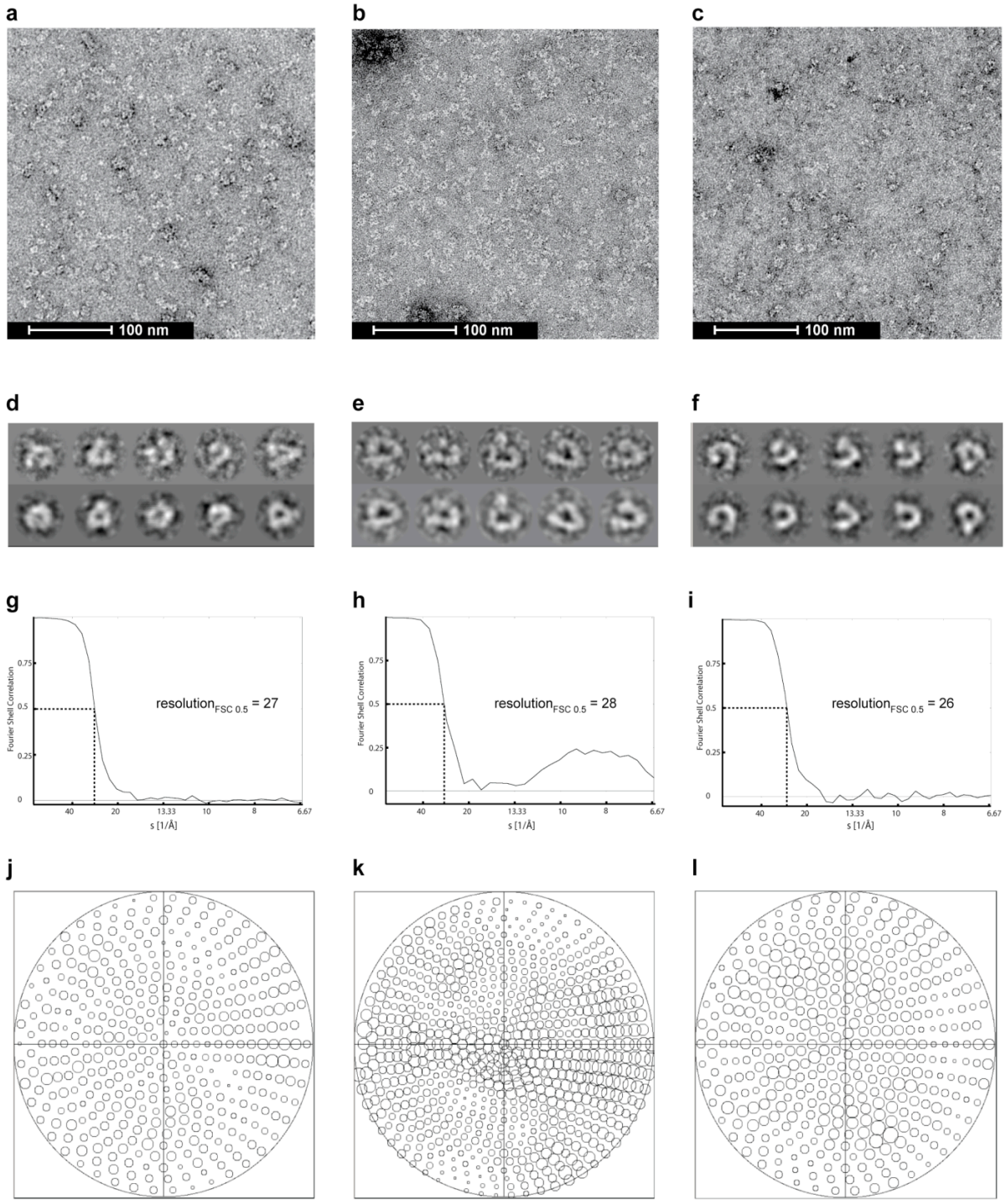


Supplementary Figure 3. Structural comparison of *Ec*TBP with other TBP structures. a, Structure of dimeric *Ec*TBP, crystallized in the absence of Mot1, in ribbon representation with highlighted and annotated secondary structure (monomers colored in lightblue and teal). **b,** A superposition of the *Ec*TBP dimer and the *Ec*Mot1^{NTD}:*Ec*TBP complex illustrates how the latch (pink) directly competes with binding of a second TBP molecule (lightblue) to a TBP molecule (blue) bound by Mot1 (yellow), thereby preventing formation of TBP dimers. **c,** Superposition (r.m.s.d. 1.27 Å) of dimeric *Ec*TBP (lightblue) to *Sc*TBP (1TBP, lightorange[2]). **d,** Superposition (r.m.s.d. 0.67 Å) of dimeric *Ec*TBP (lightblue) to *Ec*TBP^{Mot1} (blue, from the *Ec*Mot1^{NTD}:*Ec*TBP complex). **e,** Superposition (r.m.s.d 0.83 Å) of *Ec*TBP^{Mot1} (blue) to *Sc*TBP (1TBP, lightorange). **f,** Superposition (r.m.s.d 0.53 Å) of *Ec*TBP^{Mot1} (blue) to *Sc*TBP in complex with DNA (1YTB, orange[3]).



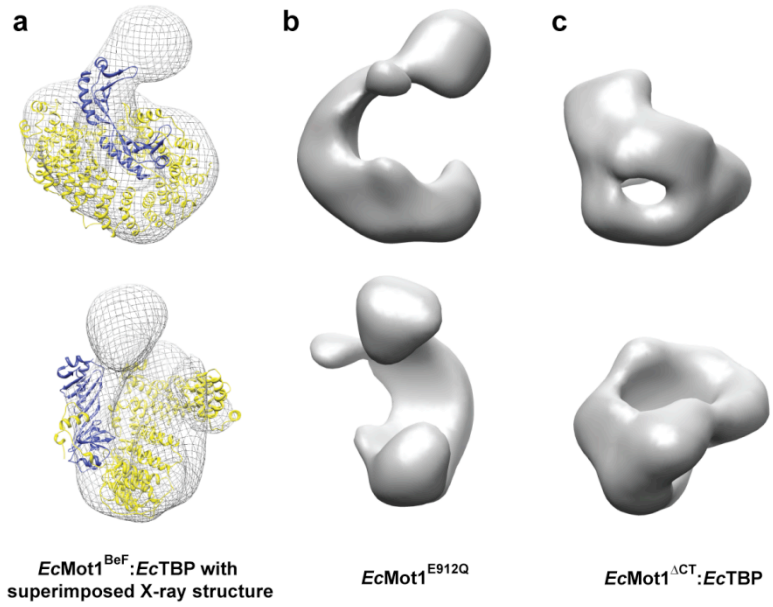
Supplementary Figure 4. Key residues involved in *Ec*Mot1:*Ec*TBP recognition and biochemical behavior of *Ec*Mot1$^{wt/NTD/NTD\Delta latch/\Delta latch}$. a, Sequence alignment showing that the key interacting residues involved in *Ec*Mot1:*Ec*TBP recognition are conserved (pink triangle). Abbreviations: *Ec*, *Encephalitozoon cuniculi*; *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Schizosaccharomyces pombe*; *Hs*, *Homo sapiens*. **b,** In size exclusion chromatography, *Ec*Mot1$^{NTD\Delta latch}$ stably binds to *Ec*TBP and forms a high molecular weight complex composed of a TBP dimer and two bound *Ec*Mot1$^{NTD\Delta latch}$. In contrast, the *Ec*Mot1^{NTD}:*Ec*TBP complex has a smaller hydrodynamic radius and a smaller molecular weight, indicating that TBP dimerization

