

1 **Phylogenomics-guided discovery of a novel conserved**
2 **cassette of short linear motifs in BubR1 essential for the**
3 **spindle checkpoint**

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16

17 **Abstract**

18 The spindle assembly checkpoint (SAC) maintains genomic integrity by preventing
19 progression of mitotic cell division until all chromosomes are stably attached to spindle
20 microtubules. The SAC critically relies on the paralogs Bub1 and BubR1/Mad3, which
21 integrate kinetochore-spindle attachment status with generation of the anaphase
22 inhibitory complex MCC. We previously reported on the widespread occurrences of
23 independent gene duplications of an ancestral 'MadBub' gene in eukaryotic evolution
24 and the striking parallel subfunctionalization that lead to loss of kinase function in
25 BubR1/Mad3-like paralogs. We now present an elaborate subfunctionalization analysis
26 that includes all known motifs in Bub1 and BubR1, and show that ancestral features are
27 consistently retained in the same functional paralog: GLEBS/CDI/CDII/kinase in the
28 Bub1-like and KEN1/KEN2/D-Box in the BubR1/Mad3-like. The recently described
29 ABBA motif can be found in either or both paralogs. We however discovered two
30 additional ABBA motifs that flank KEN2. This cassette of ABBA1-KEN2-ABBA2 forms a
31 strictly conserved module in all ancestral and BubR1/Mad3-like proteins, suggestive of
32 a specific and crucial SAC function. Indeed, deletion of the ABBA motifs in human
33 BUBR1 abrogates the SAC and affects APC/C-Cdc20 interactions. Our detailed
34 comparative genomics analyses thus enabled discovery of a conserved cassette of
35 motifs essential for the SAC and shows how this approach can be used to uncover
36 hitherto unrecognized functional protein features.

37

38 **Introduction**

39 Chromosome segregation during cell divisions in animals and fungi is monitored by a
40 cell cycle checkpoint known as the spindle assembly checkpoint (SAC) (Vleugel et al.,
41 2012; London and Biggins, 2014a; Musacchio, 2015). The SAC couples absence of stable
42 attachments between kinetochores and spindle microtubules to inhibition of anaphase
43 by assembling a four-subunit inhibitor of the anaphase-promoting complex (APC/C),
44 known as the MCC (Sacristan and Kops, 2015; Izawa and Pines, 2014; Chao et al., 2012).
45 The molecular pathway that senses lack of attachment and produces the MCC relies on
46 two related proteins known as Bub1 and BubR1/Mad3 (London and Biggins, 2014a).
47 Bub1 is a serine/threonine kinase that localizes to kinetochores and promotes
48 recruitment of MCC subunits and of factors that stimulate its assembly (Klebig et al.,
49 2009; London and Biggins, 2014b; Vleugel et al., 2015) These events are largely
50 independent of Bub1 kinase activity, however, which instead is essential for the
51 correction process of attachment errors (Kawashima et al., 2010; Klebig et al., 2009;
52 Andrews et al., 2004). BubR1/Mad3 is one of the MCC subunits, responsible for directly
53 preventing APC/C activity and anaphase onset (Tang et al., 2001; Sudakin et al., 2001;
54 Chao et al., 2012). It does so by contacting multiple molecules of the APC/C co-activator
55 Cdc20, preventing APC/C substrate access and binding of the E2 enzyme UbcH10 (Chao
56 et al., 2012; Izawa and Pines, 2014; Alfieri et al., 2016; Yamaguchi et al., 2016). The
57 BubR1/Mad3-Cdc20 contacts occur via various short linear motifs (SLiMs) known as
58 ABBA, KEN, and D-box (Alfieri et al., 2016; Chao et al., 2012; Lischetti et al., 2014; King
59 et al., 2007; DiFiore et al., 2015; Burton and Solomon, 2007b). Like Bub1, BubR1 also
60 impacts on the attachment error-correction process via a KARD motif that recruits the
61 PP2A-B56 phosphatase (Suijkerbuijk et al., 2012b; Kruse et al., 2013; Xu et al., 2013).

62 This may not however be a universal feature of BubR1/Mad3-like proteins, because
63 many lack a KARD-like motif.

64

65 Bub1 and BubR1/Mad3 are paralogs. We previously showed they originated by similar
66 but independent gene duplications from an ancestral MadBub gene in many lineages,
67 and that the two resulting gene copies then subfunctionalized in remarkably
68 comparable ways (Suijkerbuijk et al., 2012a). An ancestral N-terminal KEN motif (KEN1:
69 essential for the SAC) and an ancestral C-terminal kinase domain (essential for
70 attachment error-correction) were retained in only one of the paralogous genes in a
71 mutually exclusive manner in virtually all lineages (i.e. one gene retained KEN but lost
72 kinase, while the other retained kinase but lost KEN). One exception to this 'rule' are
73 vertebrates where both paralogs have a kinase-like domain. The kinase domain of
74 human BUBR1 however lacks enzymatic activity (i.e. is a pseudokinase) but instead
75 confers stability onto the BUBR1 protein (Suijkerbuijk et al., 2012a).

