Emergence and subsequent functional specialization of kindlins during evolution of cell adhesiveness

Julia Meller[a,*], Igor B. Rogozin[b,*], Eugenia Poliakov[c], Nahum Meller[a], Mark Bedanov-Pack[b], Edward F. Plow[a], Jun Qin[a], Eugene A. Podrez[a], and Tatiana V. Byzova[a]

[a]Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195; [b]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894; [c]Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892

ABSTRACT **Kindlins are integrin-interacting proteins essential for integrin-mediated cell adhesiveness. In this study, we focused on the evolutionary origin and functional specialization of kindlins as a part of the evolutionary adaptation of cell adhesive machinery. Database searches revealed that many members of the integrin machinery (including talin and integrins) existed before kindlin emergence in evolution. Among the analyzed species, all metazoan lineages—but none of the premetazoans—had at least one kindlin-encoding gene, whereas talin was present in several premetazoan lineages. Kindlin appears to originate from a duplication of the sequence encoding the N-terminal fragment of talin (the talin head domain) with a subsequent insertion of the PH domain of separate origin. Sequence analysis identified a member of the actin filament–associated protein 1 (AFAP1) superfamily as the most likely origin of the kindlin PH domain. The functional divergence between kindlin paralogues was assessed using the sequence swap (chimera) approach. Comparison of kindlin 2 (K2)/kindlin 3 (K3) chimeras revealed that the F2 subdomain, in particular its C-terminal part, is crucial for the differential functional properties of K2 and K3. The presence of this segment enables K2 but not K3 to localize to focal adhesions. Sequence analysis of the C-terminal part of the F2 subdomain of K3 suggests that insertion of a variable glycine-rich sequence in vertebrates contributed to the loss of constitutive K3 targeting to focal adhesions. Thus emergence and subsequent functional specialization of kindlins allowed multicellular organisms to develop additional tissue-specific adaptations of cell adhesiveness.**

Monitoring Editor
Carole Parent
National Institutes of Health

Received: Aug 21, 2014
Revised: Nov 12, 2014
Accepted: Dec 16, 2014

INTRODUCTION

The ability of cells to adhere to the extracellular matrix or to neighboring cells is essential for multicellularity. The transition from unicellular organisms to multicellular ones required the presence of transmembrane adhesion proteins such as integrins and cadherins, which facilitate cell–cell and cell–matrix adhesion and subsequent tissue formation. Integrins are transmembrane heterodimer molecules consisting of α and β subunits and are essential for cell adhesion to the extracellular matrix (ECM) or to other cells. The extracellular domains of both subunits recognize specific ligands surrounding the cell, whereas the intracellular tails of the β subunit interact with cytoskeletal and signaling proteins inside the cells. Integrins are crucial for the development and functioning of multicellular organisms, and genetic ablations of major integrin subunits in mice result in early lethality (Bouvard *et al.*, 2001). Apart from the anchorage function, integrins transduce biochemical and biomechanical signals in two directions: "inside-out" of the cell and "outside-in." Outside-in integrin signaling provides information about the cell extracellular

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-08-1294) on December 24, 2014.

*These authors contributed equally.

Address correspondence to: Tatiana V. Byzova (byzovat@ccf.org).

Abbreviations used: AFAP, actin filament–associated protein; ECM, extracellular matrix; eGFP, enhanced green fluorescent protein; FERM, four-point-one (4.1)-ezrin radixin moesin; K1, kindlin 1; K2, kindlin 2; K3, kindlin 3; PH, plecktrin homology; PINCH, particularly interesting new cysteine-histidine–rich protein.

matrix environment and initiates cellular responses to any changes occurring outside of the cell. Inside-out signaling allows modulation of cell adhesiveness through a conformational change in integrin structure, a process known as activation. In cell types that are constantly adherent to the extracellular matrix such as fibroblasts and epithelial cells, integrins are present at the sites of their ligation with extracellular matrix, known as focal adhesions (Askari *et al.*, 2010). In contrast, the integrins on circulating blood cells are normally present in the inactive conformation and are activated only upon cell stimulation with an agonist, thereby allowing cells to firmly adhere and/or aggregate. The final step of inside-out integrin activation is achieved through the binding of two immediate integrin activators, kindlin and talin, to integrin cytoplasmic domains (Byzova *et al.*, 2000; Laudanna and Bolomini-Vittori, 2009).

Talins are large proteins comprising N-terminal head and C-terminal rod domains. Both the FERM and the Rod domains of talin were shown to interact with integrins (Goksoy *et al.*, 2008; Roberts and Critchley, 2009). The talin head contains a FERM domain that is in turn divided into three subdomains, F1, F2, and F3, similar to other FERM domain–containing proteins. A portion beyond the N-terminus of the FERM domain in talins has a ubiquitin fold and is called F0 subdomain. Studies have shown that the head domain of talin is sufficient to bind integrins in vitro (Calderwood *et al.*, 1999). The head domain binds to an N-terminal membrane-proximal helix and one of the two highly conserved NXXY/F motifs in the cytoplasmic tail of β1 integrins, the membrane proximal NXXY/F. Talins are highly conserved proteins, and talin family members were common in Unikonts (Sebe-Pedros *et al.*, 2010). Similar to the β-integrin tail, the membrane-proximal NXXY/F site within adhesion molecule SibA of *Dictyostelium amoebae* interacts with talin, and this interaction is important for phagocytosis and attachment to a glass surface. Thus functions of talin in unicellular organisms include the regulation of cell adhesiveness (Cornillon *et al.*, 2006).

The kindlin-binding site involves the membrane-distal NXXY/F motif of β-integrin cytoplasmic tail (Moser *et al.*, 2009). Structurally, kindlins are similar to the head domain of talins with an addition of a pleckstrin homology (PH) domain inserted into the F2 subdomain sequence. In mammals, the family of kindlins includes three members with differential tissue distribution: kindlin 1 (K1) is restricted to epithelial cells, K2 is expressed ubiquitously, and K3 is expressed predominantly in hematopoietic and endothelial cells. Loss of K1 function in patients due to mutations in this gene leads to Kindler syndrome (KS). KS is characterized by skin atrophy and blistering (Kindler, 1954; Ashton *et al.*, 2004) and severe gastrointestinal symptoms such as ulcerative colitis (Sadler *et al.*, 2006; Kern *et al.*, 2007; Shimizu *et al.*, 2014), mucosal involvement, gingivitis, periodontitis (Ashton *et al.*, 2004; Wiebe *et al.*, 2008; Krishna *et al.*, 2014), and increased predisposition to squamous cell carcinomas (Arita *et al.*, 2007; Has *et al.*, 2010). Loss-of-function mutations in the K3 gene cause leukocyte adhesion deficiency syndrome-III (LAD-III, also known as LAD-1 variant; Malinin *et al.*, 2009, 2010; Plow *et al.*, 2009; Svensson *et al.*, 2009). LAD-III patients suffer from life-threatening infections, severe bleeding disorders, and, in some cases, anemia and osteopetrosis (Malinin *et al.*, 2009; Jurk *et al.*, 2010; McDowall *et al.*, 2010). Deletion of K2 in mice is embryonically lethal, and no cases of K2 deficiency in human patients have been described.

In this study, we analyze the evolutionary origins of kindlins, whose appearance seems to be connected with the emergence of multicellular organisms. Furthermore, we focus on the structure-based functional divergence among members of the kindlin family and map the area responsible for differential subcellular localization between K2 and K3.

RESULTS

Integrins and talins in premetazoans; emergence of kindlins in early metazoans

There is accumulating evidence that some premetazoan organisms possess homologues of metazoan integrins. Thus four β-integrin–encoding genes have been found in the genome of *Capsaspora owczarzaki* (Sebe-Pedros *et al.*, 2010; Sebe-Pedros and Ruiz-Trillo, 2010), one β-integrin in *Thecamonas trahens* (Sebe-Pedros *et al.*, 2011), and one in *Sphaeroforma arctica* (Figure 1B). The functions of integrins in these organisms are not clear; however, it was hypothesized that together with intracellular components of the cell adhesion complex (α-actinin, vinculin, and talin), these were forerunners of integrin signaling in unicellular protists (Sebe-Pedros and Ruiz-Trillo, 2010). Although it is not known how the integrin functions evolved from unicellular to multicellular organisms, it is likely that this transition required an upgrade of intracellular integrin adhesion machinery.