is prevented by the latch (see also DLS and SLS, Supplementary Table 3). Note that the *Ec*Mot1$^{\Delta latch}$:*Ec*TBP complex did not form higher molecular weight complexes compared to wildtype *Ec*Mot1:*Ec*TBP complexes. This suggests that *Ec*Mot1$^{\Delta latch}$, like *Ec*Mot1$^{NTD\Delta latch}$, fails to dissociate dimeric TBP due to the absence of the latch, but is sterically unable to bind to / dimerize via TBP dimers due to the presence of the ATPase domain. **c,** Gel mobility shift assay (EMSA) using radiolabeled TATA DNA (<1 nM), *Ec*TBP (15 nM) and *Ec*Mot1^{NTD} or *Ec*Mot1$^{NTD\Delta latch}$, added at a concentration of 15 nM or 30 nM, as indicated. **d, e,** and **f,** Quantitations of gel mobility shift assays from Figures 2e, 2f, and Supplementary Figure 4c, respectively. The bars show the relative levels of free DNA in each reaction (reactions with no added protein were normalized to 1.0). The quantitations were made by phosphorimager analysis of the EMSAs. Data represent mean and standard errors (n=2 or 3). **g,** ATPase activities of wildtype *Ec*Mot1 compared to *Ec*Mot1$^{\Delta latch}$. ATP hydrolysis rates were measured using methods described previously[1]. The reactions contained 10 nM *Ec*Mot1 or *Ec*Mot1$^{\Delta latch}$, as well as the additional components indicated below the bars including a 110 bp TATA-containing DNA fragment that supports both TBP and yeast Mot1 binding and activity[1], added to 150 nM (denoted "DNA"), a TATA-containing DNA duplex that binds TBP but is too short to support yeast Mot1 binding[1] (denoted "short DNA"), and/or 150 nM *Ec*TBP. The significantly lower ATPase activity of *Ec*Mot1$^{\Delta latch}$ compared to *Ec*Mot1 in the presence of TBP alone, may result from a different orientation of TBP on Mot1$^{\Delta latch}$, or transient TBP dimerization that impacts the function of the Swi2/Snf2 domain. We speculate that DNA stabilizes a more optimal orientation or prevents dimerization by binding to both TBP and the Swi2/Snf2 domain, rescuing the ATPase activity of the *Ec*Mot1$^{\Delta latch}$ protein. Data represent mean and standard error (n=3).

