SYMPOSIUM REPORT

Beyond the brush border: NHERF4 blazes new NHERF turf

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The Na exchanger regulatory factor (NHERF) family of epithelial-enriched PDZ domain scaffolding proteins plays important roles in maintaining and regulating epithelial cell function. The NHERFs exhibit some overlap in tissue distribution and binding partners, suggesting redundant functions. Yet, it is clear that each NHERF protein exhibits distinct properties, translating into unique cellular functions. The work summarized in this review suggests the most recently identified family member, NHERF4, is the most divergent. Additional investigation is needed, however, to understand more completely the role of NHERF4 in the context of the NHERF family.

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Epithelial cells maintain a complex three-dimensional organization to ensure directional signalling and transport critical for the normal function of many organ systems. Molecular scaffolds participate in this architecture by nucleating multiprotein complexes which enhance the efficiency and fidelity of epithelial cell function. The NHERF family of epithelial-enriched PDZ proteins functions as molecular scaffolds to coordinate a diverse range of regulatory processes for ion transport and second messenger cascades. PDZ domains mediate the majority of the known protein–protein interactions for NHERF proteins. NHERF1 (EBP50) and NHERF2 (E3KARP) each contain two PDZ domains, while NHERF3 (PDZK1, CAP70, NaPi CAP-1) and NHERF4 (IKEPP, NaPi CAP-2) possess four (Yun *et al.* 1997; Kocher *et al.* 1998; Scott *et al.* 2002). In addition to the PDZ domains, NHERF1 and NHERF2 possess COOH-terminal ERM (ezrin–radixin–moesin) binding domains which indirectly tether these proteins to the actin cytoskeleton via additional protein interactions (Bretscher *et al.* 1997; Reczek *et al.* 1997; Short *et al.* 1998; Sun *et al.* 2000). The NHERF proteins have been grouped into a family based on their overall similarity. For example, a sequence comparison of the proteins most closely related to NHERF4 reveals that the NHERF proteins are more similar to one another than to other proteins (Fig. 1*A*). Additionally, the individual PDZ domains of the NHERF proteins share a high degree of similarity; however, they are different enough to suggest unique functions (Fig. 1*B*).

To date, NHERF1 and NHERF2 have been shown to bind more than 30 proteins including cell surface receptors (both GPCR and growth factor), ion channels, transporters, and signalling proteins (see Shenolikar *et al.* (2004) for a recent in-depth review). Likewise, there is a shorter, albeit growing, list of proteins able to associate with NHERF3 and/or NHERF4 (Gisler *et al.* 2001, 2003*a*, *b*; Scott *et al.* 2002; Gentzsch *et al.* 2003; Hegedus *et al.* 2003). However, it is important to note that few published reports examine co-localization and function in relevant tissues or cell lines and many rely on heterologous expression in non-epithelial cells. Nonetheless, the data suggest that NHERFs nucleate the formation of multiprotein complexes to modulate trafficking, transport and signalling in polarized cells. As new protein associations with the NHERFs are still being described, the importance of this family in epithelial biology continues to unfold.

Studies of the NHERF family have identified overlapping and unique localizations, binding partners and functions for each family member. Therefore, it is critical to study the function of each NHERF within the context of the other three related proteins. Critical to our understanding the biology of the NHERFs is a careful examination of the tissue expression and subcellular localization of each protein. Knowing localization (under