To understand the emergence of the complex regulatory mechanism of integrin affinity modulation, we analyzed the genomic databases for talins and kindlins in different species. BLAST searches for talin detected homologues in several Opisthokonta lineages, including premetazoans (Figure 1A). In contrast, searches of the premetazoan genome database, including the organisms with fully sequenced genomes, did not detect any homologues of kindlin (Figure 1A). On the other hand, at least one kindlin gene was found in every sequenced genome of metazoans. Kindlin was also present in a recently completed genome of *Trichoplax adhaerens*, the structurally simplest metazoan and the only described representative of the phylum *Placozoa* (Dellaporta *et al.*, 2006; Srivastava *et al.*, 2008; Supplemental Figure S1). This suggests that kindlins emerged early in metazoan evolution and are likely to play a critical role in the transition to multicellularity.

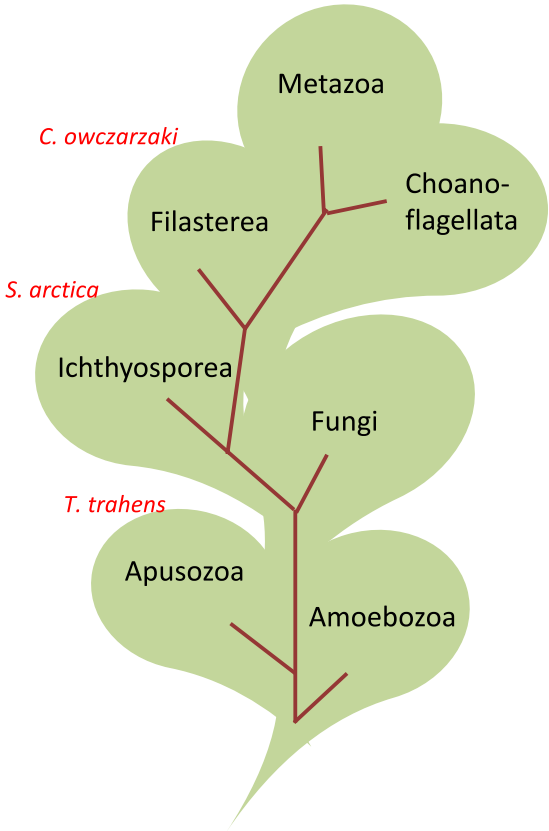
To learn when and how the interaction of kindlins with integrins evolved, we compared the sequences of β-integrin cytoplasmic domains from selected unicellular and multicellular organisms (Figure 1B). Sequence alignment showed that all of the analyzed β-integrin cytoplasmic domains contain not only the membrane-proximal NXXY/F site responsible for talin binding, but also the membrane-distal NXXY/F motif, which serves as the binding site for kindlins in metazoans (Figure 1B). Because premetazoan organisms lack kindlin, the result suggests that the membrane-distal NXXY/F motif within the β-integrin cytoplasmic domains in these organisms might have other functions but acquired a new role with the appearance of kindlins in early Metazoa. Thus it appears that the emergence of the new component of integrin signaling complex, the kindlin, allowed a more sophisticated regulation of integrin functions, which, in turn, might have been a critical step toward multicellularity.

Evolutionary origin of kindlins

The results of BLASTP searches demonstrated that kindlin is a chimeric protein consisting of the N-terminal part of talin and the fragment of a PH domain–containing protein (Figure 2). With the exception of the PH domain–containing sequence, kindlins are homologous to talins (Figure 2 and Supplemental Figure S2). Sequence similarity between human talin 1 and K2 is highly significant ($e < 10^{-18}$; Supplemental Figure S2). Moreover, in BLASTP searches for kindlins, metazoan talins appear as the next-most-similar family. This similarity suggests that kindlins originated from an ancestor of metazoan talins. This result is in agreement with another recently published

A

	Talins	Beta integrins	Kindlins
Metazoa	+	+	+
Choanoflagellata	+	-	-
Filasterea	+	+	-
Ichthyosporea	+	+	-
Apusozoa	+	+	-
Amoebozoa	+	-	-
Fungi	+	-	-



B

```
Int.β3 Homo sapiens           KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT----------
Int. β1 Hydra vulgaris        KLLATIQDRREFARFDKETKNAKWNTGENPIFKQATSTFQNPMY-GNQPQETPEEHVS
Int.β1 Capsaspora owczarzaki  KLINGMRDRREWGKFEAEREKSRWKGDDNPLFKASTTEYSNPLYNDSGK---------
Int.β2 Capsaspora owczarzaki  KLYGMLMDKREWQKFEQGRQASQWKSDNNPLFQSSVKETENPLYQGDRSH--------
Int.β3 Capsaspora owczarzaki  KIVHTIADRREWEKFQRDKESMRWKGDDNPLFKSSTKEFDNPLYNASHQQ--------
Int.β Sphaeroforma Arctica    KGIQMVMDRREWAAHNRALEGMKWNTNANPHFEPQTTETINPFYAGGP----------
Int.β Thecamonas Trahens      KIFIWKRDKQLWAKFNAQQD---WGLDANPLYKDAFDQHENPIYAENADVDDRGAAFF
```

FIGURE 1: (A) Left, table summarizing the presence of β-integrins, talins, and kindlins in metazoans and premetazoans. Database search was performed only for species with fully sequenced genomes. Right, schematic outline showing the phylogenetic positions of premetazoans expressing integrins (in red). The phylogenetic relationships are based on several recent phylogenetic studies (Sebe-Pedros *et al.*, 2010, 2011; Suga *et al.*, 2013). (B) Alignment of β-integrin cytoplasmic domains, emphasizing the membrane-proximal and the membrane-distant NPXY/F motifs responsible for interactions with talin and kindlin, respectively.

study proposing that the FERM domain originated from a protalin protein in a unicellular or proto-multicellular organism (Ali and Khan, 2014).

The PH domain–containing sequence in kindlins is most similar to the superfamily of actin filament–associated protein 1 (AFAP1)–like proteins (Figure 2 and Supplemental Figure S3). Thus AFAP1L 2 (Protein Data Bank [PDB]: 2COF) was identified as the closest BLASTP hit from the PDB database using K3 PH domain (PDB:2YS3) as a bait. In addition, BLASTP searches of the K3 PH domain (PDB:2YS3) against nonredundant protein database also resulted in identification of AFAP1 and AFAPL2 as main candidates. The secondary structures of the PH domain of kindlins and AFAP1-like proteins also show good similarity (Figure 2). The top five results from the BLASTP search were structurally aligned in the PyMOL software for further analysis. The intron/exon structure of PH domains of AFAP-1–family proteins aligned best with K2 and three PH domain intron/exon boundaries. A substantial conservation of intron positions is a general property of evolution of the exon/intron structure in deuterosotomes, and therefore intron positions can be useful phylogenetic markers for vertebrate kindlins (Rogozin *et al.*, 2012). Variability in some intron positions can be explained by intron losses/gains, whereas some other introns may not have an exact match due to the misalignment of highly diverged regions (Figure 2). For example, there is a mismatch between the first intron within the PH domain of K2 and K3, although these introns are expected to have the exact match (Supplemental Figure S3).

According to the BLASTP program, AFAP1-like proteins are reliably detected only within metazoans. With the exception of two PH domains in the middle, AFAP1-like proteins are quickly evolving (e.g., the fraction of identical residues between human and lancelet

