Developmental Cell
Short Article

The Vertebrate Mitotic Checkpoint Protein BUBR1 Is an Unusual Pseudokinase

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DOI 10.1016/j.devcel.2012.03.009

SUMMARY

Chromosomal stability is safeguarded by a mitotic checkpoint, of which BUB1 and Mad3/BUBR1 are core components. These paralogs have similar, but not identical, domain organization. We show that Mad3/BUBR1 and BUB1 paralogous pairs arose by nine independent gene duplications throughout evolution, followed by parallel subfunctionalization in which preservation of the ancestral, amino-terminal KEN box or kinase domain was mutually exclusive. In one exception, vertebrate BUBR1—defined by the KEN box—preserved the kinase domain but allowed nonconserved degeneration of catalytic motifs. Although BUBR1 evolved to a typical pseudokinase in some vertebrates, it retained the catalytic triad in humans. However, we show that putative catalysis by human BUBR1 is dispensable for error-free chromosome segregation. Instead, residues that interact with ATP in conventional kinases are essential for conformational stability in BUBR1. We propose that parallel evolution of BUBR1 orthologs rendered its kinase function dispensable in vertebrates, producing an unusual, triad-containing pseudokinase.

INTRODUCTION

Error-free chromosome segregation is dependent on the mitotic, or spindle assembly, checkpoint that prevents premature exit from mitosis (Musacchio and Salmon, 2007). The checkpoint responds to unattached kinetochores by producing a diffusible inhibitor that restrains activity of the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) toward key mitotic substrates, thereby preventing premature sister chromatid separation and mitotic exit (King et al., 1995; Sudakin et al., 1995). This inhibitor, also known as the mitotic checkpoint complex (MCC), is a three-protein complex consisting of BUB3, MAD2, and BUBR1 (known as Mad3 in budding and fission yeast) that inhibits the APC/C via interaction with the APC/C coactivator protein CDC20 (Sudakin et al., 2001). Interaction of MCC with CDC20 critically depends on an amino-terminal Lys-Glu-Asn (KEN) box in Mad3/BUBR1 (Burton and Solomon, 2007; King et al., 2007), a motif that is recognized by the APC/C as a destruction signal in bona fide APC/C substrates (Pfleger and Kirschner, 2000). A second KEN box motif in Mad3/BUBR1 is also required for APC/C inhibition by blocking substrate recruitment (Lara-Gonzalez et al., 2011). BUBR1 is in addition needed for formation of stable kinetochore-microtubule attachments (Ditchfield et al., 2003; Lampson and Kapoor, 2005).

BUBR1 shares significant similarity in both primary sequence as well as protein domain architecture with BUB1 (Figure 1A). Both contain a carboxy-terminal kinase domain, localize to unattached kinetochores via a Gle2-binding sequence (GLEBS) motif in a BUB3-dependent fashion (Taylor et al., 1998), and interact with KNL1 via a tetratricopeptide repeat (TPR) domain (Kiyomitsu et al., 2007). Although BUB1, like BUBR1, has two KEN box motifs, they are not conserved, recognized as degrons by the APC/C, and not required for cell-cycle progression (Qi and Yu, 2007). In contrast, a conserved domain (CD1) in BUB1 is required for the mitotic checkpoint and is absent from BUBR1 (Klebig et al., 2009). In addition, BUB1 protects centromeric cohesion and localizes the error-correction machinery by phosphorylating H2A (Fernius and Hardwick, 2007; Kawashima et al., 2010; Yamagishi et al., 2010; Yamaguchi et al., 2003) and regulates APC/C activity via inhibitory phosphorylation of CDC20 in human cells (Tang et al., 2001), but not in fungi (Fernius and Hardwick, 2007; Warren et al., 2002). The contribution of BUBR1 kinase activity to proper mitosis is, however, debated (reviewed in Elowe, 2011). BUBR1 orthologs in most nonvertebrate species lack a kinase domain, while possessing all other functional protein domains found in vertebrate BUBR1 (see Mad3p in Figure 1A). We set out to examine the relation between BUB1 and BUBR1 and study function of the BUBR1 kinase domain. Combined evidence from our analyses indicates that