76

77 The similar subfunctionalization of Bub1 and BubR1/Mad3-like paralogs was inferred
78 from analysis of two domains (TPR and kinase) and one motif (KEN1). We set out to
79 analyze whether any additional features specifically segregated to Bub1- or
80 BubR1/Mad3-like proteins after duplications by designing an unbiased feature
81 discovery pipeline and tracing feature evolution. The pipeline extracted all known and
82 various previously unrecognized conserved motifs from Bub1/BubR1 family gene
83 members. Two of these are novel ABBA motifs that flank KEN2 specifically in
84 BuBr1/Mad3-like proteins and we show that this highly conserved and ABBA-KEN2-
85 ABBA cassette is crucial for the SAC in human cells.

86

87 **Results and Discussion**

88 *Refined phylogenomic analysis of the MadBub gene family pinpoints 16 independent gene*
89 *duplication events across the eukaryotic tree of life.*

90 To enable detailed reconstruction of subfunctionalization events of all known functional
91 features after duplication of ancestral MadBub genes, we expanded our previously
92 published set of homologs (Suijkerbuijk et al., 2012a) through broader sampling of
93 sequenced eukaryotic genomes, focusing on sequences closely associated with
94 duplication events (**supplementary sequence file 1**). Phylogenetic analyses of a
95 multiple sequence alignment of the TPR domain (the only domain shared by all MadBub
96 family members) of 149 MadBub homologs (**supplementary discussion** and
97 **supplementary figure 1**) corroborated the ten independent duplications previously
98 described (Suijkerbuijk et al., 2012a) and allowed for a more precise determination of
99 the age of the duplications. Strikingly, we found evidence for a number of additional
100 independent duplications: Three duplications in stramenopile species of the SAR super
101 group (*Albuginaceae* {#10 in **figure 1b**}, *E. siliculosus* {#11} and *A. anophagefferens*
102 {#12}) and one at the base of basidiomycete fungi ({#4}, puccinioimycetes). The BUBR1
103 paralog in teleost fish underwent a duplication and fission event, of which the C-
104 terminus product was retained only in the lineage leading to zebra fish (*D. rerio* {#7}).
105 Lastly, through addition of recently sequenced genomes we could specify a duplication
106 around the time plants started to colonize land ({#13}, bryophytes) and an independent
107 duplication in the ancestor of higher plants ({#14}, tracheophytes), followed by a
108 duplication in the ancestor of the flowering plants ({#15}, magnoliophytes). These gave
109 rise to three MadBub homologs, signifying additional subfunctionalization of the
110 paralogs in the plant model organism *A. thaliana*. It thus seems to be the case that such

111 striking parallel subfunctionalization as we originally identified, is indeed predictive for
112 more of its occurrence in lineages whose genome sequences have since been elucidated.

113

114 *De novo discovery, phylogenetic distribution and fate after duplication of functional motifs*
115 *in the MadBub gene family.*

116 Previous analyses revealed a recurrent pattern of mutually exclusive retention of an N-
117 terminal KEN-box and a C-terminal kinase domain after duplication of an ancestral
118 MadBub (Suijkerbuijk et al., 2012a; Murray, 2012). These patterns suggested the
119 hypothesis of paralog subfunctionalization towards either inhibition of the APC/C in the
120 cytosol (retaining the KEN-box) or attachment-error correction at the kinetochore
121 (retaining the kinase domain). Given the extensive sequence divergence of MadBub
122 homologs and a scala of different known functional elements, we reasoned that a
123 comprehensive analysis of MadBub gene duplicates would provide opportunities for the
124 discovery of novel and co-evolving ancestral features. For clarity we refer to the Bub1-
125 like paralog (C-terminal kinase domain) as BUB and the BubR1/Mad3-like paralog (N-
126 terminal KEN box) as MAD throughout the rest of this manuscript.

127

128 To capture conserved ancestral features of diverse eukaryotic MadBub homologs, we
129 constructed a sensitive de novo motif and domain discovery pipeline (ConFeaX:
130 conserved feature extraction) similar to our previous approach used to characterize
131 KNL1 evolution (Tromer et al., 2015). In short, the MEME algorithm (Bailey et al., 2009)
132 was used to search for significantly similar gapless amino acid motifs, and extended
133 motifs were aligned by MAFFT (Kato and Standley, 2013). Alignments were modeled
134 using HMMER (Eddy, 2011) and sensitive profile HMM searches were iterated and
135 specifically optimized using permissive E-values/bit scores until convergence

136 (**methods** and **figure 1a**). Due to the degenerate nature of the detected short linear
137 motifs, we manually scrutinized the results for incorrectly identified features and
138 supplemented known motif instances, when applicable. We preferred ConFeaX over
139 other de novo motif discovery methods (e.g. Davey et al., 2012 Nguyen Ba et al., 2012)
140 as it does not rely on high quality full length alignment of protein sequences and allows
141 detection of repeated or dynamic non-syntenic conserved features (which is a common
142 feature for SLiMs). It is therefore better tuned to finding conserved features over long
143 evolutionary distances in general and specifically in this case where recurrent
144 duplication and subfunctionalization hamper conventional multiple sequence alignment
145 based analysis.