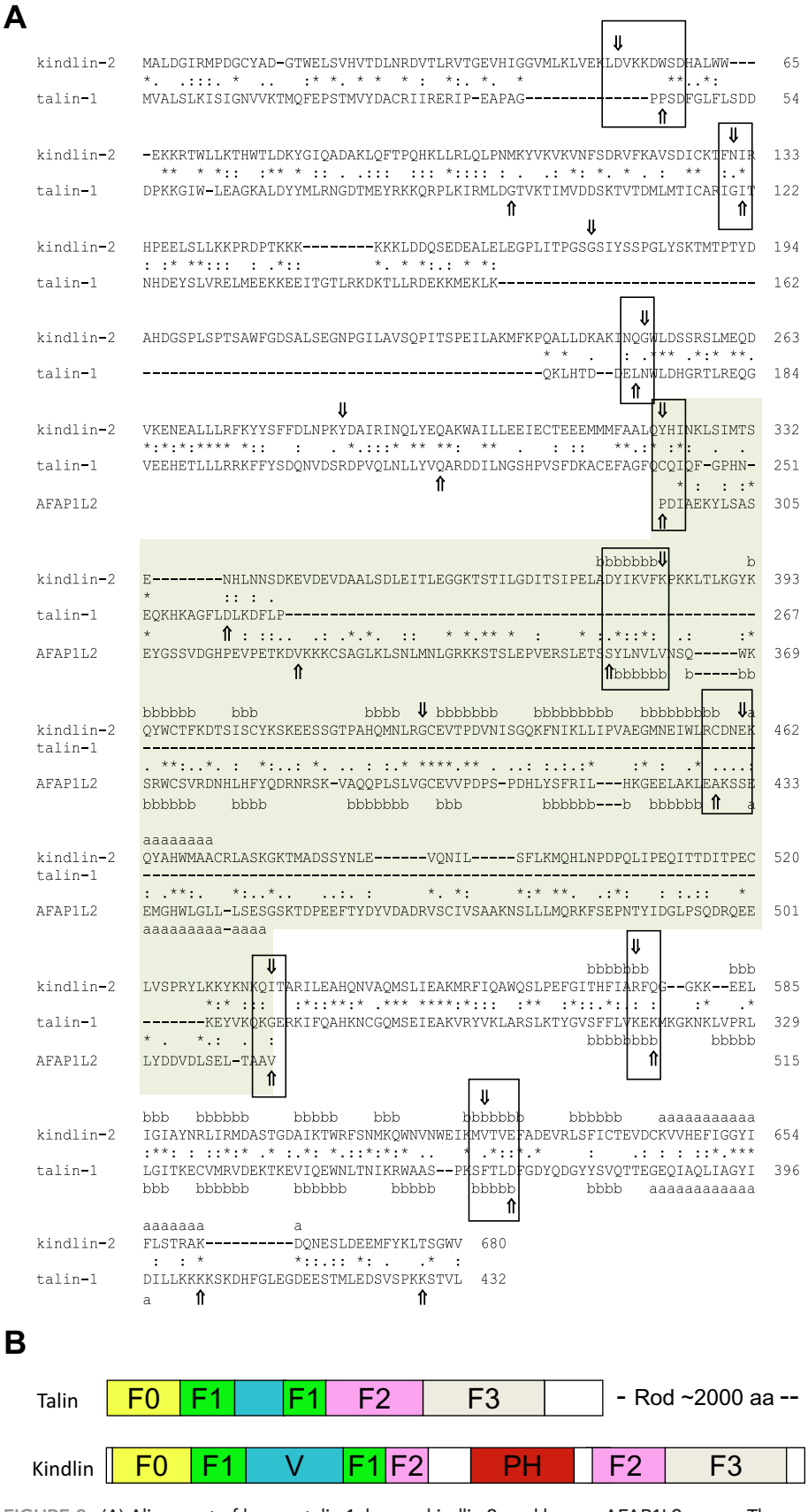


FIGURE 2: (A) Alignment of human talin 1, human kindlin 2, and human AFAP1L2 genes. The region of similarity with AFAP1L2 is shown in green. Intron positions are shown by arrows. aa, α-helixes; bb, β-strands. Asterisks indicate identity of amino acids in a position of the alignment; dots indicate functional similarity of amino acids in a position. (B) Domain structure of talins and kindlins, F, FERMT domain; PH, pleckstrin homology domain; V, variable loop.

Branchiostoma floridae closest AFAP1-like homologues is <28%). Therefore their non-metazoan homologues are likely to diverge beyond recognition by any conventional database search engines (Rost, 1999). It is likely that insertion of a fragment originated from an AFAP1-superfamily ancestor into the N-terminal part of a talin gene, which, possibly, was already truncated, resulting in the emergence of kindlins early in metazoan evolution. This hypothesis is supported by the sequence/structural similarities of PH domains of AFAP1 proteins and kindlins (Figure 2 and Supplemental Figure S3). Another feature that supports the close similarity between the AFAP1 superfamily and kindlins is a stretch of six/seven identical residues (Supplemental Figure S3), likely corresponding to a β-strand within kindlins and AFAP1proteins.

Of interest, HMM searches (see *Materials and Methods*) suggested that kindlins contain two PH domains (Figure 2), and the second PH domain is not recognized by CDD PHI-BLASTP searches (unpublished results). The second PH domain is not detected by PHI-BLASTP, possibly due to high variability, which is a characteristic feature of PH domains (Musacchio *et al.*, 1993).

Phylogenetic analysis of the kindlin superfamily

A maximum-likelihood (ML) phylogenetic tree of the kindlin superfamily is shown in Figure 3 and Supplemental Figure S4. The ML tree is rooted using a kindlin sequence from *T. adhaerens* (Figure 3). In addition, neighbor-joining (NJ), minimum evolution (ME), and maximum parsimony (MP) trees are shown in Supplemental Figures S5–S7. The topologies of ML, NJ, MP, and ME trees are slightly different; however, these differences do not alter the results or the conclusions of the phylogenetic analysis presented.

Most invertebrate species have one copy of the kindlin gene, with an exception of flatworms and *Drosophila*, whose genomes contain two copies of kindlin (Supplemental Figure S4). Sea urchin, acorn worm, and lancelet (all deuterostomes) were grouped together with insects. Likewise, flatworms and roundworms were grouped together, representing a substantial deviation from the species tree (Creevey *et al.*, 2011). This might be a result of a long-branch attraction—the grouping of two or more long branches as sister groups due to methodological artifacts (Reyes *et al.*, 2000; Philippe *et al.*, 2005).

Vertebrate kindlins form a well-supported clade (Figure 3). Of note, the K1 and K3 families in jawed vertebrates form a group,

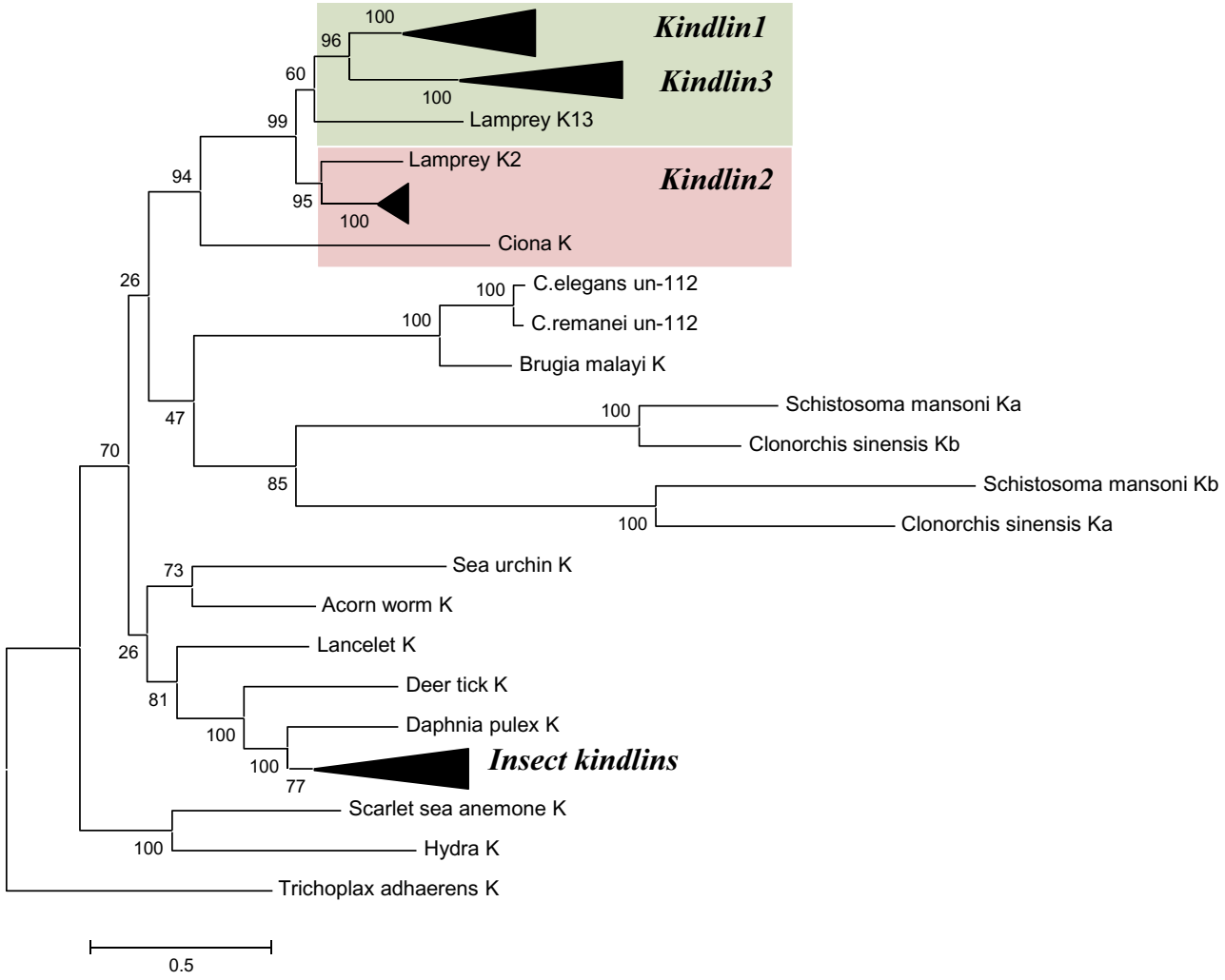


FIGURE 3: Maximum likelihood phylogenetic tree (JTT substitution model, the "all sites" option and the "gamma2 + invariant sites" option as implemented in the MEGA5 program). Some clades were collapsed using MEGA5 tree editor (the complete version of the tree is shown in Supplemental Figure S4). Numbers for interior branches refer to bootstrap values with 1000 pseudoreplicates. K, kindlin. The tree is rooted using the kindlin sequence from *T. adhaerens*.