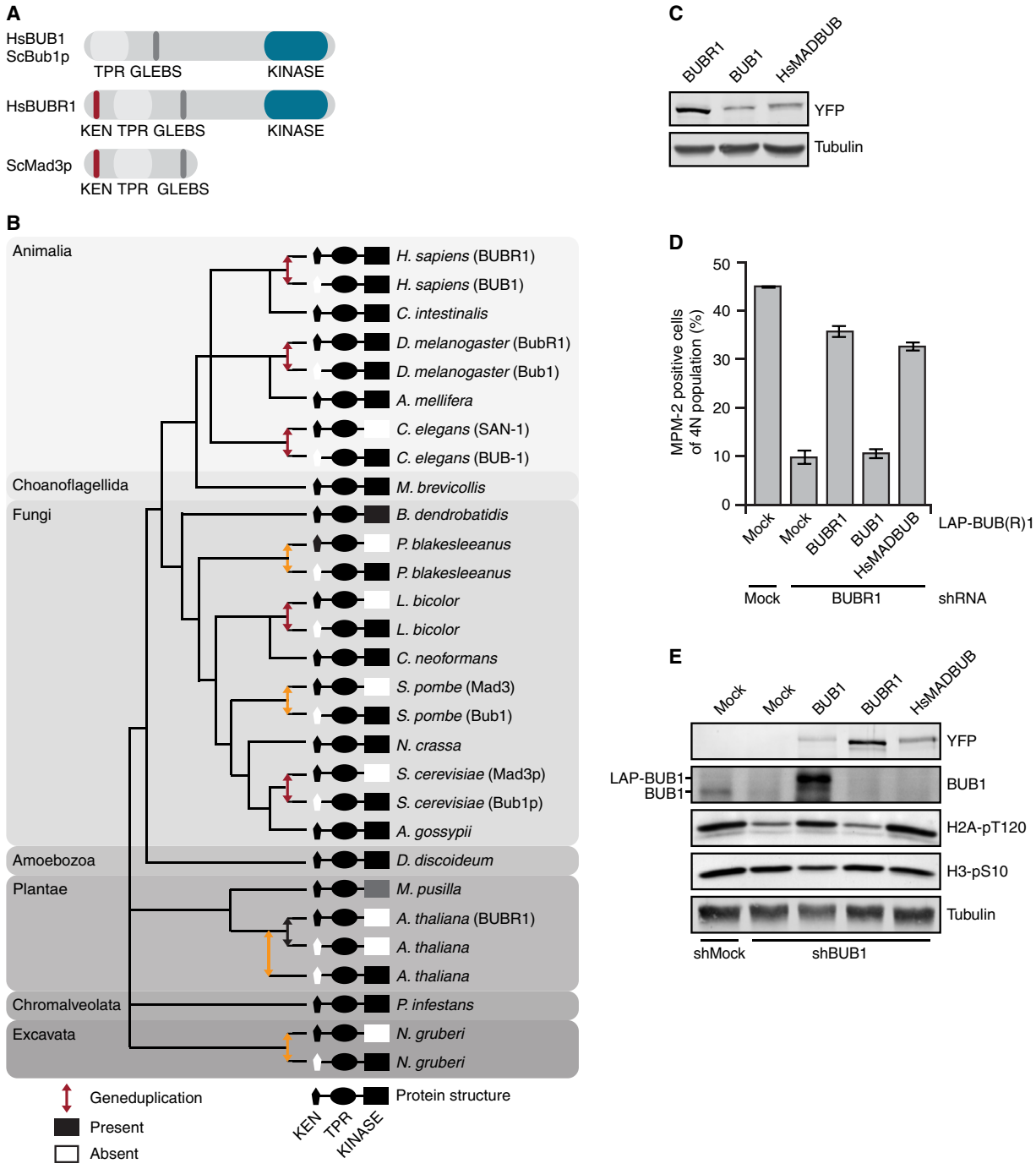

Figure 1. BUBR1/Mad3 and BUB1 Paralogous Pairs Show Widespread Parallel Evolution

(A) Schematic representation of human (Hs) and budding yeast (Sc) BUB1, BUBR1, and Mad3p.

(B) Reconciled tree of the BUB protein family. The presence (black), absence (white), and partial presence (gray) of the KEN box, TPR, and kinase domain are depicted. Arrows indicate gene duplications: high confidence, red; likely, orange; additional, black.

(C and D) Analysis of HsMADBUB, a fusion of HsBUBR1 (aa 1–714), and HsBUB1 (aa 734–1,085). Immunoblot of lysates (C) and flow cytometric analysis (D) of nocodazole-treated U2OS cells transfected with indicated shRNAs in combination with LAP-BUB(R)1. Graph represents the percentage of MPM-2-positive cells of 4N population (average of three experiments, ±SEM).

(E) Immunoblot of lysates of U2OS cells treated and transfected as in (C) and (D) selected for transfection.

See also Figure S1, Table S1, and Alignment S1.

vertebrate BUBR1 is a pseudokinase of an unusual kind: it contains the catalytic triad characteristic of conventional kinases but uses it for structural rather than catalytic purposes.

RESULTS

Mad3/BUBR1 and BUB1 Paralogous Pairs Arose by Gene Duplications on at Least Nine Independent Occasions

To identify the relation between BUBR1- and BUB1-like genes, sensitive homology searches were used to find protein sequences containing TPR and GLEBS motifs as well as fulfilling best-hit criteria, in a collection of eukaryotic genomes (van Dam et al., 2009). Although not ubiquitously present in all taxa, candidate orthologs containing these motifs were identified in all super groups of eukaryotic life that contain sequenced genomes, indicating that the BUB family possibly originated in, or before, the hypothetical Last Eukaryotic Common Ancestor (LECA). Species with BUB family genes contain either a single (e.g., *C. intestinalis*) or multiple (e.g., two in *H. sapiens* or three in *A. thaliana*) BUB-like genes. Phylogenetic analysis revealed that distinct BUB paralogs arose by gene duplication of single ancestral genes (Figure 1B; see Figure S1, Table S1, and Supplemental Alignment available online). Importantly, we found evidence for nine independent gene duplication events: in fungi (four), metazoans (three), plants (one) and excavates (one) (Figure 1B). The duplication in *S. cerevisiae* was previously identified by an independent method to be the result of the yeast Whole Genome Duplication event (Kellis et al., 2004). Our phylogenetic analysis indicates that the hypothetical ancestral LECA protein likely contained all essential protein domains of the present-day Mad3/BUBR1 and BUB1 paralogs. Moreover, the ancestral domain organization has been preserved for orthologous proteins (hereafter referred to as MADBUB) in organisms that have not undergone a duplication of an ancestral gene (Figure 1B). This raised the hypothesis that in organisms in which no gene duplication occurred, MADBUB proteins combine both Mad3/BUBR1-specific and BUB1-specific functions. In support of this, HsMADBUB, an ancestral-like protein assembled from the NH_2 terminus of human BUBR1 (aa 1–714) and the kinase domain of human BUB1 (aa 734–1,085), was able to replace BUBR1 in the mitotic checkpoint and BUB1 in phosphorylation of H2A-pT120 in human cells (Figures 1C–1E). Due to lack of specific functional domains (e.g., CD1), however, it is likely that HsMADBUB will not be able to provide full functional replacement of BUBR1 and BUB1. Nevertheless, these data show that HsMADBUB can support some paralog-specific functions and that joining paralog-specific domains does not interfere with those processes.