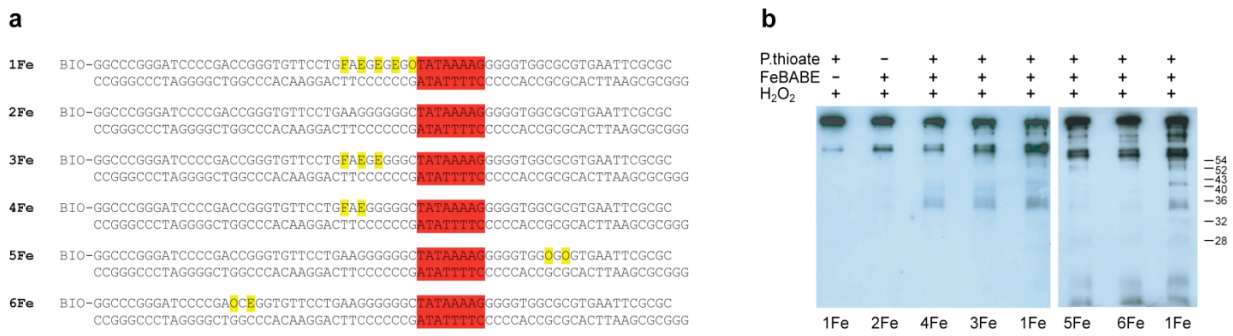


Supplementary Figure 5. EM raw data. Negative stain images of (**a**) *Ec*Mot1^{BeF}:*Ec*TBP, a complex of *Ec*Mot1 and *Ec*TBP in the presence of ADP-BeF_3^-, a non-hydrolyzable ATP analogue; **b**, *Ec*Mot1^{E912Q}, a Walker B mutant of *Ec*Mot1, was used so that ATP could be added

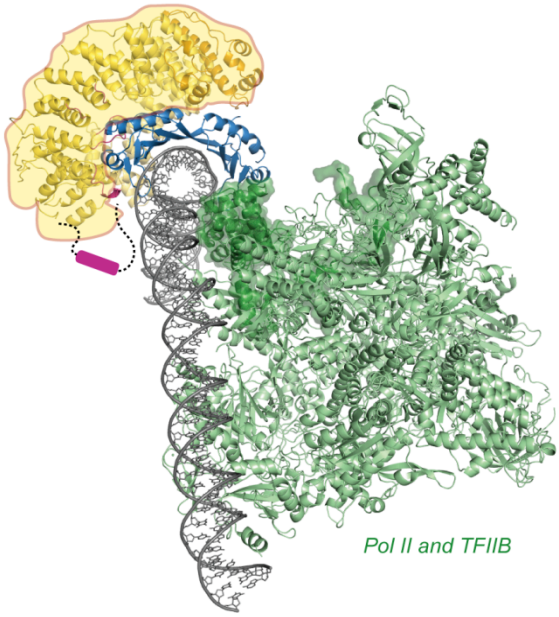
without being hydrolyzed; and **c**, the *Ec*Mot1$^{\Delta CT}$:*Ec*TBP complex. *Ec*Mot1$^{\Delta CT}$ lacks the C-terminal half of the two-lobed Swi2/Snf2 domain. Initial class averages of *Ec*Mot1^{BeF}:*Ec*TBP (**d**, upper row), *Ec*Mot1^{E912Q} (**e**, upper row) and *Ec*Mot1$^{\Delta CT}$:*Ec*TBP (**f**, upper row) obtained by multivariate statistical analysis. Each class contains approximately 10 images. The lower row shows reprojections of the initial 3D structure in the Euler angle directions assigned to the class averages. Fourier shell correlation curves of *Ec*Mot1^{BeF}:*Ec*TBP (**g**), *Ec*Mot1^{E912Q} (**h**) and *Ec*Mot1$^{\Delta CT}$:*Ec*TBP (**i**). The resolution of the map estimated by Fourier Shell Correlation with a 0.5 correlation cut-off and no masking is given. Euler angle distribution plots for *Ec*Mot1^{BeF}:*Ec*TBP (**j**), *Ec*Mot1^{E912Q} (**k**) and *Ec*Mot1$^{\Delta CT}$:*Ec*TBP (**l**) after refinement with projection matching in EMAN. Discrete directions with 5º or 7º azimuthal equidistant angular spacing are represented by circles, the size which corresponds to the number of particles matching that direction. The polar angle (Θ) increases from the center of the circle to the outside and the azimuthal angle (Φ) increases along the circumference of the circle.