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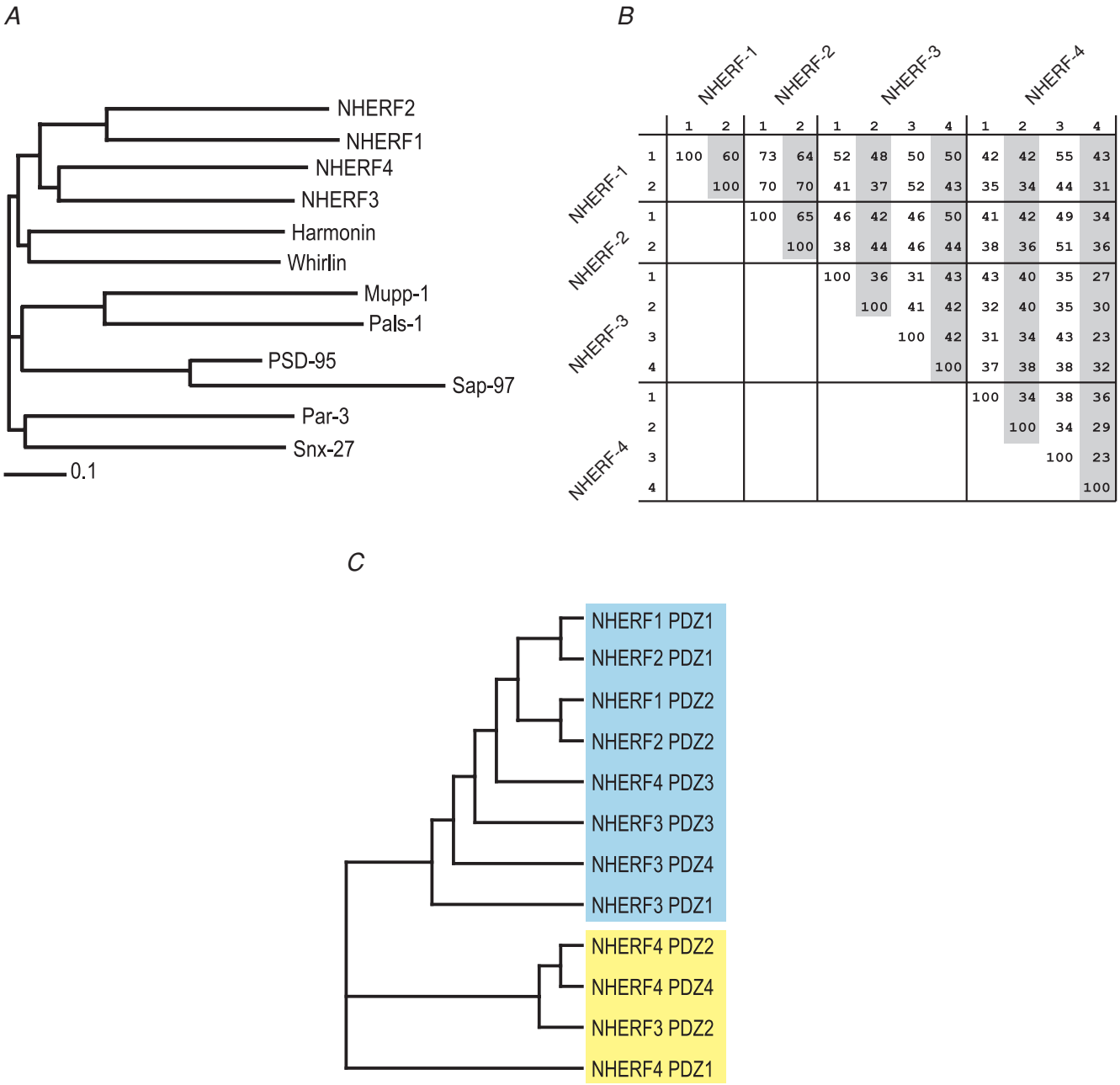
normal conditions and in various disease states) will allow us to focus on physiologically relevant binding partners that can then be studied in the appropriate context. While we know multiple physiologically important associations for NHERF1, NHERF2 and NHERF3, our knowledge of NHERF4 is far less complete. Nonetheless, published and preliminary data indicate that NHERF4 has unique biochemical and cell biological characteristics. In this review, we will provide a brief overview of the NHERF family with an emphasis on NHERF4.