146

147 ConFeaX identified known functional motifs and domains and in some cases extended
148 their definition: KEN1 (Murray and Marks, 2001), KEN2 (Burton and Solomon, 2007a),
149 GLEBS (Taylor et al., 1998), KARD (Suijkerbuijk et al., 2012b; Xu et al., 2013; Kruse et al.,
150 2013), CDI (Klebig et al., 2009), D-box (Burton and Solomon, 2007a), CDII (a co-
151 activator domain of BUB1 (Kang et al., 2008; Klebig et al., 2009)) and the recently
152 discovered ABBA motif (termed ABBA3 in **figure 3**) (DiFiore et al., 2015; Lischetti et al.,
153 2014) (**figure 1a, supplementary table II** and **supplementary sequence file 2**). The
154 TPR and the kinase domain were annotated using profile searches of previously
155 established models (Suijkerbuijk et al., 2012a) and excluded from de novo sequence
156 searches. KEN1 and KEN2 could be discriminated by differentially conserved residues
157 surrounding the core KEN box (**figure 1a**). Those surrounding KEN1 are involved in the
158 formation of the helix-turn-helix motif that positions yeast Mad3 towards Cdc20 (Chao
159 et al., 2012), while two pseudo-symmetrically conserved tryptophan residues with
160 unknown function specifically defined KEN2. Furthermore, we found that the third

161 position of the canonical ABBA motif is often occupied by a proline residue and the first
162 position in ascomycetes (fungi) is often substituted for a polar amino acid [KRN] (**figure**
163 **1a**), signifying potential lineage-specific changes in Cdc20-ABBA interactions. Last, we
164 also discovered a novel motif predominantly associated with the MAD paralog in
165 basidiomycetes, plants, amoeba and stramenopiles but not metazoa, which we termed
166 conserved motif I (CMI) (**figure 1a**).

167

168 Projection of the conserved ancestral features onto the MadBub gene phylogeny
169 provided a highly detailed overview of MadBub motif evolution (**figure 1b**,
170 **supplementary figure 1b**). We found that the core functional motifs and domains
171 (TPR, KEN1, KEN2, ABBA, D-box, GLEBS, CMI, CDI, CDII and kinase) are present
172 throughout the eukaryotic tree of life, representing the core features that were likely
173 part of the SAC signaling network in the last eukaryotic common ancestor (LECA). Of
174 note are lineages (nematodes, flatworms (*S. mansoni*), dinoflagellates (*S. minutum*) and
175 early-branching fungi (microsporidia and *C. coronatus*)) for which multiple features
176 were either lost or considerably divergent (**supplementary figure 1b**). Especially
177 interesting is *C. elegans* in both KEN boxes and the GLEBS domain appeared to have
178 been degenerated (ceMAD=san-1) and the CDI domain is lost (ceBUB=bub-1), indicating
179 extensive rewiring or a less essential role of the SAC in nematode species, as has been
180 suggested recently (Davey and Morgan, 2016; Moyle et al., 2014).

181

182 Our motif discovery analyses revealed the CDC20/Cdh1-interacting ABBA motif to be
183 much more abundant than the single instances that were previously reported for
184 BUBR1 and BUB1 in humans (Lischetti et al., 2014; DiFiore et al., 2015). We observed
185 three different contexts for the ABBA motifs (**figure 1b, supplementary figure 1b**): (1)

186 in repeat arrays (e.g. MAD of *P. patens*, basidiomycetes and stramenopiles), (2) in the
187 vicinity of CDI (many instances) and/or D-box/KEN (e.g. human), and (3) as two highly
188 conserved ABBA motifs flanking KEN2 (virtually all species). Because of the positional
189 conservation of the latter, we have termed these, ABBA1 and ABBA2. Any additional
190 ABBA motifs were pooled in the category 'ABBA-other'.

191

192 In order to track the fate of the features discovered using ConFeaX, we quantified their
193 co-presences and absences, as a proxy for co-evolution, by calculating the Pearson
194 correlation coefficient (r) for the profiles of each domain/motif pair of 16 duplicated
195 MadBub homologs (**figure 1b**) (Wu et al., 2003). Subsequent average clustering of the
196 Pearson distance ($d=1-r$) revealed two sets of co-segregating and anti-correlated
197 conserved features (**figure 2a+b**) consistent with our hypothesis that MadBub gene
198 duplication caused parallel subfunctionalization of features towards the kinetochore
199 (mainly BUB) and the cytosol (MAD) (Suijkerbuijk et al., 2012a). GLEBS, CDI, ABBA-
200 other, KARD, CDII and the kinase domain formed a coherent cluster of features with
201 bona fide function at the kinetochore. For a detailed discussion on several intriguing
202 observations regarding presence/absence of these motifs in several eukaryotic lineages,
203 and what this may mean for BUB/MAD and SAC function in these lineages, see
204 **Supplementary Discussion**. A second cluster contained known motifs that bind and
205 interact with (multiple) CDC20 molecules, including KEN1, KEN2, and (to a lesser
206 extent) the D-box. Our newly discovered ABBA motifs that flank KEN2 were tightly
207 associated with KEN2 and KEN1 (**figure 2**). As such, the ABBA1-KEN2-ABBA2 cassette
208 (**Figure 3a**) co-segregated with MAD function during subfunctionalization of MadBub
209 gene duplicates. Although the D-box often co-occurs with the KEN-ABBA cluster, this
210 motif was occasionally lost (e.g. archeplastids, *S. pombe* and *A. anophagefferens*).