Parallel Subfunctionalization of BUB Paralogs

Domain analysis of all BUB paralogous pairs showed that gene duplication was invariably followed by parallel subfunctionalization, at the domain and protein level, of both duplicates. One copy, hereafter referred to as BUB1-like, lost the amino-terminal KEN box that is critical for checkpoint activity (Burton and Solomon, 2007; King et al., 2007) but retained the kinase domain in all nine cases. The other, hereafter referred to as Mad3/BUBR1-like, kept that KEN box but lost the kinase domain in all branches except insects and vertebrates (Figure 1B). BUB1 is a bona fide kinase with critical enzymatic functions in mitosis (Kang et al., 2008; Kawashima et al., 2010; Klebig et al., 2009; Warren et al., 2002; Yamagishi et al., 2010), but the necessity of enzymatic activity of BUBR1 for error-free chromosome segregation is controversial (reviewed in Elowe, 2011). Our phylogenomics studies indicated a striking tendency in orthologs of Mad3/BUBR1 (defined by the NH_2-terminal KEN box; this study; Murray and Marks, 2001), but not BUB1, to shed the kinase domain (following seven of the nine duplication events). This loss may be the result of selectively neutral reciprocal loss of domains because the other duplicate (BUB1) can fulfill essential functions, or may be indicative of a selective disadvantage of retaining kinase activity in KEN-box-containing copies.

Nonconserved Degeneration of Kinase Motifs in Vertebrate BUBR1

The strong correlation between preservation of the amino-terminal KEN box and loss of the kinase domain urged us to examine the two exceptions more closely. Comparison of critical motifs in the kinase domain of human BUBR1 and BUB1 duplicate genes and homology modeling based on the known BUB1 crystal structure (Kang et al., 2008) revealed that, whereas BUB1 contains all features of an active kinase, the domain in BUBR1 strongly diverged from both BUB1 and from canonical kinases. This was most striking in two critical kinase motifs (Figures 2A and 2B). First, the Gly-rich loop ($G_{50}xG_{52}xxG_{55}xV_{57}$, conventional nomenclature, according to PKA sequence) contains several negatively charged and bulky residues, including an aspartate at position $G_{52}{}^{PKA}$ and a leucine at $G_{55}{}^{PKA}$ (Figures 2A and 2B); both highly conserved positions. Second, the highly conserved $K_{168}{}^{PKA}$ and $N_{171}{}^{PKA}$ in the catalytic loop ($HRD_{166}xK_{168}xxN_{171}{}^{PKA}$) are substituted by a serine and cysteine, respectively (Figures 2A and 2B). Strikingly, the serine replacing $K_{168}{}^{PKA}$ in human BUBR1 ($S_{884}{}^{BUBR1}$) was found phosphorylated in mitosis (Figures 2A and S2A), effectively resulting in a charge reversal in that position. Combined, the extensive alterations and modifications in these motifs may be incompatible with catalytic ability. Interestingly, the sequence of the deviant BUBR1 kinase motifs is not conserved in the entire vertebrate lineage: various residues in both the Gly-rich and catalytic loops differ considerably among vertebrate BUBR1 species, both in sizes and charges of side chains. In contrast, similar motifs in animal BUB1 orthologs and in MADBUB proteins are highly conserved (Figures 2C and 2D). Unlike vertebrates, BubR1 in *D. melanogaster*, the only other exception in our data set that retained the BUBR1 kinase domain after gene duplication, has intact kinase features highly resembling BUB1 (Figure 2D), indicating that, for reasons unknown, DmBubR1 kinase has not degenerated and was likely under different selective pressure than its vertebrate counterpart.

Of the human kinome, 10% is predicted to be inactive because of the absence of one or more of three essential catalytic residues: $K_{72}{}^{PKA}$, $D_{166}{}^{PKA}$, and $D_{184}{}^{PKA}$ (Manning et al., 2002). Such proteins are referred to as pseudokinases. Although these residues are present in human BUBR1 (K_{795}, D_{882}, D_{911}), the degeneration of its enzymatic motifs is very similar to many pseudokinases (Scheeff et al., 2009). We thus examined whether BUBR1 can perform phosphate transfer, by monitoring autophosphorylation and phosphorylation of histone H1. As