	NHERF-1		NHERF-2		NHERF-3				NHERF-4			
	1	2	1	2	1	2	3	4	1	2	3	4
NHERF-1 1	100	60	73	64	52	48	50	50	42	42	55	43
NHERF-1 2		100	70	70	41	37	52	43	35	34	44	31
NHERF-2 1			100	65	46	42	46	50	41	42	49	34
NHERF-2 2				100	38	44	46	44	38	36	51	36
NHERF-3 1					100	36	31	43	43	40	35	27
NHERF-3 2						100	41	42	32	40	35	30
NHERF-3 3							100	42	31	34	43	23
NHERF-3 4								100	37	38	38	32
NHERF-4 1									100	34	38	36
NHERF-4 2										100	34	29
NHERF-4 3											100	23
NHERF-4 4												100

Figure 1. PDZ domains of NHERF proteins
A, the most similar proteins to NHERF4 were determined by BLAST search, compared by multiple sequence alignment (Notredame *et al.* 2000), and the phylogenetic tree was created using TreeView (Page, 1996). *B*, the amino acid sequences of the individual PDZ domains of the NHERF family members were aligned using T-coffee (Notredame *et al.* 2000) and the phylogenetic tree was created using TreeView (Page, 1996). The PDZ domains have been grouped (Group A, blue box; Group B, yellow box in *C*) based on where each PDZ domain originates from the base of the tree. The most divergent PDZ domains in the NHERF family, Group B, largely belong to NHERF4. *C*, a comparison of the identity of each NHERF PDZ domain at the amino acid level. Identity values were calculated based on T-coffee alignments.

NHERF4

Our understanding of the cellular function of NHERF4 is based on interaction with a limited number of binding partners. Our laboratory cloned NHERF4 in a yeast two-hybrid screen using the conserved COOH-terminus of the receptor guanylyl cyclase, GCC, as the bait. Based on the domain organization and mRNA distribution, we originally named the GCC interacting protein intestine and kidney enriched PDZ protein (IKEPP). IKEPP was also described as NaPi Cap-2 when it was simultaneously identified as a protein interacting with the type IIa sodium–phosphate co-transporter (Gisler *et al.* 2001). However, we have changed the name to NHERF4 to reflect the high degree of similarity with the other NHERF proteins. GCC is expressed at the apical cell surface of intestine and kidney epithelia (Nandi *et al.* 1997; Potthast *et al.* 2001). When activated by ligand, GCC increases intracellular cGMP, initiating a signalling cascade that stimulates salt and water secretion. GCC is potently activated by the *E. coli* heat-stable enterotoxin STa, resulting in secretory diarrhoea and severe dehydration (Schulz *et al.* 1990). Early studies on the NHERF proteins suggest that NHERFs are required for the apical membrane localization of cystic fibrosis transmembrane conductance regulator (CFTR) and the multidrug resistance protein 2 (MRP2) (Moyer *et al.* 1999; Harris *et al.* 2001). Therefore, we speculated that an association with NHERF4 or other related NHERFs may regulate the polarized membrane trafficking of GCC. However, truncated GCC proteins, lacking the COOH-terminal four amino acids required for NHERF binding, were efficiently targeted to the apical cell surface (Scott *et al.* 2002). More recent data suggest that NHERF proteins do not play critical roles in apical localization, as many NHERF binding proteins (including CFTR and MRP2) retain their apical localization in the absence of NHERF binding (Nies *et al.* 2002; Benharouga *et al.* 2003; Ostedgaard *et al.* 2003; Milewski *et al.* 2005). Although an interaction with NHERFs is not required for the apical sorting of GCC, we find that the interaction of NHERF4 with GCC significantly inhibits heat-stable toxin-induced cGMP synthesis in heterologous over-expression systems (Scott *et al.* 2002). Although the mechanism by which NHERF4 inhibits cGMP production by activated GCC is unknown, it may do so by recruiting inhibitory factors to the activated receptor or by inhibiting GCC oligomerization.

In addition to GCC and the type IIa sodium–phosphate co-transporter, NHERF4 can also interact with the multidrug resistance-related protein 2 (MRP2 or cMOAT) *in vitro* (Hegedus *et al.* 2003). Based on these interactions, we predict that NHERF4 functions to regulate epithelial transport and signalling, similar to the other NHERF proteins. However, understanding the precise cellular role of NHERF4 will require identifying additional binding partners and carefully analysing NHERF4 subcellular localization. Preliminary observations suggest that NHERF4 has unique PDZ domain primary structure and a distinctive subcellular localization making it quite different from the other NHERF proteins.