which is supported by the high bootstrap value (96). Of interest, the primitive jawless fish lamprey has two distinct kindlins: the first is a putative orthologue of K2 (Figure 3), and the second is an orthologue of a putative common ancestor of both K1 and K3 (Figure 3 and Supplemental Figure S7). This further supports the grouping of K1 and K3 separately from K2 in vertebrates. However, K1 and K3 families of jawed vertebrates could not be grouped together with lamprey K1/3 based on the results of distance-based methods (NJ and ME). Therefore lamprey K1/3 appears as a separate branch (Supplemental Figures S5 and S6). Taking the results together, in agreement with a previous study (Khan *et al.*, 2011), our analysis suggests that K2 is likely to possess the ancestral features and function of the kindlin family, whereas K1 and K3 seem to be derived from the K2 gene duplication, followed by its divergence. Analysis of the mean sequence divergence of the kindlin family from jawed vertebrates using MEGA5 software shows that K3 is the most divergent member. The Poisson distance for K3 was 0.344; for K1, 0.283; and for K2, 0.076.

Structure-based analysis of functional divergence among kindlin paralogues

From the functional perspective, mammalian K3 also stands apart from other kindlins. K1 and K2 function in cells constitutively adherent to extracellular matrix, where integrins serve as anchoring points for an assembly of focal adhesion complexes (Larjava *et al.*, 2008; Malinin *et al.*, 2010). Although all three kindlins are able to directly interact with integrins (Ma *et al.*, 2008; Moser *et al.*, 2008; Harburger *et al.*, 2009), only K1 and K2 localize to and function as components of focal adhesions (Bialkowska *et al.*, 2010). Although K3 can localize to podosomes, the specialized transient adhesion structures of hematopoietic cells (Ussar *et al.*, 2006), it is not able to localize to focal adhesions, even when it is expressed in adherent cells (Bialkowska *et al.*, 2010; Figure 4B). K3 distribution is also distinct from that of K2 in the context of the same cell, such as endothelial cells (Bialkowska *et al.*, 2010).

To identify region(s) within kindlins responsible for localization to focal adhesions, K2 and K3, as well as a series of their chimeras, were expressed as enhanced green fluorescent protein (eGFP) fusion proteins, and their localization to focal adhesions was assessed based on their colocalization with a focal adhesion marker, vinculin (Figure 4). The study was performed in endothelial cells that express both K2 and K3 (Supplemental Figure S8) and therefore are likely to possess the required binding partners for both paralogues (Bialkowska *et al.*, 2010).

As anticipated, eGFP-K2 exhibited substantial colocalization with vinculin (colocalization coefficient $R = 0.4$). At the same time, the R values for eGFP-K3 or eGFP alone were eightfold lower (Figures 4, B–D, and 5A). Of interest, replacement of the N-terminal 241 or 441 amino acids of K3 with the corresponding sequences from K2 (1–265, construct 1; and 1– 461, construct 2; respectively) did not add to the extent of the vinculin colocalization of the resultant chimeras as compared with eGFPK3 or eGFP alone (Figures 4B and 5A). This result emphasized the importance of the remaining 261 C-terminal residues of K2 in the localization to focal adhesions. Indeed, when this C-terminal segment of K2 was fused to the N-terminal 441 amino acids of K3, the resultant chimeric protein (construct 6) gained the ability to colocalize with vinculin ($R = 0.4$; Figures 4C and 5A).

To further narrow the area responsible for the localization of K2 to focal adhesions from the C-terminal end, we replaced the C-terminal 66 or 116 amino acids of K2 of construct 4 with the corresponding sequences from K3 (resulting in constructs 7 and 8, respectively). This replacement caused a relatively mild to moderate decrease of 5 and 25%, respectively, in the extent of colocalization with vinculin (Figures 4C and 5A). However, replacement of larger C-terminal areas of K2 (523–680 and 466–680) with the corresponding K3 sequence (constructs 11 and 12, respectively) resulted in loss of the ability to localize to focal adhesions (Figures 4C and 5A). Thus we concluded that the region between amino acids (aa) 461 and 563 is required for focal adhesion localization. K2 chimera containing 100 residues of K2 sequence (461–563) surrounded by the complementary K3 areas (N-442 and 542-C) exhibited more than one-third of the ability of full-length K2 to colocalize with vinculin ($R = 0.15$; Figures 4 and 5A, construct 10).

A summary of our results is shown in Figure 5B. The N-terminal (1–265) and C-terminal (aa 613–680) regions do not seem to contribute >5% for K2 recruitment/localization to focal adhesions. The region between aa 420 and 563 (see chimera N9) appears to play a crucial role in K2 localization to focal adhesions. The C-terminal subdomain of the F2 domain of this segment (aa 461–563) appears to play a substantial role in 420-563–mediated recruitment of K2 to focal adhesions. Indeed, in K3, the corresponding segment exhibits only 51% of identity to K2 due to the presence of a variable glycine-rich insertion specific for K3 (see later discussion).

Together the inability of K3 to constitutively localize to focal adhesions, functionally distinguishing it from K2 and K1, is dependent on the C-terminal part of F2 domain.

Evolution of the C-terminal part of the kindlin F2 domain

To understand further the divergence of kindlin functions, we built a phylogenetic tree for this C-terminal subdomain of the F2 domain (Supplemental Figure S9). The resulting tree is similar to the tree of the full-length kindlin (Figure 3). In both cases, the trees have noticeable long branches leading to mammalian K3.

The most prominent feature of the homologue of the focal adhesion localization segment in K3 is a variable glycine-rich insertion, which is characteristic for mammalian K3 (Supplemental Figures S10 and S11). Similar insertions are observed in some other species (e.g., roundworms and flat worms; Supplemental Figure S10). It appears that these insertions have their own independent origins in different lineages, since multiple independent losses in a large number of deuterostome lineages are very unlikely (Supplemental Figures S10 and S11). This insertion might serve as an additional protein-binding motif accommodating interactions with the components of integrin signaling machinery.