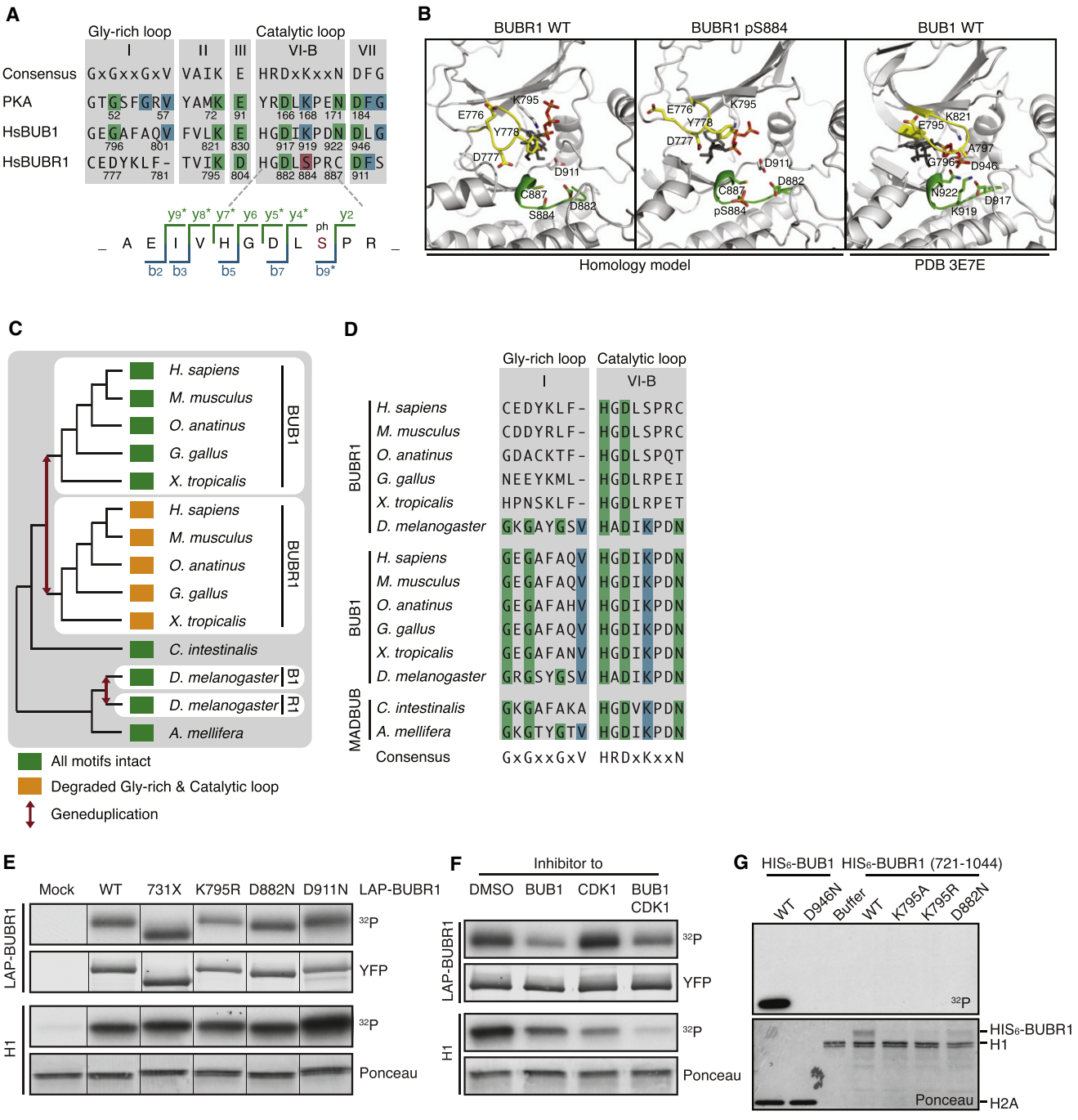

Figure 2. Nonconserved Degeneration of Kinase Motifs in Vertebrate BUBR1

(A) Multiple sequence alignment of critical motifs in PKA, HsBUB1, and HsBUBR1. Invariant (green), highly conserved (blue), and phosphorylated (red) residues are indicated. The sequence of the pS884-containing peptide of BUBR1 (aa 876–885) is shown; an asterisk marks b and y ions with a neutral loss of phosphoric acid (ph).

(B) Homology model of the WT (left) and pS884 (middle) BUBR1 sequence based on the BUB1 structure (right). The Gly-rich loop (yellow), catalytic loop (green), and relevant residues are indicated.

(C and D) Reconciled tree (C) and multiple sequence alignment (D) of the chordate and insect BUB family. Intact (green) or degenerated (orange) loops and invariant (green) or highly conserved (blue) residues are indicated.

(E and F) In vitro ^{32}P kinase assay with WT and mutant LAP-BUBR1, in the presence of DMSO, or inhibitors of BUB1 (100 μM) or CDK1 (10 μM) (F). Titration of BUBR1 was performed to obtain comparable protein levels (E).

(G) In vitro ^{32}P kinase assay with HIS_6-BUBR1 (aa 721–1,044) or HIS_6-BUB1.

See also Figure S2.

previously reported (Mao et al., 2003; Taylor et al., 1998), kinase activity of BUBR1 immunoprecipitates was observed in vitro (Figure 2E). This activity, however, was not due to BUBR1 because mutation of either one of the three catalytic residues or removal of the kinase domain did not significantly impact substrate phosphorylation (Figures 2E and S2B), although dimerization of exogenous BUBR1 with endogenous cannot be excluded. Please note that for reasons detailed below, cellular expression of the mutants was titrated to ensure comparable levels to WT in the kinase reaction. The BUBR1-unrelated kinase activity present in BUBR1 immunoprecipitations was greatly reduced by addition of inhibitors to BUB1 and CDK1 (Figures 2F, S2C, and S2D). CDK1 is known to phosphorylate H1 (Arion et al., 1988) and BUBR1 (Elowe et al., 2007), and BUB1 binds (Kops et al., 2010; Taylor et al., 2001) and phosphorylates (Figure S2E) BUBR1. Collectively, these data indicate that most likely it is additional copurifying kinases that are responsible for the observed activity in BUBR1 kinase assays.