211 Finally, CMI co-segregated with the Cdc20-interacting motifs (Figure 2a), suggesting a
212 MAD-specific role for this newly discovered motif (possibly in MCC function and/or
213 Cdc20-binding) in species harboring it such as plants, basidiomycetes and
214 stramenopiles.

215

216 *The conserved ABBA1-KEN2-ABBA2 cassette is essential for proper SAC signaling in*
217 *human cells*

218 The strong correlation of the ABBA1-KEN2-ABBA2 cassette with KEN1 and the D-box,
219 urged us to examine the role of these motifs in BUBR1-dependent SAC signaling in
220 human cells. We therefore generated stable isogenic HeLa FlpIn cell lines expressing
221 doxycyclin-inducible versions of LAP-tagged BUBR1 (Suijkerbuijk et al., 2010). These
222 included: Δ ABBA1, Δ ABBA2, Δ ABBA1+2, alanine-substitutions of the two KEN2-flanking
223 tryptophans (W1-A, W2-A and W1/2-A), KEN1-AAA KEN2-AAA, Δ ABBA3 and Δ D-box
224 (**figure 3a-c**). The SAC was severely compromised in cells depleted of endogenous
225 BUBR1 by RNAi, as measured by inability to maintain mitotic arrest upon treatment
226 with S-trityl-L-cysteine (STLC) (Ogo et al., 2007) (median(m) = 50 minutes (min.) from
227 NEBD to mitotic exit, compared control ($m > 500$ min.)) (**figure 3d-e**). SAC proficiency
228 was restored by expression of siRNA-resistant LAP-BUBR1 ($m > 500$ min.). As shown
229 previously (Burton and Solomon, 2007a; Elowe et al., 2010; Lara-Gonzalez et al., 2011),
230 mutants of KEN1, KEN2 and the D-box strongly affected the SAC. Importantly, BUBR1
231 lacking ABBA1 or ABBA2 or both, or either of the two tryptophans, could not rescue the
232 SAC (**figure 3e**). We observed a consistently stronger phenotype for the mutated motifs
233 on the N-terminal side of KEN2 (Δ ABBA1 ($m = 65$ min.) and W1-A ($m = 165$ min.))
234 compared to those on the C-terminal side (Δ ABBA2 ($m = 200$ min.) and W2-A ($m = 260$
235 min.)). The double ABBA (1/2) and tryptophan (1/2) mutants were however further

236 compromised ($m = 50$ and 110 min., respectively), suggesting non-redundant functions.
237 As expected from the interaction of ABBA motifs with the WD40 domain of CDC20 and
238 CDH1 (DiFiore et al., 2015), BUBR1 lacking ABBA1 and/or ABBA2 was less efficient in
239 binding APC/C-Cdc20 in mitotic human cells, to a similar extent as mutations in KEN1
240 (**figure 3f**). In our hands ABBA1 and 2 mutants were more strongly deficient in SAC
241 signaling and APC/C-Cdc20 binding than the previously described ABBA motif (ABBA3)
242 (**figure 3d-e**). In conclusion therefore, the ABBA1-KEN2-ABBA2 cassette in BUBR1 is
243 essential for APC/C inhibition by the SAC.

244

245 We here discovered a symmetric cassette of SLiMs containing two Cdc20-binding ABBA
246 motifs and KEN2. This cassette strongly co-occurs with KEN1 in MAD-like and MadBub
247 proteins throughout eukaryotic evolution and has important contributions to the SAC in
248 human cells. Our co-precipitation experiments along with the known roles for ABBA-
249 like motifs and KEN2 and their recent modeling into the MCC-APC/C structure (Alfieri et
250 al., 2016; Yamaguchi et al., 2016) strongly suggest that the ABBA1-W-KEN2-W-ABBA2
251 cassette interacts with one or multiple Cdc20 molecules. Together with KEN1, these
252 interactions likely regulate affinity of MCC for APC/C or its positioning once bound to
253 APC/C. The constellation of interactions between Cdc20 molecules and the various
254 Cdc20-binding motifs in one molecule of BUBR1 (3x ABBA, 2x KEN and a D-box) is not
255 immediately obvious, and will have to await detailed atomic insights. The symmetric
256 arrangement of the cassette may be significant in this regard, as is the observation that
257 (despite a highly conserved WD40 structure of Cdc20) the length of spacing between
258 the ABBA motifs and KEN2 is highly variable between species. A more detailed
259 understanding of SAC function may be aided by ConFeaX-driven discovery of lineage-

260 specific conserved features in the MadBub family when more genome sequences

261 become available, as well as of features in other SAC proteins families.