The NHERF PDZ domains

Based on a phylogenetic analysis, we have clustered the NHERF PDZ domains into two distinct groups. Group A is largely made up of the PDZ domains of NHERF1, NHERF2 and NHERF3, which are the most highly conserved in the family (Fig. 1*C*, blue box). The PDZ domains of NHERF4 make up the majority of Group B, and are more divergent (Fig. 1*C*, yellow box). The exceptions to this general grouping are PDZ3 of NHERF4, which is more closely related to the Group A PDZ domains, and PDZ2 of NHERF3, which is a Group B PDZ domain. Interestingly, all of the known NHERF binding partners interact with Group A PDZ domains, while none have been identified for the Group B domains. For example, the first, third and fourth PDZ domains of NHERF3 (Group A) can all interact with CFTR *in vitro*, while PDZ2 (Group B) cannot (Wang *et al.* 2000). These sequence analyses highlight several important details regarding the NHERF family. First, our present understanding of the NHERF family is based on proteins that interact with only the PDZ domains listed in Group A. The PDZ domains in Group B represent 1/3 of the family, yet almost nothing is known about their binding partners. Identifying the proteins that interact with the Group B PDZ domains is critical for understanding the complete functional significance of the NHERF family. Second, it is clear from the phylogenetic analysis that NHERF4 is the most divergent family member. Only PDZ3 of NHERF4, which interacts with guanylyl cyclase C (Scott *et al.* 2002), the type IIa NaPi co-transporter (Gisler *et al.* 2001), and MRP2 (Hegedus *et al.* 2003), clusters with the PDZ domains of the other NHERF family members.

The differences among the PDZ domains of the NHERF family members also extend to amino acids critical for binding specificity. Nearly all of the published binding partners of NHERF proteins contain the type I PDZ binding consensus S/T-X-Φ-COOH (where Φ is hydrophobic). A conserved histidine in class I PDZ domains coordinates the hydroxyl group of the serine or threonine residue in the PDZ ligand (Doyle *et al.* 1996; Morais Cabral *et al.* 1996; Songyang *et al.* 1997). Interestingly, only NHERF3 and NHERF4 contain PDZ domains that lack the conserved histidine residue and instead posses a tyrosine (PDZ4 of NHERF3 and PDZ1 of NHERF4) or an aspartic acid (PDZ4 of NHERF4) (Fig. 2). The fourth PDZ domain of NHERF3 can interact with the type I PDZ binding motif of CFTR, demonstrating that the presence of the conserved histidine does not define a class I

PDZ domain (Wang *et al.* 2000). Alternatively, we find that the fourth PDZ domain of NHERF4 interacts with class II PDZ ligands *in vitro* (W. R. Thelin, C. A. Hodson and S. L. Milgram unpublished observation), again distinguishing NHERF4 from the other family members.