To confirm that BUBR1 has no detectable catalytic activity in vitro, WT and mutant variants of the BUBR1 kinase domain were expressed in *E. coli* and purified (Figure S2F). A kinase activity assay using as substrates histones H1 and H2A, and BUBR1 itself, in the presence of various potential metal cofactors, showed no sign of phosphorylation activity for any of the BUBR1 variants in any condition (Figures 2G and S2G). At the same time, BUB1 was able to efficiently phosphorylate H2A in the presence of Mg^{2+}.

BUBR1 Kinase Activity Is Dispensable for Error-Free Chromosome Segregation

Lack of detectable BUBR1-specific in vitro kinase activity does not exclude that BUBR1 is a kinase under physiological conditions. To determine whether the conserved catalytic residues are needed for function of BUBR1 in mitosis, the four kinase-deficient mutants were analyzed for their ability to complement BUBR1 function in an shRNA-based reconstitution system (Suijkerbuijk et al., 2010). Mitotic checkpoint activity and fidelity of chromosome segregation were examined by measuring mitotic index upon treatment of cells with spindle poisons, and by real-time imaging of chromosome segregation during unperturbed mitotic progression. Crucially, mutation of the catalytic aspartate $D_{882}{}^{BUBR1}$ to asparagine or alanine did not affect BUBR1 function (Figures 3A–3C and S3C), nor did removal of the kinase domain (see also Suijkerbuijk et al., 2010), whereas mutation of the amino-terminal KEN box abrogated checkpoint activity (Figure S3A). In contrast, as previously shown by others (Huang et al., 2008; Kops et al., 2004; Malureanu et al., 2009; Mao et al., 2003), mutation of $K_{795}{}^{BUBR1}$ caused a slight reduction of checkpoint activity and chromosome missegregations (misalignments and lagging chromosomes) due to unstable kinetochore-microtubule attachments and accelerated mitotic exit (Lampson and Kapoor, 2005; Meraldi et al., 2004). A similar defect was observed for substitution of $D_{911}{}^{BUBR1}$ for asparagine (Figures 3A–3C). Because both the catalytic aspartate mutants and kinase-truncation mutant fully restored BUBR1 function, these data suggest that K795R and D911N may affect BUBR1 functionality in a catalysis-independent way. Indeed, we found that these mutants, but not D882N/A and 731X, were present in cells in lower levels than WT (Figures 3D and S3B). In contrast, mutation of the analogous residues in BUB1, $K_{821}{}^{BUB1}$ and $D_{946}{}^{BUB1}$, did not affect BUB1 levels (Figure 3E), showing that the impact on protein level of these mutations was specific to BUBR1.

Residues Lining the Nucleotide-Binding Pocket Are Essential for BUBR1 Conformational Stability

Unlike conventional kinases (Akamine et al., 2003; Fukuda et al., 2011), the two pseudokinases ILK and VRK3 require $K_{72}{}^{PKA}$ to maintain structural integrity, regardless of ATP binding (Fukuda et al., 2011; Scheeff et al., 2009). Three lines of evidence suggest that $K_{795}{}^{BUBR1}$ fulfills a similar function. First, the more drastic $K_{795}{}^{BUBR1}$ to alanine substitution (K795A) reduced cellular BUBR1 protein levels more than the conservative exchange with arginine (Figure 3F). Second, under close to physiological conditions, recombinant BUBR1 kinase domain (aa 721–1,044) resulted in less soluble K795A/R protein compared to WT (Figure S2F), suggesting specific destabilization or aggregation of mutant BUBR1. Third, an in vitro thermal denaturation assay showed that recombinant K795A protein had significantly lower denaturing temperature than WT protein (34.7°C versus 39.1°C, respectively) (Figures 3G and S3D), indicative of compromised intrinsic thermal stability of K795A. In agreement with this, growth of K795A-expressing cells at 27°C or 33°C for 24 hr restored protein levels of the K795A mutant (Figures 3H and S3F), and checkpoint function of these cells (Figure S3E), respectively. We thus conclude that $K_{795}{}^{BUBR1}$ is required for structural integrity. Because D882N/A is fully functional, this strongly suggests that malfunction of K795A/R, and most likely also D911N, in cells was caused by structural rather than catalytic problems.

Given their role in kinase function, the structural role of $K_{795}{}^{BUBR1}$ and $D_{911}{}^{BUBR1}$ may be related to the nucleotide-binding site. In support of this, substitution of $V_{793}{}^{BUBR1}$ for arginine (V793R) in the VAIK motif (TVIK in BUBR1), which displaces ATP from the active site of kinases or disrupts the c-spine fusion in pseudokinases that do not bind ATP (Hu et al., 2011; Kornev et al., 2008; Zeqiraj et al., 2009), also caused BUBR1 protein instability (Figure 3I) and accompanying mitotic defects (Figures 3A–3C). Interestingly, presence of AMP-PNP/Mg^{2+} had little, if any, effect on thermal stability of recombinant BUBR1 kinase domain. Instability of K795A/R and D911N in cells may therefore not, or only partly, have been due to lack of ATP binding (Figures S3G and S3H). Note that the unfolding temperature of the kinase domain is higher than that of the full-length protein, suggesting that additional domains in BUBR1 may contribute to the relative protein instability observed with the full-length protein. It furthermore indicates that the observed lack of kinase activity of the recombinant BUBR1 kinase domain in Figure 2G is not due to poorly folded protein. Collectively, these observations argue that residues lining the nucleotide-binding pocket are required to preserve structural integrity of BUBR1.