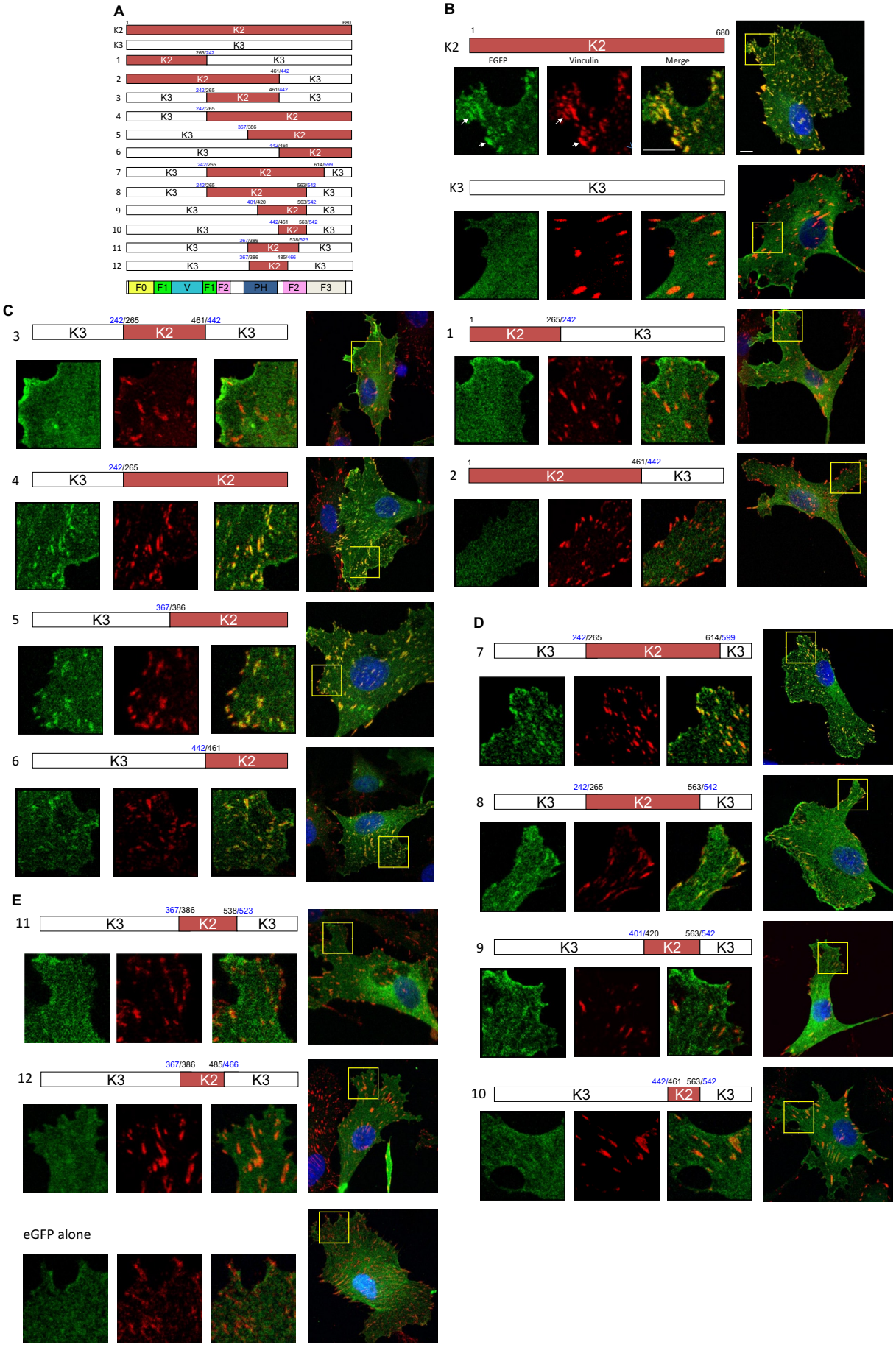
Next we used the DIVERGE2 method to analyze functionally important residues in jawed vertebrate K3 (see *Materials and Methods*). This analysis identified 26 residues that were substantially functionally diverged from jawed vertebrate K1 and K2 clades (divergence value >1; Figure 6 and Supplemental Figure S12). Almost half of these functionally diverged residues (12 residues) are located in the C-end portion of the segment (117 residues from the F3 domain; Figure 6 and Supplemental Figure S12). The N-end and the middle of the K3 protein contain 14 functionally diverged residues in 586 residues of the alignment (Figure 6 and Supplemental Figure S12). The probability of observing 12 out of 26 residues within the small fragment of the alignment (~17%) is extremely low ($p = 0.00042$ according to the binomial test). This result suggests that functional divergence of three vertebrate kindlin families is largely focused at the C-end of kindlin proteins.

DISCUSSION

Until recently, it was believed that the presence of integrins, along with the cell adhesion machinery, is a characteristic feature distinguishing multicellular organisms from their unicellular ancestors. However, several recent studies revealed that several integrin subunits are present on the surface of unicellular ancestors of metazoans (Sebe-Pedros *et al.*, 2010, 2013; Sebe-Pedros and Ruiz-Trillo, 2010; Suga *et al.*, 2013). Moreover, some protists, such as *C. owczarzaki*, seem to have not only integrin subunits, but also an entire intracellular complex connecting integrins to the actin cytoskeleton (also known as an adhesome), containing vinculin, paxillin, focal adhesion kinase, integrin-linked kinase, talin, α-actinin, PINCH, and parvin (Suga *et al.*, 2013). Of interest, a study showed that *C. owczarzaki*, which has a distinctive aggregative multicellular stage during its life cycle, is able to change expression of various proteins depending on its adhesive or aggregative status (Sebe-Pedros *et al.*, 2013). Thus it seems that this unicellular ancestor of metazoans already exhibits a prototype of outside-in integrin signaling.

In this study, we focused on the origin and evolution of inside-out integrin signaling and the key proteins, talins and kindlins, involved in the final events of this process. Our database searches revealed that whereas talin is present in many premetazoans, kindlin appears to serve an additional function that is specific for metazoans. All metazoan lineages have at least one member of kindlin family. Of interest, a recent study using fluorescence fluctuation imaging methods showed that during cell adhesion, K2 is recruited to focal adhesions before talin (Bachir *et al.*, 2014). It is possible that the emergence of kindlin allowed organisms to acquire new adhesive functions critical for their transition to multicellularity.

Our results suggest that kindlins originated from a duplication of a talin head domain–encoding sequence with a subsequent insertion of a PH domain originated from AFAP-1L2 proteins. Undoubtedly, the combination of functions of these two functionally versatile proteins provided an immediate evolutionary advantage. PH domains are widely associated with the membrane-targeting function and provide a connection to phosphatidyl-inositol signaling cascade (Lemmon and Ferguson, 2000). Besides the PH domain, kindlins possess other membrane-binding regions, including the F0 (ubiquitin-like N-terminal domain) and the variable loop within the F1 domain (Perera *et al.*, 2011; Bouaouina *et al.*, 2012). Moreover, our analysis suggests that the F3 domain of kindlins is able to interact with lipids as well, based on its high similarity to the PH domains. It appears that the insertion of an AFAP1-L2 PH domain allowed kindlins to acquire additional specificity in membrane binding. Alternatively, the third membrane-binding segment could provide kindlins with additional flexibility during rearrangements of interactions


A
K2
K3
1
2
3
4
5
6
7
8
9
10
11
12
F0
F1
V
F1
F2
PH
F2
F3
B
EGFP
Vinculin
Merge
C
D
E
eGFP alone

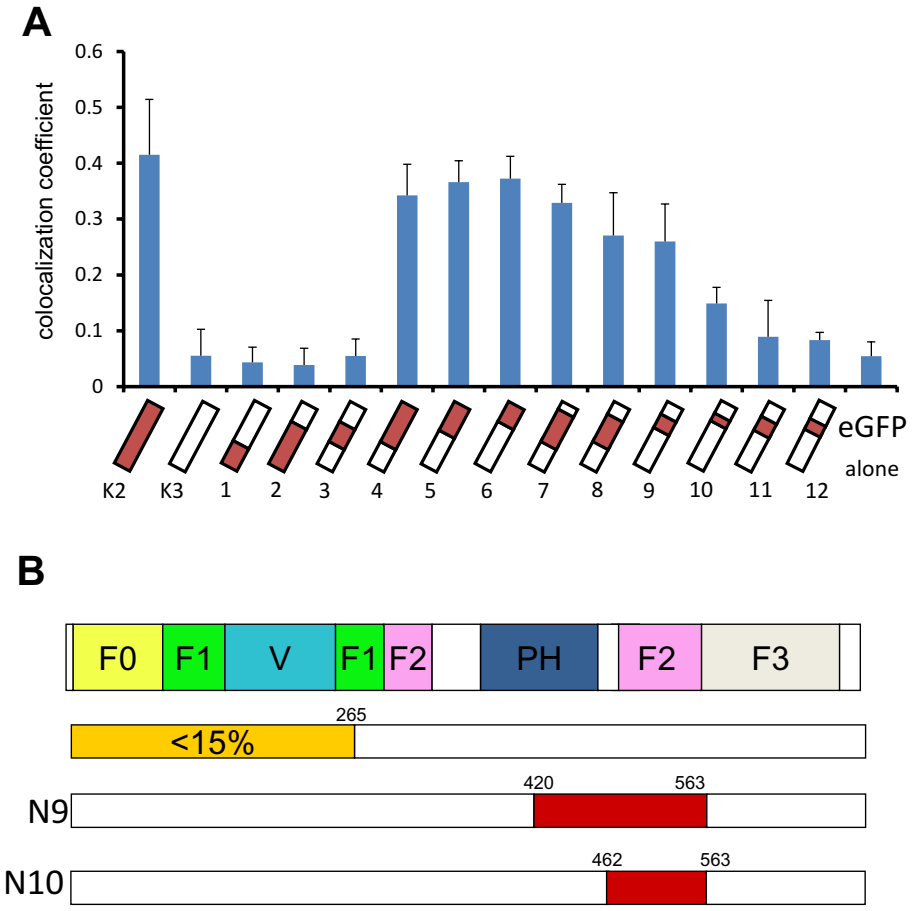


FIGURE 5: (A) Localization of K2, K3, and K2/3 chimeric proteins to focal adhesions was evaluated by colocalization with vinculin. Colocalization coefficient values ± SEM from different cells. Chimeras are numbered as in Figure 4A. (B) Diagram summarizing the contributions of different regions within K2 to its localization to focal adhesions based on the results in A. The colocalization coefficient of full-length K3 was subtracted from the coefficient of full-length K2, and the resulting value was assigned 100%. Kindlin with its domain structure is shown on top. F, FERM domain; PH, pleckstrin homology domain; V, variable domain.