Supplementary Figure 6. Surface representation of EM negative stained *Ec*Mot1:*Ec*TBP complex densities. a, Two views of the surface representation of the *Ec*Mot1^{BeF}:*Ec*TBP density with overlaid crystal structure of *Ec*Mot1^{NTD}:*Ec*TBP. The good fit indicates that ADP-BeF_3^- binding to the ATPase domain does not strongly alter the HEAT repeat structure in the absence of DNA. **b**, Density maps for *Ec*Mot1^{E912Q} using the views of (**a**) show that the lateral protrusion interpreted with TBP is missing. **c**, Density map for *Ec*Mot1$^{\Delta CT}$ (an *Ec*Mot1 variant lacking the C-terminal part of the Swi2/Snf2 ATPase), using the views in (**a**), shows that the globular protrusion is missing.



Supplementary Figure 7. Localization of the Mot1 ATPase domain to upstream DNA. a, DNA probes used for FeBABE mediated cleavage of Mot1. Conjugated phosphorothioates are marked in yellow (F = A-phosphorothioate, O = C-phosphorothioate, E = G-phosphorothioate, Z = T-phosphorothioate), the TATA boxes are highlighted in red. **b**, FeBABE-mediated cleavage of Mot1 analyzed by Western blot[4]. Approximate sizes of the cleavage products in kDa are shown in the right. Control experiments demonstrated that Mot1 binding to the DNA depends on prior incubation with TBP as expected (not shown). Additional control experiments (not shown) indicate that the very small polypeptides (<28 kD) at the very bottom of the right-hand blot in panel **b** are not specific cleavage products; they are detectable in all reactions with sufficient Western blot exposure.



Supplementary Figure 8. Implications for PIC recognition. Model of how Mot1 (yellow) could theoretically bind to a minimal preinitiation complex (PIC, based on a closed promoter complex model, 3K1F, green[5]). The Mot1 latch is omitted from the structure and indicated by a cartoon (magenta). TBP is colored in blue and a modeled DNA is shown in grey.

Supplementary Tables

Supplementary Table 1. Summary of crystallographic data and refinement statistics

	*Ec*Mot1^{NTD}:*Ec*TBP SeMet	*Ec*Mot1^{NTD}:*Ec*TBP Native	*Ec*TBP Native
Data collection			
Space group	$P2_1$	$P2_1$	H3
Cell dimensions			
a, *b*, *c* (Å)	99.0, 146.8, 102.8	99.3, 147.8, 103.4	104.4, 104.4, 129.1
α, β, γ (°)	90.0, 90.0, 94.6	90.0, 90.0, 94.5	90.0, 90.0, 120.0
Wavelength (Å)	0.9796	0.8726	1.0000
Resolution (Å)*	50.0 – 3.3 (3.4 – 3.3)	50.0 – 3.1 (3.2 – 3.1)	30.0 – 1.9 (2.0 – 1.9)
R_{sym} (%)*	11.8 (36.8)	6.7 (60.0)	5.5 (39.0)
$I/\sigma I$*	9.9 (2.4)	17.1 (2.4)	15.1 (3.4)
Completeness (%)*	95.2 (81.1)	99.7 (97.2)	90.2 (84.8)
Redundancy*	4.2 (2.2)	4.3 (4.2)	3.0 (2.9)
Refinement			
Resolution (Å)		3.1	1.9
No. reflections		54011	36954
R_{work}/ R_{free} (%)		18.7 / 24.2	17.9 / 21.5
No. atoms			
Protein		14984	3027
Ligand/ion		24	16
Water		29	305
B-factors			
Protein		79.5	30.6
Ligand/ion		111.9	45.7
Water		54.7	40.6
R.m.s deviations			
Bond lengths (Å)		0.005	0.007
Bond angles (°)		0.868	1.080
Ramachandran (%)			
favoured		91.1	98.3
allowed		8.4	1.7
outlier		0.5	0.0