Two Bona Fide Vertebrate BUBR1 Pseudokinases

Because substitution of $D_{882}{}^{BUBR1}$ did not lower BUBR1 levels or compromise BUBR1 function in cells, the D882N/A mutants uncoupled catalytic ability from structural integrity. Importantly, the genomes of the green lizard *A. carolinensis* as well as the zebrafish *D. rerio* encode BUBR1 proteins with substitution of $D_{882}{}^{BUBR1}$ for asparagine or glycine, respectively, which we

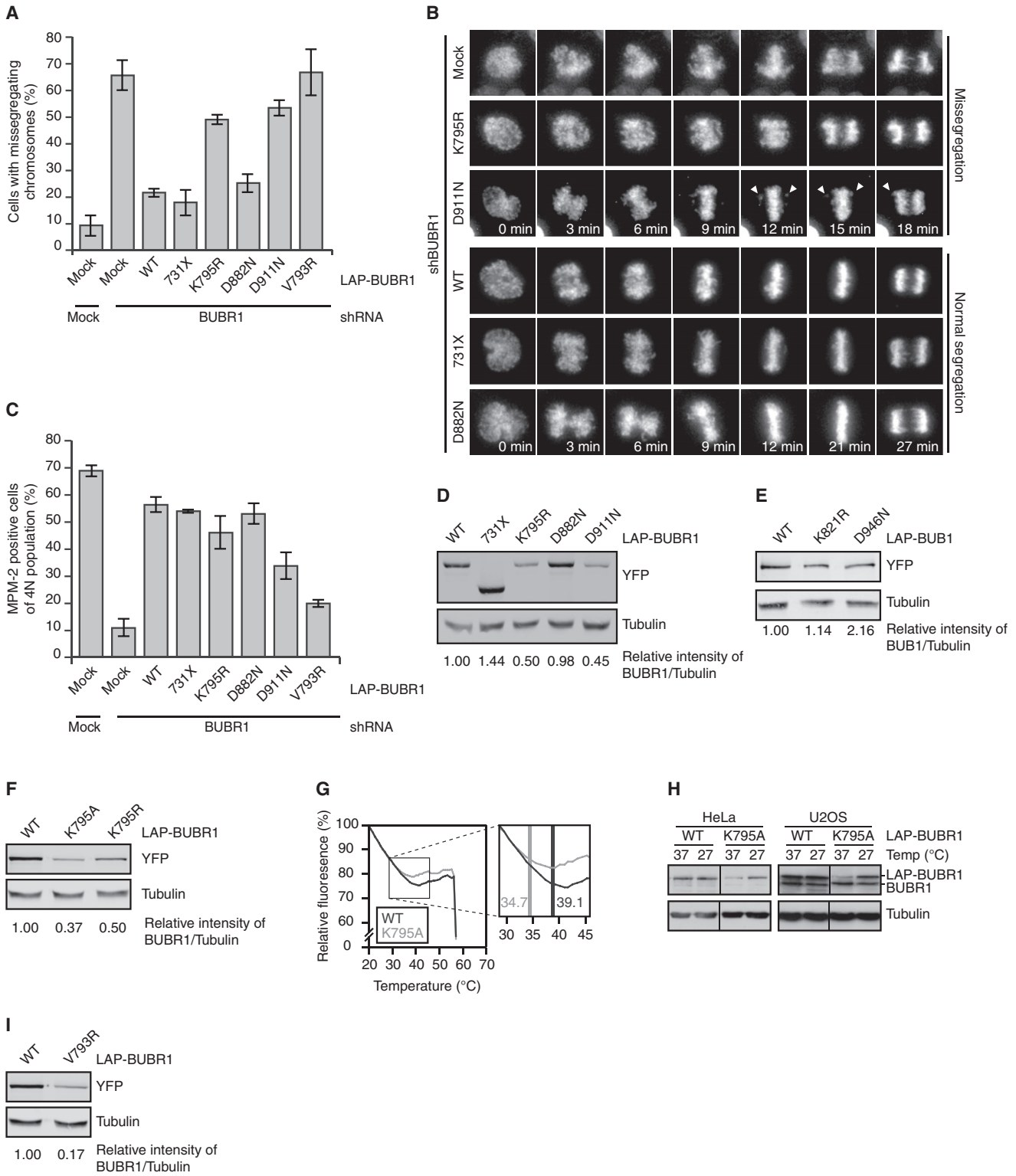

Figure 3. BUBR1 Kinase Activity Is Dispensable for Error-Free Chromosome Segregation in Human Cells

(A and B) Analysis of chromosome segregation by live imaging of HeLa cells transfected with H2B-EYFP and control (Mock) or BUBR1 shRNA in combination with control or LAP-BUBR1. (A) indicates the percentage of cells with chromosome missegregations (average of at least 100 cells in three experiments, ±SEM). Arrowheads in (B) point to missegregating chromosomes.

(C) Flow cytometric analysis of nocodazole-treated U2OS cells transfected as in (A). Graph represents the percentage of MPM-2-positive cells of 4N population (average of three experiments, ±SEM).

verified by sequencing (N_{883} lizard and G_{590} zebrafish, Figures 4A and 4B). Thus, at least two vertebrate BUBR1 orthologs are bona fide pseudokinases, with a mutation in the only residue that is structurally tolerated in human BUBR1.