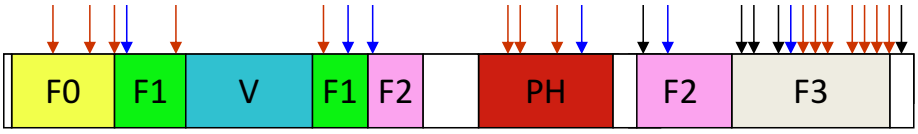


FIGURE 6: Analysis of functionally diverged residues in kindlins using the DIVERGE2 program (Piatigorsky *et al.*, 1988). A full-length kindlin with its subdomains is shown. F, FERM domain; PH, pleckstrin homology domain; V, variable domain. Blue arrows indicate the residues within K3, which are divergent from K2 and K1. Red arrows indicate residues within both K1 and K3, which are divergent from K2. Black arrows indicate the residues common in K2 and K3 but divergent in K1 (for details see Supplemental Figure S11).

between numerous regulatory proteins in the proximity of the membrane. In either scenario, the insertion of the PH domain is likely to provide an extra level of regulation for the cell adhesion machinery.

Whereas some invertebrates have two kindlins, all of the jawed vertebrates possess three kindlin paralogues. Phylogenetic analysis of vertebrate kindlins suggested that K2 has the lowest rate of evolution and is likely to maintain the ancestral function, whereas K3 is the most rapidly evolving paralogue. It is likely that the increase in the number of kindlin paralogues resulted from recurrent gene duplications (Khan *et al.*, 2011). There are numerous models of evolution of gene duplications (reviewed by Innan and Kondrashov, 2010). On the basis of our functional divergence analysis, we propose a functional specialization model (Hughes, 1994) as a plausible mechanism of gene duplication in kindlins. This model suggests that the original gene had two functions, which could not be independently developed and improved. Therefore, after duplication, each gene copy could be driven by natural selection to specialize and to improve one of the two original functions (Piatigorsky *et al.*, 1988). The improvement could be due to either differentiation of expression patterns (Oakley *et al.*, 2006) or substantial improvement of one of its functions (James and Tawfik, 2003).

In this study, we assessed the functional divergence between kindlin paralogues using the sequence swap (chimera) approach, followed by analysis by DIVERGE2 software. Comparison of K2/K3 chimeras revealed that the F2 subdomain, in particular its C-terminal part, is crucial for the different properties of K2 and K3. The presence of this segment enables K2 but not K3 to localize to focal adhesions along with other components, such as vinculin.

The corresponding segment within K3 contains a variable region, which suggests that K3 might have lost its original ability to localize to focal adhesion due to a loss of essential targeting sequence. Of note, in all mammalian K3 proteins, this region contains a variable glycine-rich insertion, possibly encoding an extra site for protein binding or a flexible linker. It is tempting to speculate that the function of this flexible linker in K3 is to prevent a constitutive association with focal adhesions while still allowing for conditional (transient) recruitment to those sites. Because K3 is the only kindlin family member expressed in hematopoietic cells, which are characterized by rapidly activated adhesiveness, it is likely that the

FIGURE 4: Functional divergence between K2 and K3. (A) Diagram showing K2, K3, and K2/K3 chimeric proteins used to identify areas responsible for localization of K2 to focal adhesions. Residue numbers at the fusion sites are indicated (number of the residue within K2 in black, corresponding number within K3 in blue). Bottom, domain structure of kindlins. F, FERMT domain; PH, pleckstrin homology domain; V, variable loop. (B–E) BAECs were transfected with GFP-K2, GFP-K3, or GFP-K2/3 chimeras and replated on fibronectin-coated coverslips (5 µg/ml) 24 h after the transfection. The cells were fixed 16 h later and stained for vinculin. Representative images were taken using fluorescence microscopy. Right, the corresponding schematic representations of the chimeras used. White arrows indicate focal adhesions. Scale bars, 5 µm.

loss of permanent associations with focal adhesions might be essential for kindlin function in these cells. Similar insertions were found in several nonvertebrate species (e.g., *Ciona*, roundworms, and flatworms); however, they are likely to have an independent evolutionary origin.

Previous studies showed that the integrin-binding motif in the F3 subdomain is required for K2 localization to focal adhesions (Shi *et al.*, 2007). The integrin-binding site in the F3 domain, however, is not likely to account for the dramatic difference in K2 and K3 localization, since all three kindlins, including K3, are able to bind integrins. Indeed, according to our results, the lack of K3 in focal adhesions largely is not due to sequence variation in the F3 subdomain. Thus, whereas integrin binding is essential for K2 localization to focal adhesions, it is likely that the different localization properties between K2 and K3 result from their differential binding to other focal adhesion proteins. Recently an independent study reported that the F2 subdomain of K2 is largely responsible for the targeting of K2 to integrin-linked kinase (ILK) and subsequently to focal adhesions (Huet-Calderwood *et al.*, 2014). However, another study showed that it is K2 that recruits ILK to focal adhesions, not vice versa (Montanez *et al.*, 2008). Although the overall role of the F2 subdomain in K2 focal adhesion targeting is consistent with our results, our study provides finer mapping and reveals the predominant importance of the C-terminal portion within the F2 subdomain. It remains to be established, however, what proteins bind specifically to that region to recruit K2 to focal adhesions.

Of interest, the sequence of ~70 aa at the C-terminal end of K2 does not appear to substantially contribute to focal adhesion localization, since its replacement with the corresponding sequence from K3 nearly did not alter its subcellular distribution. However, as shown by DIVERGE2-assisted analysis, the same area within K2 is heavily enriched in residues functionally divergent from K1 and K3. Therefore it is likely that the C-terminal region of K2 (K2 vs. both K1 and K3) has functions distinct from those in K1 and K3 and that these functions are not related to the subcellular localization.

In conclusion, the emergence of kindlins is likely to serve as an essential step toward adaptation of integrin signaling machinery to more complex functions associated with the transition of premetazoans to early metazoans. During further metazoan evolution and tissue specialization, kindlins paralogues (kindlin 3 in particular) underwent additional changes, which allowed kindlins to perform their unique functions in different tissues.

MATERIALS AND METHODS

Data sets, BLAST searches, three-dimensional searches, secondary structure prediction, phylogenetic analysis

Protein sequences were downloaded from the National Center for Biotechnology Information (NCBI), ENSEMBL, and Broad Institute of Harvard and the Massachusetts Institute of Technology, Origins of Multicellularity Project (www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html), databases. Similar searches were performed using the nonredundant protein sequence database at the NCBI and the gapped BLAST program. Protein domains were analyzed using HHMPred at the Pfam website (pfam.sanger.ac.uk). Multiple protein sequence alignments were constructed using the Muscle program (Edgar, 2004) and then adjusted by hand (details available upon request [rogozin@ncbi.nlm.nih.gov]). Structural alignments of PH domains were performed using the PyMOL software (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). Phylogenetic trees based on multiple alignments of protein sequences were constructed using the ML, NJ, minimum-economy, and MP methods as implemented in MEGA (Kumar *et al.*, 2008), FASTTREE (Price *et al.*, 2010), and PAUP* programs (Wilgenbusch, Swofford, 2003). An optimal model of substitutions for phylogenetic reconstructions was chosen using the ModelGenerator program (Keane *et al.*, 2006). A statistical method for estimating type II (cluster-specific) functional divergence of protein sequences implemented in the DIVERGE2 program (Gu, 2006) was used for analysis of functionally important residues (vertebrate K3 and K1 clades were used for analysis). DIVERGE2 was designed to detect functional divergence between member genes of a protein family based on (site-specific) shifted evolutionary rates after gene speciation or duplication. Posterior analysis results in a site-specific profile for predicting amino acid residues that are responsible for functional divergence (Gu, 2006). Protein secondary structure was predicted using JPRED (Jones, 1999).