*Highest resolution shell is shown in parenthesis.

Supplementary Table 1. Summary of crystallographic data and refinement statistics.

*Sc*Mot1	*Ec*Mot1		*Ec*TBP	*Sc*TBP
D365	D212	—	R48	R90
F363	F210	⋰		
Y368	D215	—	R65	R107
D369	D216	—	R96	K138
		╲	K99	R141
D462	D290	╲		
D464	D292	—	K103	K145
W420	W255	⋰		
E421	Q256	—	R46	H88
Y366	F213	·····	I100	I142
		⋱	L104	I146
I164	M109	·····	F57	R90
R165	V110	⋰		
A168	I113	·····	I61	I103
		⋱	L72	L114
K169	A114	·····	V29	V71
Q175	E120	—	K159	K240
M179	F123	·····	Q116	Q158
D184	F129	·····	F74	F116
		⋱	A58	A100

Supplementary Table 2. Contact residues of *Ec*Mot1^{NTD} and *Ec*TBP. Contact residues between *Ec*Mot1^{NTD} and *Ec*TBP, along with corresponding residues in *Saccharomyces cerevisiae* (*Sc*). Solid lines indicate a bonded interaction whereas dashed lines represent hydrophobic interactions. Residues known to influence the interaction of Mot1 and TBP are highlighted in blue[6].

Dynamic Light Scattering	**Hydrodynamic radius (nm)**		**Corresponding MW of a globular protein (kDa)**	**Expected MW from sequence content (kDa)**	**Oligomeric state**
***Ec*Mot1^{NTD}**	6.4		262	92	Homodimer
***Ec*Mot1^{NTD}:*Ec*TBP**	5.2		162	116	Heterodimer
***Ec*Mot1$^{NTD\Delta latch}$**	6.2		245	88	Homodimer
***Ec*Mot1$^{NTD\Delta latch}$:*Ec*TBP**	6.1		233	112	Dimer of heterodimer
Static Light Scattering	**M_n**	**M_w**	**Polydispersity (M_w / M_n)**	**Expected MW from sequence content (kDa)**	**Oligomeric state**
***Ec*TBP**	63.7	64.5	1.012	24	Homodimer
***Ec*Mot1$^{NTD\Delta latch}$**	173.9	174.2	1.001	88	Homodimer
***Ec*Mot1^{NTD}:*Ec*TBP**	128.2	129.8	1.013	116	Heterodimer
***Ec*Mot1$^{NTD\Delta latch}$:*Ec*TBP**	224.0	225.6	1.007	112	Dimer of heterodimer
***Ec*Mot1**	161.0	161.5	1.003	147	Monomer

Supplementary Table 3. Dynamic light scattering and static light scattering. The table summarizes results from dynamic light scattering (DLS) and static light scattering (SLS) experiments and includes most probable oligomeric states of the proteins. The absolute molecular weights in dynamic light scattering can substantially differ from the calculated molecular weights since they rely on globular shaped proteins. The obtained molecular weights from static light scattering are in good agreement with the estimated molecular weights from analytical size exclusion chromatography. *Ec*Mot1^{NTD} and *Ec*Mot1$^{NTD\Delta latch}$ form homodimers. In contrast, the *Ec*Mot1^{NTD}:*Ec*TBP complex has a reduced hydrodynamic radius / molecular weight, consistent with a 1:1 heterodimer, whereas the *Ec*Mot1$^{NTD\Delta latch}$:*Ec*TBP complex forms a heterotetramer. Together with size exclusion experiments, this analysis shows that the Mot1 latch is needed for preventing the formation of TBP dimers.

Supplementary References

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6. Darst, R. P. *et al.* Mot1 regulates the DNA binding activity of free TATA-binding protein in an ATP-dependent manner. *The Journal of biological chemistry* **278**, 13216-13226, (2003).