262

263 **Supplementary information**

264 Supplementary information included Supplementary discussion, 2 figures, 3 tables and

265 2 sequence files.

266

267 **Methods**

268 *Phylogenomic analysis*

269 We performed iterated sensitive homology searches with jackhammer (Finn et al.,
270 2011) (based on the TPR, kinase, CDI, GLEBS and KEN boxes) using a permissive E-
271 value and bitscore cut-off to include diverged homologs on uniprot release 2016_08
272 and Ensemble Genomes 32 (<http://www.ebi.ac.uk/Tools/hmmer/search/jackhammer>).
273 Incompletely predicted genes were searched against whole genome shotgun contigs
274 (wgs, <http://www.ncbi.nlm.nih.gov/genbank/wgs>) using tblastn. Significant hits were
275 manually predicted using AUGUST (Stanke et al., 2006) and GENESCAN (Burge and
276 Karlin, 1997). In total we used 152 MadBub homologs (**supplementary sequence file**
277 **1**). The TPR domains of 148 sequences were aligned using MAFFT-LINSI (Katoh and
278 Standley, 2013); only columns with 80% occupancy were considered for further
279 analysis. Phylogenetic analysis of the resulting multiple sequence alignment was
280 performed using RAxML (Stamatakis, 2014) (**supplementary figure 1a**). Model
281 selection was performed using Prot Test (Darriba et al., 2011) (Akaike Information
282 Criterion); LG+G was chosen as the evolutionary model.

283

284 *Conserved Feature Extraction and subfunctionalization analysis*

285 ConFeaX starts with a probabilistic search for short conserved regions (max 50) using
286 the MEME algorithm (option: any number of repeats) (Bailey et al., 2009). Significant
287 motif hits are extended on both sides by 5 residues to compensate for the strict
288 treatment of alignment information by the MEME algorithm. Next, MAFFT-LINSI (Katoh
289 and Standley, 2013) introduces gaps and the alignments are modeled using the HMMER
290 package (Eddy, 2011) and used to search for hits that are missed by the MEME

291 algorithm. Subsequent alignment and HMM searches were iterated until convergence.
292 For short linear motifs with few conserved positions, specific optimization of the
293 alignments and HMM models using permissive E-values/bit scores was needed (*e.g.*
294 ABBA motif and D-box). Sequence logos were obtained using weblogo2 (Crooks et al.,
295 2004). Subsequently, from each of the conserved features, a phylogenetic profile was
296 derived (present is '1' and absent is '0') for all duplicated MadBub sequences as
297 presented in figure 1. For all possible pairs, we determined the correlation using
298 Pearson correlation coefficient (Wu et al., 2003). Average clustering based on Pearson
299 distances ($d=1-r$) was used to indicate sub-functionalization.

300

301 *Cell culture, transfection and plasmids*

302 HeLa FlpIn T-rex cells were grown in DMEM high glucose supplemented with 10% Tet-
303 free FBS (Clontech), penicillin/streptomycin (50 mg ml⁻¹), alanyl-glutamine (Sigma; 2
304 mM). pCDNA5-constructs were co-transfected with pOgg44 recombinase in a 10:1 ratio
305 (Klebig et al., 2009) using FuGEHE HD (Roche) as a transfection reagent. After
306 transfection, the medium was supplemented with puromycin (1 µg ml⁻¹) and blasticidin
307 (8 µg ml⁻¹) until cells were fully confluent in a 10cm culture dish. siBUBR1 (5'-
308 AGAUCCUGGCUAACUGUUCUU-3' custom Dharmacon) was transfected using Hiperfect
309 (Qiagen) at 40 nM for 48h according to manufacturer's guidelines. RNAi-resistant LAP
310 (YFP)-BUBR1 was sub cloned from plC58 (Suijkerbuijk et al., 2010) into pCDNA5.1-puro
311 using AflII and BamHI restriction sites. To acquire mutants, site-directed mutagenesis
312 was performed using the quickchange strategy (for primer sequences see
313 **supplementary table III**).

314

315 *Live cell imaging*

316 For live cell imaging experiments, the stable HeLa-FlpIn-TREx cells were transfected
317 with 40nM siRNA (start and at 24 hrs). After 24 hrs, the medium was supplemented
318 with thymidine (2.5 mM) and doxycyclin (2 $\mu\text{g ml}^{-1}$) for 24 hrs to arrest cells in early S-
319 phase and to induce expression of the stably integrated construct, respectively. After 48
320 hrs, cells were released for 3 hrs and arrested in prometaphase of the mitotic cell cycle
321 (after ~8-10 hrs) by the addition of the Eg5 inhibitor S-trityl-L-cysteine (STLC, 20 μM).
322 HeLa cells were imaged (DIC) in a heated chamber (37 °C, 5% CO₂) using a CFI S Plan
323 Fluor ELWD 20x/NA 0.45 dry objective on a Nikon Ti-Eclipse wide field microscope
324 controlled by NIS software (Nikon). Images were acquired using an Andor Zyla 4.2
325 sCMOS camera and processed using NIS software (Nikon) and ImageJ.