Construct number	Fusion sites (aa residues)	Fusion sites (nucleotides)
1	K2/K3: 265/242	K2/K3: 981/875
2	K2/K3: 461/442	K2/K3: 1569/1475
3	K3/K2: 242/265	K3/K2: 875/981
	K2/K3: 461/442	K2/K3: 1569/1475
4	K3/K2: 242/265	K3/K2: 875/981
5	K3/K2: 366/386	K3/K2: 1250/1344
6	K3/K2: 442/461	K3/K2: 1475/1569
7	K3/K2: 242/265	K3/K2: 875/981
	K2/K3: 613/598	K2/K3: 2028/1946
8	K3/K2: 242/265	K3/K2: 875/981
	K2/K3: 563/542	K2/K3: 1875/1792
9	K3/K2: 400/420	K3/K2: 1352/1446
	K2/K3: 563/542	K2/K3: 1875/1792
10	K3/K2: 442/461	K3/K2: 1475/1569
	K2/K3: 563/542	K2/K3: 1875/1792
11	K3/K2: 366/386	K3/K2: 1250/1344
	K2/K3: 537/523	K2/K3: 1800/1718
12	K3/K2: 366/386	K3/K2: 1250/1344
	K2/K3: 485/467	K2/K3: 1641/1547

Kindlin2, GenBank accession #BC017327 (GI 33878545); kindlin3, GenBank accession #NM178443 (GI 215983054).

TABLE 1: Amino acid and nucleotide residues at sites of fusion between kindlin2 and kindlin3 for each chimeric construct.

Plasmids

All kindlin constructs were cloned into peGFP C2 vector and expressed as N-terminal eGFP-linked proteins. The points of junction between K2 and K3 were chosen at the areas that are identical between the proteins, in order to avoid formation of artificial sequences. The sites of fusion between K2 and K3 are listed in Table 1.

Cell culture, transfection, microscopy, and colocalization analysis

Primary bovine aortic endothelial cells (BAECs) were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, and penicillin and streptomycin at 37°C with 5% CO_2. The cells were transfected with GFP-K2, GFP-K3, or GFP-K2/3 chimeras using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA)

according to the manufacturer's instructions. At 24 h after the transfection, the cells were replated on fibronectin-coated coverslips 5 (μg/ml) and fixed 16 h later. For vinculin colocalization studies, the cells were stained for vinculin with mouse monoclonal antibody (clone 7F9; eBioscence, San Diego, CA), and Alexa 564–conjugated anti- mouse secondary antibody. Confocal fluorescent images from the bottom cell surfaces were captured on green (for GFP) and red (for vinculin) channels using a Leica TCS SP5 II laser scanning confocal microscope with 63× oil objective (Leica Microsystems, Wetzlar, Germany). The same settings were applied to all samples. Only cells within a narrow range of eGFP expression level were included in the analysis. The expression level for eGFP K2 was 5 ± 1 times that of endogenous kindlin 2, as quantified using kindlin 2 antibody; the expression level of the rest of the eGFP fusion proteins was matched to that of eGFP K2 based on eGFP intensity. On average, there was no significant difference in eGFP intensity levels between analyzed samples. For colocalization analysis, areas of individual cells were selected by tracing the cell edge, and colocalization coefficient (*R*) values for red and green channels were determined using the colocalization threshold function of Fiji software (National Institutes of Health, Bethesda, MD). At least six cells for each construct from two independent experiments were analyzed. Percentage of *R* values for different constructs, *R*(*X*), from the difference between the *R* values of K2 and K3 was calculated as $100\% \times [R(X) - R(\mathrm{K3})]/[R(\mathrm{K2}) - R(\mathrm{K3})]$. For evaluation of kindlin expression levels, the cells were stained with rabbit polyclonal kindlin 2 and kindlin 3 antibodies, followed by Alexa 564–conjugated anti-rabbit secondary antibody, as described previously (Bialkowska *et al.*, 2010).

ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health Intramural Research Program of the National Heart, Lung, and Blood Institute (grant HL073311) and by National Eye Institute and the Intramural Research Program of the National Library of Medicine at the National Institutes of Health (U.S. Department of Health and Human Services).

REFERENCES

Ali RH, Khan AA (2014). Tracing the evolution of FERM domain of Kindlins. Mol Phylogenet Evol 80, 193–204.

Arita K, Wessagowit V, Inamadar AC, Palit A, Fassihi H, Lai-Cheong JE, Pourreyron C, South AP, McGrath JA (2007). Unusual molecular findings in Kindler syndrome. Br J Dermatol 157, 1252–1256.

Ashton GH, McLean WH, South AP, Oyama N, Smith FJ, Al-Suwaid R, Al-Ismaily A, Atherton DJ, Harwood CA, Leigh IM, *et al.* (2004). Recurrent mutations in kindlin-1, a novel keratinocyte focal contact protein, in the autosomal recessive skin fragility and photosensitivity disorder, Kindler syndrome. J Invest Dermatol 122, 78–83.

Askari JA, Tynan CJ, Webb SE, Martin-Fernandez ML, Ballestrem C, Humphries MJ (2010). Focal adhesions are sites of integrin extension. J Cell Biol 188, 891–903.

Bachir AI, Zareno J, Moissoglu K, Plow EF, Gratton E, Horwitz AR (2014). Integrin-associated complexes form hierarchically with variable stoichiometry in nascent adhesions. Curr Biol 24, 1845–1853.

Bialkowska K, Ma YQ, Bledzka K, Sossey-Alaoui K, Izem L, Zhang X, Malinin N, Qin J, Byzova T, Plow EF (2010). The integrin co-activator Kindlin-3 is expressed and functional in a non-hematopoietic cell, the endothelial cell. J Biol Chem 285, 18640–18649.

Bouaouina M, Goult BT, Huet-Calderwood C, Bate N, Brahme NN, Barsukov IL, Critchley DR, Calderwood DA (2012). A conserved lipid-binding loop in the kindlin FERM F1 domain is required for kindlin-mediated alphaIIb-beta3 integrin coactivation. J Biol Chem 287, 6979–6990.

Bouvard D, Brakebusch C, Gustafsson E, Aszodi A, Bengtsson T, Berna A, Fassler R (2001). Functional consequences of integrin gene mutations in mice. Circ Res 89, 211–223.

Byzova TV, Goldman CK, Pampori N, Thomas KA, Bett A, Shattil SJ, Plow EF (2000). A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Mol Cell 6, 851–860.

Calderwood DA, Zent R, Grant R, Rees DJ, Hynes RO, Ginsberg MH (1999). The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J Biol Chem 274, 28071–28074.

Cornillon S, Gebbie L, Benghezal M, Nair P, Keller S, Wehrle-Haller B, Charette SJ, Bruckert F, Letourneur F, Cosson P (2006). An adhesion molecule in free-living Dictyostelium amoebae with integrin beta features. EMBO Rep 7, 617–621.

Creevey CJ, Muller J, Doerks T, Thompson JD, Arendt D, Bork P (2011). Identifying single copy orthologs in Metazoa. PLoS Comput Biol 7, e1002269.

Dellaporta SL, Xu A, Sagasser S, Jakob W, Moreno MA, Buss LW, Schierwater B (2006). Mitochondrial genome of Trichoplax adhaerens supports placozoa as the basal lower metazoan phylum. Proc Natl Acad Sci USA 103, 8751–8756.

Edgar RC (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113.

Goksoy E, Ma YQ, Wang X, Kong X, Perera D, Plow EF, Qin J (2008). Structural basis for the autoinhibition of talin in regulating integrin activation. Mol Cell 31, 124–133.

Gu X (2006). A simple statistical method for estimating type-II (cluster-specific) functional divergence of protein sequences. Mol Biol Evol 23, 1937–1945.

Harburger DS, Bouaouina M, Calderwood DA (2009). Kindlin-1 and -2 directly bind the C-terminal region of beta integrin cytoplasmic tails and exert integrin-specific activation effects. J Biol Chem 284, 11485–11497.

Has C, Burger B, Volz A, Kohlhase J, Bruckner-Tuderman L, Itin P (2010). Mild clinical phenotype of Kindler syndrome associated with late diagnosis and skin cancer. Dermatology 221, 309–312.

Huet-Calderwood C, Brahme NN, Kumar N, Stiegler AL, Raghavan S, Boggon TJ, Calderwood DA (2014). Differences in binding to the ILK complex determines kindlin isoform adhesion localization and integrin activation. J Cell Sci 127, 4308–4321.

Hughes AL (1994). The evolution of functionally novel proteins after gene duplication. Proc Biol Sci 256, 119–124.