DISCUSSION

Recent work has caused a redefinition of the boundary between active kinases and pseudokinases. CASK and WNK1, originally classified as pseudokinases based on the absence of D_{184}^{PKA} or K_{72}^{PKA}, respectively, were shown to use alternative strategies for catalysis (Min et al., 2004; Mukherjee et al., 2008). Based on the combined phylogenomic, structure prediction, biochemical, and functional evidence presented here, we propose that vertebrate BUBR1 represents an unusual pseudokinase, which contains all catalytic residues but most likely lacks catalytic ability and does not require catalysis for function. In most eukaryotic species and at least two vertebrates, the kinase was shed or inactivated during evolution, via subfunctionalization after gene duplication. In other vertebrates including humans, the kinase domain and key residues lining the nucleotide-binding pocket were preserved for structural purposes, whereas many other motifs were allowed strong, nonconserved degeneration.

We can only speculate as to the reasons for the high frequency of convergent subfunctionalization events of BUB paralogs or the preservation of the kinase domain in vertebrate BUBR1 orthologs. The presence of a single MADBUB copy in many evolutionary branches may indicate that the subfunctionalization is only beneficial when coinciding with external or parallel internal reasons or instead has a neutral character. Similar reasons may underlie preservation of the kinase domain in vertebrates, but more pragmatic ones such as functional coupling or proximity of the kinase domain to other features of the BUBR1 protein cannot be excluded. Indeed, previous studies suggest that kinase-proximal sequences are required for stability of kinetochore-microtubule interactions (Elowe et al., 2007; Suijkerbuijk et al., 2010), a function of BUBR1 that is not reported in species in which Mad3/BUBR1 lacks a kinase domain (e.g., *S. cerevisiae*, *C. elegans*). Conversely, loss of the kinase domain after most duplications might be related to sensitivity of this domain to structural destabilization (this study; Suijkerbuijk et al., 2010). If the kinase domain of one paralog becomes nonessential during subfunctionalization but has low tolerance for amino acid substitutions, selective pressures may have favored nonsense mutations that truncate the kinase domain altogether rather than maintaining it with risk of destabilization of the full-length protein by single substitutions within this domain.

Previous studies have reported a contribution of enzymatic activity of vertebrate BUBR1 to the mitotic checkpoint (Huang et al., 2008; Kops et al., 2004; Malureanu et al., 2009; Mao et al., 2003). These studies made use of a mutation in the BUBR1 kinase domain (equivalent of human K_{795}^{BUBR1}) that we here show reduces conformational stability. Because this likely

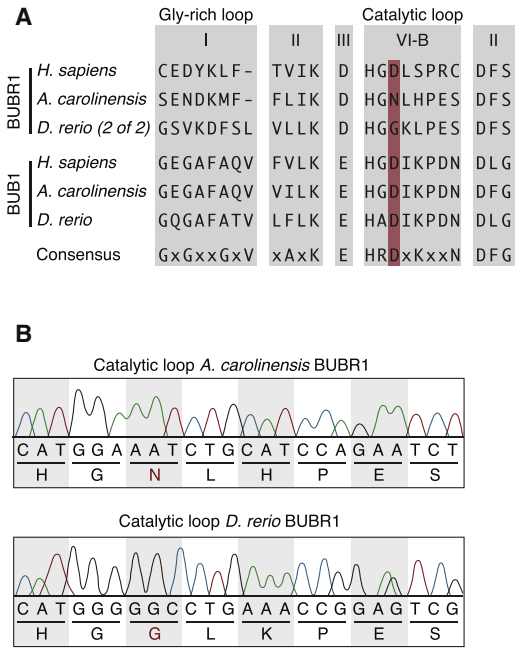

Figure 4. The BUBR1 Orthologs of the Vertebrates *A. carolinensis* and *D. rerio* Are Bona Fide Pseudokinases

(A) Multiple sequence alignment of critical kinase motifs with the mutated catalytic residue indicated in red. Zebrafish expresses two copies; the one with the kinase domain is shown.

(B) Sequence traces and corresponding nucleotide and amino acid sequences of the catalytic loop from *A. carolinensis* (lizard) genomic DNA and *D. rerio* (zebrafish) cDNA.

results in at least partly unfolded protein, it may significantly reduce the level of functional BUBR1 beyond the observed reduction in total protein (e.g., by partial unfolding of residual protein and enhanced binding of chaperone protein, like that observed for unstable disease-associated BUBR1 mutants; Suijkerbuijk et al., 2010). Retrospectively, this hinders conclusive interpretations of experiments using that mutation. Our data on the D882N/A mutations show that substituting this residue uncouples effects on kinase activity from effects on conformational stability. The D882N/A mutants restore full functionality to BUBR1-depleted cells, providing strong support for the hypothesis that BUBR1 kinase activity is dispensable for error-free chromosome segregation. We furthermore consider it unlikely that putative kinase activity may be utilized for nonmitotic processes or that the kinase domain has nonenzymatic relevance (Baas et al., 2003; Fukuda et al., 2009): *D. rerio* and *A. carolinensis* do not possess BUBR1 homologs with enzymatic activity, and the BUBR1-731X mutant that lacks the kinase domain is dispensable for mitosis, respectively. Because it remains possible that the kinase domain provides nonenzymatic functionality in other processes or under altered growth conditions, generation of

(D–F and I) Quantitative immunoblot of lysates of U2OS cells transfected with WT or mutant LAP-BUB(R)1 and selected for transfection. Band intensity of BUB(R)1 (corrected for tubulin) relative to WT is indicated (average of at least three experiments).