326

327 *Immuno-precipitation and Western blot*

328 HeLa-FlpIn-TREx cells were induced with doxycyclin (2 $\mu\text{g ml}^{-1}$) 48 hrs before
329 harvesting. Synchronization by thymidine (2 mM) for 24 hrs and release for 10 hrs into
330 Taxol (2 μM) arrested cells in prometaphase. Cells were collected by mitotic shake-off.
331 Lysis was done in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% NP40, 1mM EDTA, 1mM
332 DTT, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 2 and 3
333 (Sigma). Complexes were purified using GFP-Trap beads (ChromoTek) for 15 min at
334 4°C. Precipitated proteins were washed with lysis buffer and eluted in 5x SDS sample
335 buffer. Primary antibodies were used at the following dilutions for western blotting:
336 BUBR1 (A300-386A Bethyl) 1:2000, alpha-Tubulin (T9026 Sigma) 1:5000, GFP
337 (Custom) 1:10 000, APC1 (A301-653A Bethyl) 1:2500, APC3 (gift from Phil Hieter)
338 1:2000, MAD2 (Custom) 1:2000, CDC20 (A301-180A Bethyl) 1:1000. Western blot
339 signals were detected by chemiluminescence using an ImageQuant LAS 4000 (GE
340 Healthcare) imager.

341 **Author contributions**

342 ET performed the motif search, phylogenetic analysis and SAC assays. DB performed the
343 immunoprecipitation and western blot analyses. GK, BS conceived and managed the
344 project. ET, BS and GK wrote the manuscript.

345

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353

354 **Competing Financial Interests**

355 The authors declare no competing financial interests.

356

357 **Figure Legends**

358 **Figure 1 – Fate of conserved functional sequence features after 16 independent** 359 **duplications of the MADBUB gene family throughout eukaryotic evolution (A)**

360 Overview of the de novo sequence discovery pipeline ConFeaX including the ancestral
361 conserved features of a search against the eukaryotic MADBUB gene family. The
362 consensus sequences of the detected conserved motifs are depicted as a sequence logo
363 (colors reflect distinct amino acid properties and height of the letters indicates
364 conservation of amino acids). Each features is assigned a differently colored shape. (B)
365 Cartoon of the evolutionary scenario of 16 independent duplications of the MADBUB
366 gene family throughout eukaryotic evolution, including a projection of conserved
367 features onto the linear protein representation (on scale). Gene duplications are
368 indicated by an arrow (red: high confidence, orange: uncertain). The subfunctionalized
369 paralogs MAD and BUB are color brown and blue, respectively. Numbers indicate the
370 clades in which the duplications occurred: {1}-mucorales; {2}- saccharomycetaceae; {3}-
371 schizosaccharomycetes; {4}-pucciniomycetes; {5}-agaricomycetes (excluding early-
372 branching species); {6}-vertebrates; {7}-teleost fish; {8}-nematodes; {9}-diptera (flies);
373 {10}-albuginaceae (oomycete); {11}-ectocarpales (brow algae); {12}-aureococcus
374 (harmful algae bloom); {13}-bryophytes (mosses); {14}-tracheophytes (vascular
375 plants); {15}-magnoliaphytes (flowering plants); {16}-naegleria;

376

377 **Figure 2 – Co-evolution of conserved features signify subfunctionalization of MAD**
378 **and BUB after MADBUB duplication (A)** Average clustering based on pearson
379 distances of conserved ancestral feature correlation matrix ($d=1-r$) of MADBUB
380 paralogs. Blue and red indicate co-presence or absence of features to be present in the

381 same paralog. (B) Evolutionary scenario of MADBUB subfunctionalization: MAD
382 (cytosol) as a SAC effector and BUB (kinetochore) involved in SAC signal formation and
383 kinetochore microtubule attachment.

384

385 **Figure 3 - The evolutionary conserved cassette ABBA1-KEN2-ABBA2 in BUBR1 is**

386 **essential for SAC signaling** (A) Alignment of ABBA1-KEN2-ABBA2 cassette (red).

387 Linkers (black) between ABBA motifs and KEN2 are indicated by {n}. The sequence logo

388 on top is representative for all eukaryotic sequences (colors reflect distinct amino acid

389 properties and height of the letters indicates conservation of amino acids). (B)

390 Schematic representation of LAP-hBUBR1 mutants. Color-coding is consistent for each

391 mutant in this figure (C) Immunoblots of BUBR1 and tubulin of mitotic lysates of HeLa

392 FlpIn cell lines stably expressing LAP-tagged BUBR1 proteins. Cells were treated with

393 siRNA (40nM) for 48 hours and cells were released and arrested into taxol after double

394 thymidine block. (E) Time-lapse analysis of HeLa FlpIn cells expressing hBUBR1

395 mutants, treated with 20 μ M STLC. Data (n=50, N=3) indicate the mean of cumulative

396 fraction of cells that exit mitosis after nuclear envelope breakdown. Transparent

397 regions represent the standard error of the mean. Values between accolades {} indicate

398 the median value. Cells were score by cell morphology using DIC imaging (D); see for

399 examples of SAC deficient (Δ ABBA1/2) and proficient cells (wildtype). (F) Immunoblots

400 of GFP, APC3 and CDC20 in LAP-BUBR1 precipitations (LAP-pulldown) in whole cell

401 lysates of mitotic HeLa FlpIn cells stably expressing LAP-BUBR1 mutant constructs.