NHERF protein phosphorylation

While many studies have identified NHERF binding partners, we are just beginning to appreciate how NHERF protein interactions are regulated. Post-translational modifications such as phosphorylation are clearly important in regulating NHERF proteins. Under basal conditions, NHERF1 is constitutively phosphorylated (Reczek *et al.* 1997). NHERF1 may be hyper-phosphorylated during specific stages of the cell cycle or in response to extracellular signals (He *et al.* 2001; Deliot *et al.* 2005). While the significance of this is not clear, it appears that phosphorylation regulates NHERF1 oligomerization (Hall *et al.* 1999; He *et al.* 2001; Fouassier *et al.* 2005). Interestingly, phosphorylation can promote or inhibit NHERF1 oligomerization in a site-specific manner. Phosphorylation of NHERF1 on serine 289 by G protein-coupled receptor kinase 6a, or on serines 337 and 338 by protein kinase C, enhances oligomerization (Hall *et al.* 1999; Fouassier *et al.* 2005). Conversely, the phosphorylation of NHERF1 on serines 297 and 301 by cyclin-dependent kinase 2 (Cdc2) inhibits oligomerization (He *et al.* 2001). Although the importance of NHERF oligomerization is not well understood, it probably impacts on protein interactions and complex formation. For example, parathyroid hormone treatment leads to increased NHERF1 phosphorylation, which correlates with a decreased association between NHERF1 and the sodium phosphate co-transporter IIa (Deliot *et al.* 2005). Unlike NHERF1, NHERF2 is not phosphorylated by Cdc2, suggesting that the NHERF proteins respond differently to cell signalling events (He *et al.* 2001). Thus, unique post-translational modifications may give rise to unique functions for each NHERF family member. Much less is known regarding the phosphorylation state of the other NHERF proteins. By 2-D gel electrophoresis, we find that NHERF4 migrates as six distinct spots, suggesting multiple phosphorylation sites (W. R. Thelin, C. A. Hodson and S. L. Milgram, unpublished observation). In future studies, it will be critical to consider how cell signalling events impact on NHERF protein phosphorylation and regulate the protein complexes they organize.

The localization of NHERF proteins

NHERFs are co-expressed in a variety of epithelia but may also be expressed individually in certain cell types. NHERF1 and NHERF2 appear to be the most broadly distributed. While NHERF1 is widely recognized as a brush border protein in the kidney proximal tubule and gastrointestinal tract, it is additionally found in the airway epithelium, liver and parietal cells of the stomach (Mohler *et al.* 1999; Ingraffea *et al.* 2002). NHERF2 is highly expressed in lung and is co-expressed with NHERF1 in kidney where it is found in proximal tubules and in the renal corpuscle (Wade *et al.* 2001; Ingraffea *et al.* 2002; Capuano *et al.* 2005). Similar to NHERF1, NHERF2 co-localizes with NHE3 in the brush border of intestinal