(G) Temperature-dependent denaturation of full-length WT and K795A GST-BUBR1; inset highlights the denaturation range.

(H) Immunoblot of lysates HeLa and U2OS cells transfected with WT or K795A LAP-BUBR1, grown at 27°C and 37°C for 24 hr.

See also Figure S3.

a vertebrate organism expressing the equivalent of BUBR1-731X will be needed to provide conclusive insight into a potential non-catalytic role of the BUBR1 kinase domain.

Hereditary kinase domain mutations in BUBR1 cause the cancer predisposition syndrome mosaic variegated aneuploidy (MVA; OMIM 257300) (Hanks et al., 2004). We recently showed that the primary cause for BUBR1 malfunction in patient cells carrying these mutations is reduced BUBR1 protein levels (Suijkerbuijk et al., 2010). One of the mutations affects isoleucine 909 (I909T), a residue in close proximity to D_{911}^{BUBR1}. It will be interesting to examine whether this mutation, as well as other disease-associated BUBR1 mutations, affects BUBR1 conformational stability in a manner similar to the K_{795}^{BUBR1} and D_{911}^{BUBR1} mutants. Structural, biophysical, and biochemical insight into the unusual pseudokinase domain of BUBR1 may reveal why stability of this kinase domain is particularly sensitive to various substitutions throughout the domain, and will aid understanding of the molecular causes of MVA.

EXPERIMENTAL PROCEDURES

All sections have additional information in the Supplemental Experimental Procedures.

Phylogenomics

Sensitive homology searches using BLAST, PSI-BLAST, and HMMER were used to search for BUB1 homologous sequences in eukaryotic genomes. The sequences were aligned using MAFFT LINSI. Phylogenetic analysis on the Multiple Sequence Alignment was performed using PhyML, RaxML, and MrBayes. The evolutionary model (LG) for the maximum likelihood methods was selected using ProtTest, and the mixed model setting for MrBayes was used.

Mass Spectrometry

LAP-BUBR1 purified from mitotic HeLa cells was analyzed on an LTQ-Orbitrap Velos (Thermo Fisher Scientific) after in-gel digestion and stage-tip purification.

Live-Cell Imaging

For live-cell imaging, cells were plated in 8-well chambered glass-bottomed slides (LabTek II), transfected, and imaged on an Olympus IX-81 microscope.

Flow Cytometry

Cells were released from a 24 hr thymidine-induced block into nocodazole for 16 hr, all cells were harvested, fixed, immunostained, and the fraction of mitotic cells was determined by flow cytometric analysis of transfected cells on a BD FACSCalibur benchtop flow cytometer.

Kinase Assays

Twenty-five microliter reactions with LAP-BUBR1, purified from mitotic 293T cells, and HIS_6-BUBR1 (aa 721–1,044), purified from *E. coli*, were performed in kinase buffer supplemented with 100 μM ATP, 5 μCi ^{32}P-γ-ATP, 2.5 μg H1 (Roche) for 30 min at 30°C. Similar conditions were used for LAP-BUB1, purified from mitotic 293T cells, except that substrates were 2.5 μg H2A (NEB) and LAP-BUBR1-731X, purified from thymidine-treated 293T cells. HIS_6-BUB1, purified from insect cells, was incubated with 100 μM ATP, 2.5 μg H2A (NEB M2502S) at 30°C for 45 min, and immunoblotted for BUB1 and pH2A-T120.

Thermal Denaturation and Circular Dichroism

Thermal denaturation was analyzed by following temperature-dependent changes in intrinsic protein fluorescence. The experiments were preformed for Purified GST-BUBR1 full-length WT or K795A (0.75 μM) and for HIS_6-BUBR1 (aa 721–1,044) in a PerkinElmer LS55 fluorimeter. Temperature-dependent unfolding was measured by far-UV circular dichroism of HIS_6-BUBR1 (aa 721–1,044) on a Jasco-32 spectropolarimeter.

ACCESSION NUMBERS

The Ensemble accession number for the BUBR1 (*A. carolinensis*) sequence reported in this paper is ENSACAT00000014492. The NCBI accession number for the BUBR1 (*D. rerio*) sequence reported in this paper is Gene ID 567931. Identification numbers of the sequences of candidate BUBR1 and BUB1 orthologs used in the reconciled evolutionary tree are depicted in the Supplemental Alignment file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, one alignment, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2012.03.009.

ACKNOWLEDGMENTS

We are grateful to M. Tollis, S. Boissinot, F. Bos, S. Schulte-Merker, N. Gray, K. Shokat, and C. Zhang for reagents, to D. Littler, T. Heidebrecht, A. van den Dikkenberg, V. Groenewold, A. McAinsh, B. Rowland, and J. Haarhuis for technical assistance, to P. Meraldi for suggesting the name MADBUB, to P. Hogeweg, P. Maddox, A. Kornev, S. Lens, N. Jelluma, and T. Dansen for valuable suggestions and critical reading of the manuscript, and to the Kops, Lens, and Medema laboratories for valuable discussions. This work was supported by the following grants: KWF UU-2009-4516 and ERC-StG KINSIGN (to G.J.P.L.K.), EMBO long-term fellowship to N.C.H. and E.v.C., Marie-Curie Excellence Grant, NWO-VIDI and UU-High Potential to S.G.D.R., and SNSF (PBBSP3-133408) to E.v.C.

Received: December 9, 2011
Revised: February 17, 2012
Accepted: March 18, 2012
Published online: June 11, 2012

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