402

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Figure 1

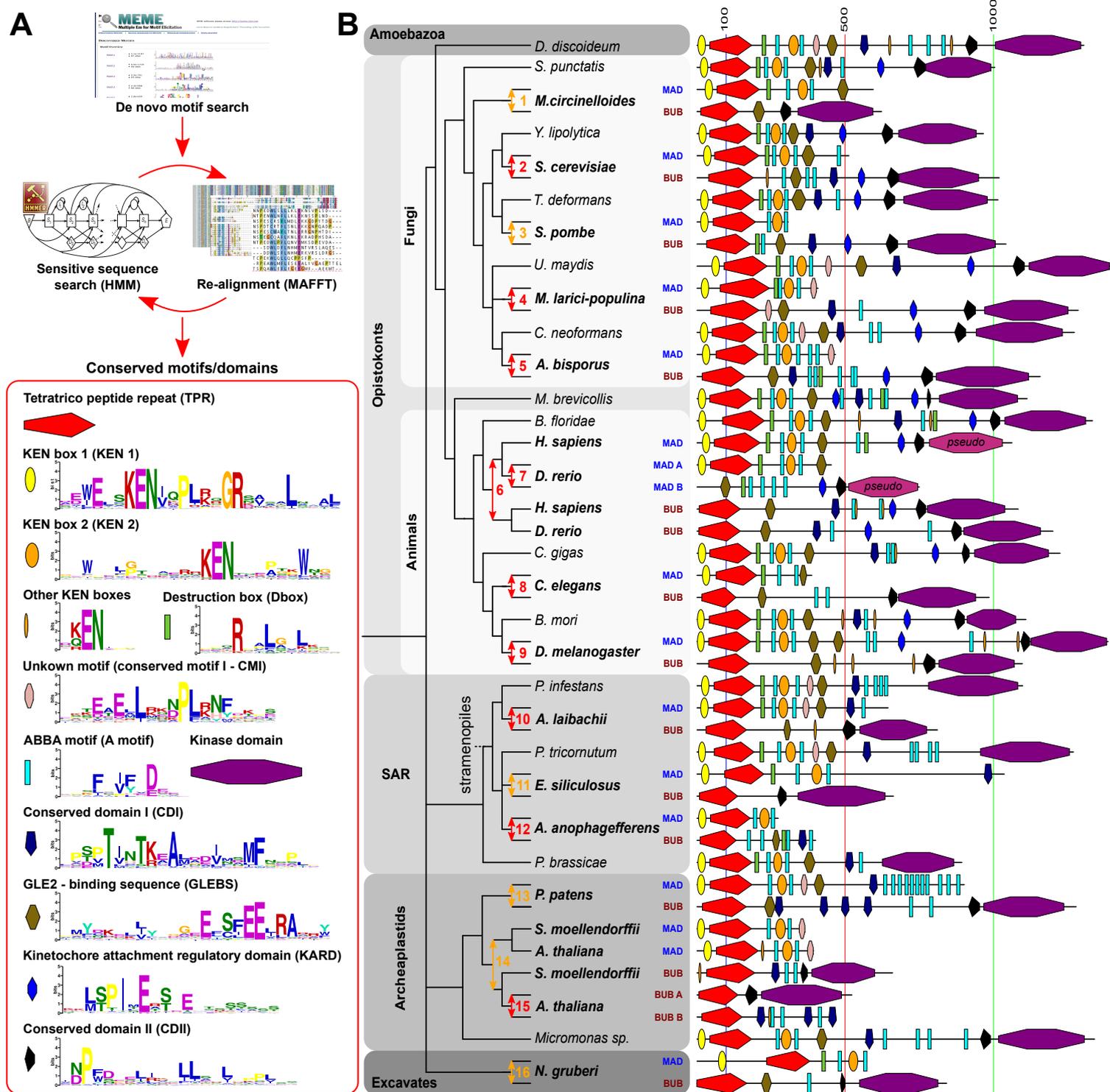


Figure 2 Tromer et al. 2016