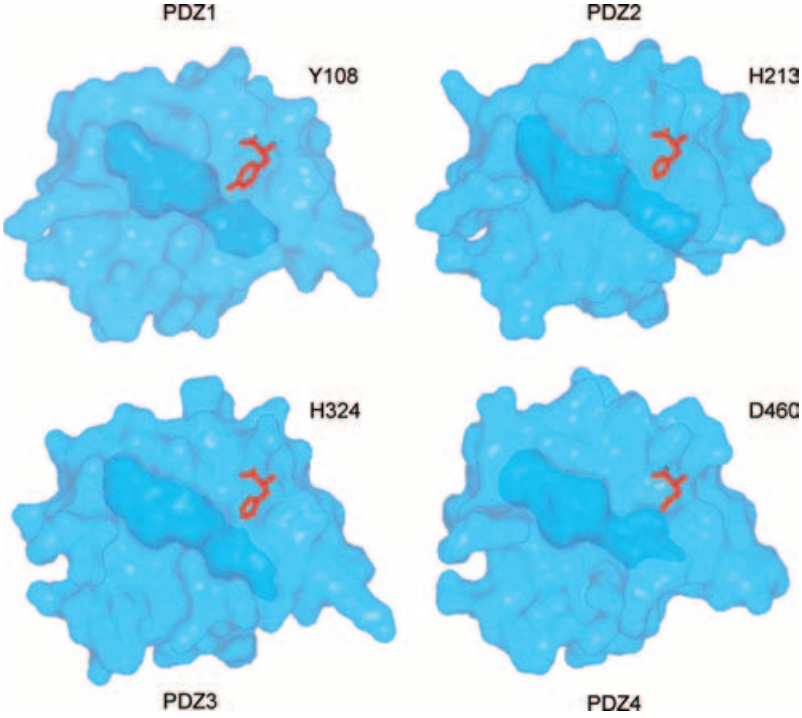


Figure 2. Amino acid differences in the PDZ binding pocket of NHERF4 may give rise to unique binding specificities
The crystal structure of PDZ1 of NHERF1 (Karthikeyan *et al.* 2001) was used as a template to model the four PDZ domains of NHERF4 using 3-D PSSM (Fischer *et al.* 1999; Kelley *et al.* 2000; Bates *et al.* 2001). Interestingly, PDZ1 and PDZ4 of NHERF4 lack histidine residues in the $\alpha\beta1$ position (highlighted in red), characteristic of class I PDZ domains. These differences (tyrosine for PDZ1 and aspartic acid for PDZ4 of NHERF4) may give rise to unique binding partners compared with the other members of the NHERF family, which tend to bind class I ligands.

tissue (Lamprecht *et al.* 2002). NHERF3 shares much of the tissue distribution with NHERF1 and NHERF2 including the proximal tubule and small intestine and is also present in liver (Custer *et al.* 1997; Gisler *et al.* 2001). NHERF4 is distinct from the other family members and possesses the most restricted tissue expression of all the NHERFs, being found only at significant levels in the gastrointestinal tract and kidney.

Detailed microscopic analysis is uncovering significant differences in the subcellular localization of the NHERF proteins, which may explain the co-expression of such seemingly similar proteins within tissues such as the proximal tubule and gastrointestinal tract. NHERF4 has been reported to localize to a subapical region of the kidney proximal tubule and is largely absent from the brush border (Gisler *et al.* 2001). On the contrary, NHERF1 and NHERF3 are highly localized to the brush border while the targeting of NHERF2 varies somewhat between apical and more diffuse among different tissues (Sun *et al.* 2000; Ingraffea *et al.* 2002). Although the NHERFs are similar in terms of nucleating protein complexes via PDZ domains, differences in subcellular localization patterns probably translate into unique biological roles for each protein.

Summary

It is clear that NHERFs have unique and overlapping cellular functions. Thus far, over 30 different binding partners have been identified for the NHERF family, many of which interact with multiple family members. For example, NHERF1, NHERF3 and NHERF4 all interact with the type IIa sodium–phosphate co-transporter (Gisler *et al.* 2001; Hernando *et al.* 2002). While overlapping binding partners suggests functional redundancy, the evidence suggests that each NHERF protein retains a unique cellular function. Both NHERF1 and NHERF2 can mediate inhibition of NHE-3 via a cAMP-dependent process; however, only NHERF2 can do so in response to elevated cGMP (Yun *et al.* 1997; Zizak *et al.* 1999; Cha *et al.* 2005). Not all NHERF-interacting proteins bind to multiple family members. For instance, phospholipase Cβ1 interacts with NHERF1 specifically (Suh *et al.* 2001). Furthermore, differences in the subcellular distribution and/or level of expression of each scaffolding protein and each binding partner will probably drive the formation of unique multiprotein complexes in cells.

To distinguish the unique and overlapping functions of the NHERF proteins we must study each member individually and in the context of the rest of the family. Presently, our understanding of the physiological roles of NHERF proteins relies heavily upon *in vitro* binding assays and functional experiments in heterologous expression systems. Thus, it will be important to extend these studies into physiologically relevant model systems including epithelial cell lines and knockout mice as described in the accompanying reviews. Based on the subtle phenotypes observed under basal conditions in the NHERF1 and NHERF3 knockout mice, it is reasonable to assume that there may be significant functional redundancy by other NHERF proteins. Therefore it will be critically important to cross the individual NHERF knockout mice to further dissect the unique and overlapping roles of this protein family.

At first glance, each of the NHERF family of epithelial PDZ proteins appears quite similar to one another. Closer inspection, however, reveals many differences in cellular expression, subcellular localization and biochemical properties. These differences probably affect *in vivo* function and will help explain the unique aspects of the NHERF proteins. Although much less is known regarding the localization and the binding partners of NHERF4, our initial observations support the idea that NHERF4 is distinctive from other NHERFs. Future studies will reveal how the unique biochemical properties and localization pattern of NHERF4 translate into distinctive physiological roles.

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