Innan H, Kondrashov F (2010). The evolution of gene duplications: classifying and distinguishing between models. Nat Rev Genet 11, 97–108.

James LC, Tawfik DS (2003). Conformational diversity and protein evolution—a 60-year-old hypothesis revisited. Trends Biochem Sci 28, 361–368.

Jones DT (1999). Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol 292, 195–202.

Jurk K, Schulz AS, Kehrel BE, Rapple D, Schulze H, Mobest D, Friedrich WW, Omran H, Deak E, Henschler R, *et al.* (2010). Novel integrin-dependent platelet malfunction in siblings with leukocyte adhesion deficiency-III (LAD-III) caused by a point mutation in FERMT3. Thromb Haemost 103, 1053–1064.

Keane TM, Creevey CJ, Pentony MM, Naughton TJ, McLnerney JO (2006). Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6, 29.

Kern JS, Herz C, Haan E, Moore D, Nottelmann S, von Lilien T, Greiner P, Schmitt-Graeff A, Opitz OG, Bruckner-Tuderman L, *et al.* (2007). Chronic colitis due to an epithelial barrier defect: the role of kindlin-1 isoforms. J Pathol 213, 462–470.

Khan AA, Janke A, Shimokawa T, Zhang H (2011). Phylogenetic analysis of kindlins suggests subfunctionalization of an ancestral unduplicated kindlin into three paralogs in vertebrates. Evol Bioinformatics Online 7, 7–19.

Kindler T (1954). Congenital poikiloderma with traumatic bulla formation and progressive cutaneous atrophy. Br J Dermatol 66, 104–111.

Krishna CV, Parmar NV, Has C (2014). Kindler syndrome with severe mucosal involvement in childhood. Clin Exp Dermatol 39, 340–343.

Kumar S, Nei M, Dudley J, Tamura K (2008). MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings Bioinformatics 9, 299–306.

Larjava H, Plow EF, Wu C (2008). Kindlins: essential regulators of integrin signalling and cell-matrix adhesion. EMBO Rep 9, 1203–1208.

Laudanna C, Bolomini-Vittori M (2009). Integrin activation in the immune system. Wiley Interdiscip Rev Syst Biol Med 1, 116–127.

Lemmon MA, Ferguson KM (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem J 350, 1–18.

Malinin NL, Plow EF, Byzova TV (2010). Kindlins in FERM adhesion. Blood 115, 4011–4017.

Malinin NL, Zhang L, Choi J, Ciocea A, Razorenova O, Ma YQ, Podrez EA, Tosi M, Lennon DP, Caplan AI, *et al.* (2009). A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. Nat Med 15, 313–318.

Ma YQ, Qin J, Wu C, Plow EF (2008). Kindlin-2 (Mig-2): a co-activator of beta3 integrins. J Cell Biol 181, 439–446.
McDowall A, Svensson L, Stanley P, Patzak I, Chakravarty P, Howarth K, Sabnis H, Briones M, Hogg N (2010). Two mutations in the KINDLIN3 gene of a new leukocyte adhesion deficiency III patient reveal distinct effects on leukocyte function in vitro. Blood 115, 4834–4842.
Montanez E, Ussar S, Schifferer M, Bosl M, Zent R, Moser M, Fassler R (2008). Kindlin-2 controls bidirectional signaling of integrins. Genes Dev 22, 1325–1330.
Moser M, Legate KR, Zent R, Fassler R (2009). The tail of integrins, talin, and kindlins. Science 324, 895–899.
Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R (2008). Kindlin-3 is essential for integrin activation and platelet aggregation. Nat Med 14, 325–330.
Musacchio A, Gibson T, Rice P, Thompson J, Saraste M (1993). The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem Sci 18, 343–348.
Oakley TH, Ostman B, Wilson AC (2006). Repression and loss of gene expression outpaces activation and gain in recently duplicated fly genes. Proc Natl Acad Sci USA 103, 11637–11641.
Perera HD, Ma YQ, Yang J, Hirbawi J, Plow EF, Qin J (2011). Membrane binding of the N-terminal ubiquitin-like domain of kindlin-2 is crucial for its regulation of integrin activation. Structure 19, 1664–1671.
Philippe H, Zhou Y, Brinkmann H, Rodrigue N, Delsuc F (2005). Heterotachy and long-branch attraction in phylogenetics. BMC Evol Biol 5, 50.
Piatigorsky J, O'Brien WE, Norman BL, Kalumuck K, Wistow GJ, Borras T, Nickerson JM, Wawrousek EF (1988). Gene sharing by delta-crystallin and argininosuccinate lyase. Proc Natl Acad Sci USA 85, 3479–3483.
Plow EF, Qin J, Byzova T (2009). Kindling the flame of integrin activation and function with kindlins. Curr Opin Hematol 16, 323–328.
Price MN, Dehal PS, Arkin AP (2010). FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS One 5, e9490.
Reyes A, Pesole G, Saccone C (2000). Long-branch attraction phenomenon and the impact of among-site rate variation on rodent phylogeny. Gene 259, 177–187.
Roberts GC, Critchley DR (2009). Structural and biophysical properties of the integrin-associated cytoskeletal protein talin. Biophys Rev 1, 61–69.
Rogozin IB, Carmel L, Csuros M, Koonin EV (2012). Origin and evolution of spliceosomal introns. Biol Direct 7, 11.
Rost B (1999). Twilight zone of protein sequence alignments. Protein Eng 12, 85–94.
Sadler E, Klausegger A, Muss W, Deinsberger U, Pohla-Gubo G, Laimer M, Lanschuetzer C, Bauer JW, Hintner H (2006). Novel KIND1 gene mutation in Kindler syndrome with severe gastrointestinal tract involvement. Arch Dermatol 142, 1619–1624.
Sebe-Pedros A, de Mendoza A, Lang BF, Degnan BM, Ruiz-Trillo I (2011). Unexpected repertoire of metazoan transcription factors in the unicellular holozoan Capsaspora owczarzaki. Mol Biol Evol 28, 1241–1254.
Sebe-Pedros A, Irimia M, Del Campo J, Parra-Acero H, Russ C, Nusbaum C, Blencowe BJ, Ruiz-Trillo I (2013). Regulated aggregative multicellularity in a close unicellular relative of metazoa. Elife 2, e01287.
Sebe-Pedros A, Roger AJ, Lang FB, King N, Ruiz-Trillo I (2010). Ancient origin of the integrin-mediated adhesion and signaling machinery. Proc Natl Acad Sci USA 107, 10142–10147.
Sebe-Pedros A, Ruiz-Trillo I (2010). Integrin-mediated adhesion complex: cooption of signaling systems at the dawn of Metazoa. Commun Integr Biol 3, 475–477.
Shimizu S, Nishie W, Tsuchiya K, Inokuma D, Koguchi-Yoshioka H, Kikuchi K, Motoya S, Nakamura H, Shimizu H (2014). Kindler syndrome with severe intestinal involvement: a 31-year follow-up. Acta Dermatol Venereol 94, 351–352.
Shi X, Ma YQ, Tu Y, Chen K, Wu S, Fukuda K, Qin J, Plow EF, Wu C (2007). The MIG-2/integrin interaction strengthens cell-matrix adhesion and modulates cell motility. J Biol Chem 282, 20455–20466.
Srivastava M, Begovic E, Chapman J, Putnam NH, Hellsten U, Kawashima T, Kuo A, Mitros T, Salamov A, Carpenter ML, *et al.* (2008). The Trichoplax genome and the nature of placozoans. Nature 454, 955–960.
Suga H, Chen Z, de Mendoza A, Sebe-Pedros A, Brown MW, Kramer E, Carr M, Kerner P, Vervoort M, Sanchez-Pons N, *et al.* (2013). The Capsaspora genome reveals a complex unicellular prehistory of animals. Nat Commun 4, 2325.
Svensson L, Howarth K, McDowall A, Patzak I, Evans R, Ussar S, Moser M, Metin A, Fried M, Tomlinson I, *et al.* (2009). Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. Nat Med 15, 306–312.
Ussar S, Wang HV, Linder S, Fassler R, Moser M (2006). The Kindlins: subcellular localization and expression during murine development. Exp Cell Res 312, 3142–3151.
Wiebe CB, Petricca G, Hakkinen L, Jiang G, Wu C, Larjava HS (2008). Kindler syndrome and periodontal disease: review of the literature and a 12-year follow-up case. J Periodontol 79, 961–966.
Wilgenbusch JC, Swofford D (2003). Inferring evolutionary trees with PAUP*. Curr Protoc Bioinformatics, Chapter 6, Unit 6.4.