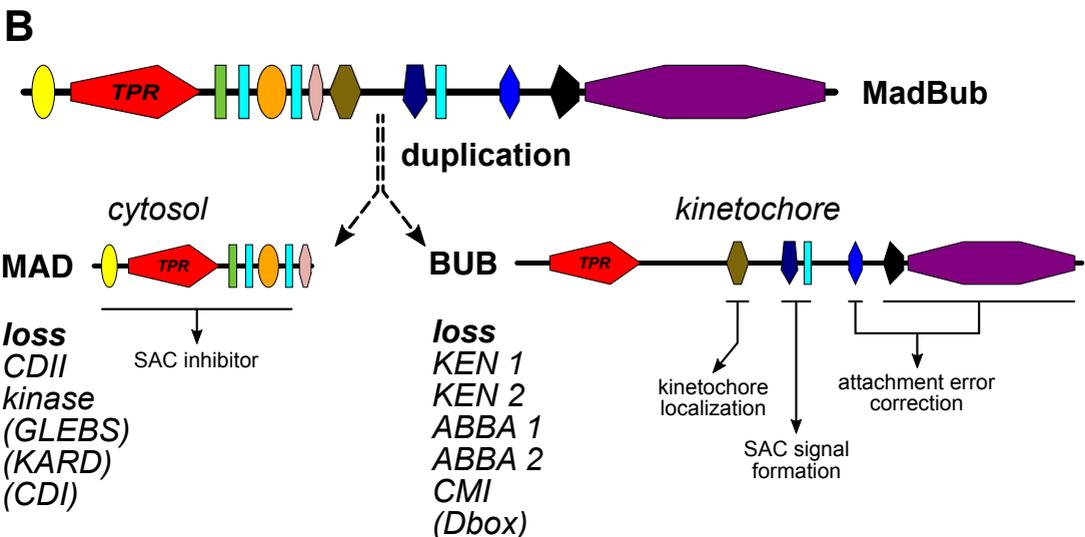
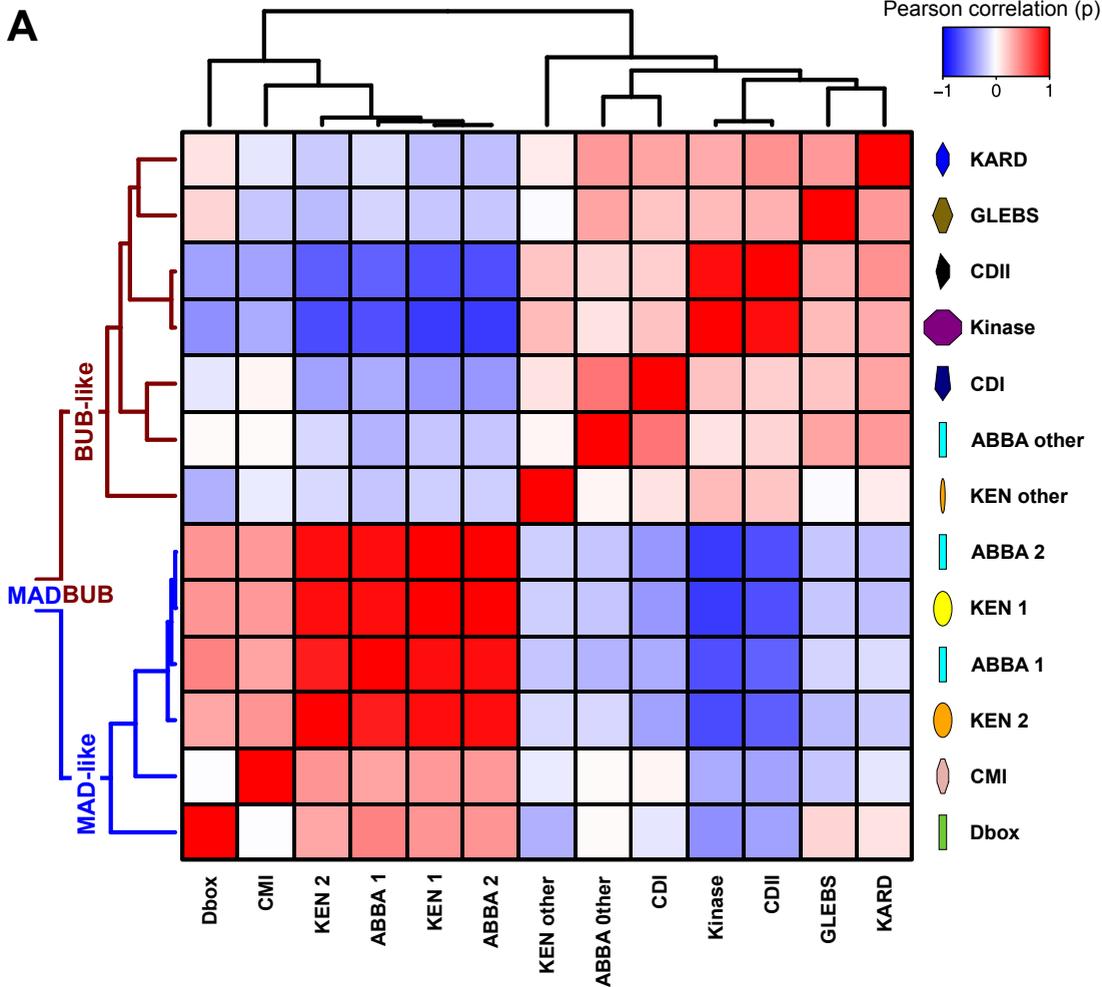


Figure 3 Tromer et al. 2016

