CYRI (FAM49) is a Rac1 interacting local inhibitor of actin-based protrusion

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Abstract

Actin-based protrusions are reinforced through positive feedback, but it is unclear what restricts their size, or limits positive signals when they retract or split. We identify an evolutionarily conserved regulator of actin-based protrusion: CYRI (**CY**FIP-related **R**ac **i**nteractor). CYRI binds activated Rac1 via a Domain of Unknown Function DUF1394, shared with CYFIP, defining DUF1394 as a Rac1-binding module. CYRI-depleted cells have broad lamellipodia enriched in Scar/WAVE, but reduced protrusion-retraction dynamics. Pseudopods induced by optogenetic Rac1 activation in CYRI-depleted cells are larger and longer-lived. Conversely, CYRI overexpression suppresses recruitment of active Scar/WAVE to the cell edge, resulting in short-lived, unproductive protrusions. CYRI thus focusses protrusion signals and regulates pseudopod complexity by inhibiting Scar/WAVE-induced actin polymerization. It thus behaves like a "local inhibitor" predicted in widely accepted mathematical models, but not previously identified in cells. CYRI therefore regulates chemotaxis, cell migration and epithelial polarisation by controlling polarity and plasticity of protrusions.

Introduction

Cell migration is an ancient and fundamental mechanism whereby cells exert control over interactions with their environment. The actin cytoskeleton is the main driver of cell migration, with dozens of proteins controlling actin organisation[1]. Actin protrusions, or pseudopods, govern migration, however the feedback loops controlling assembly, splitting and disassembly of these structures is an area of active debate[2].

The Scar/WAVE complex is the main driver of Arp2/3-mediated branched actin networks underlying pseudopod generation. The complex consists of five subunits CYFIP, NCKAP1, Scar/WAVE, ABI, HSPC300 (nomenclature in Supplementary Table 1). The main Arp2/3 activating subunit, Scar/WAVE, is autoinhibited until signals trigger a conformational change, exposing an Arp2/3 activation sequence [3, 4]. The Scar/WAVE complex is recruited to acidic phospholipids in the plasma membrane via a patch of basic charges[3] and via interaction with the small GTPase Rac1[5-7].

Many motile cell types steer by splitting pseudopods into two or more daughters; selecting pseudopods from the split for retraction/maintenance provides a directional bias steering cells up chemotactic gradients[8]. Actin and associated signal transduction networks form excitable systems that propagate in waves and self-limit to drive protrusion and retraction[1, 9, 10]. Actin and associated cytoskeletal components likely control their own excitability in concert with signaling lipids, but dynamic interplay between "on" and "off" signals is essential for migration to be plastic and responsive.

Negative regulators of Arp2/3 complex include Gadkin, which sequesters Arp2/3 at the trans Golgi network and endosomes[11]. Another inhibitor, Arpin mimics the activating sequence of Scar/WAVE but inhibits the Arp2/3 complex [12]. Here, we describe a negative regulator of the Scar/WAVE complex, CYRI (encoded by the *FAM49* gene), an evolutionarily conserved protein that mimics the Rac1 interaction domain of CYFIP and promotes dynamic pseudopod splitting.

Results

CYRI is an evolutionarily conserved N-myristoylated protein with homology to CYFIP

We sought new Scar/WAVE complex interactors by precipitating GFP-fused NAP1 (for nomenclature see Supplementary **Table 1**) from *napA* knockout rescued *Dictyostelium* cells. Reversible formaldehyde crosslinking *in cellulo*[13] stabilised transient interactions and GFP-Trap immunocapture recovered Scar/WAVE, ABI, HSPC300 and PIR121. Another interactor was identified as Fam49 (FAMily of unknown function 49; **Fig 1a** and Supplementary **Table 2**). Although FAM49 did not co-precipitate with the Scar/WAVE complex in the absence of crosslinking, we focused on it for two reasons. Firstly, *FAM49* is highly conserved across evolution and is roughly co-conserved with the Scar/WAVE complex[14,15] (**Supplementary Fig.1a**). Secondly, Pfam and InterPro identified FAM49 as uniquely sharing a DUF1394 domain with the Scar/WAVE

complex subunit CYFIP (**Fig. 1b and Supplementary Fig.1b**). FAM49 proteins comprise mostly DUF1394, while CYFIP proteins contain a cytoplasmic fragile X interaction domain[16] **(Fig. 1b)**. We renamed FAM49 to CYRI for **CY**FIP-related **R**ac1 **I**nteractor, in mammals, represented by CYRI-A (FAM49-A) and CYRI-B (FAM49-B) and henceforth we use this nomenclature.

The DUF1394 region of CYFIP, highlighted in red (PDB 3P8C and **Fig. 1c)** partly overlaps with the published Scar/WAVE complex Rac1 interaction site, in particular R190 in CYFIP1[3] (**Fig. 1c**, Black arrow and blue balls). Modeling the structure of the DUF1394 of CYRI-B using Phyre2, reveals structural similarities with CYFIP **(**PDB 3P8C and **Fig. 1d)**. The analogous R161 of CYRI **(Fig 1d** blue sidechains, and **e** red box**)** is part of a highly conserved 33-amino acid stretch (>75% similarity) across diverse phyla (**Supplementary Fig. 1b-c**). R160 is also conserved in CYRI but replaced by lysine in CYFIP (**Fig. 1d-e and Supplementary Fig. 1b-c)**.

The N-terminal glycine-2 of CYRI proteins encodes a putative myristoylation site[17-19] **(Fig. 1f)**, which is not conserved in CYFIP. We confirmed the myristoylation of CYRI-B by assessing the incorporation of myristate analogue (C14:0-azide) onto G2 using CLICK chemistry *in cellulo*. Mutation of this glycine to alanine abolishes the CLICK signal **(Fig. 1 g-h)**.

In summary, we have defined CYRI, an evolutionarily conserved protein with a putative Rac1-binding DUF1394 module. Furthermore, N-terminal myristoylation suggests CYRI may dynamically associate with the plasma membrane[20], where active Rac1 stimulates the Scar/WAVE complex to catalyse lamellipodial expansion.

CYRI interacts directly with activated Rac1 *in vitro*

Homology between CYRI and CYFIP **(Supplementary Fig. 1b)**, suggested potential interaction with Rac1. Yeast two-hybrid screening with $Rac1^{G12V}$ as bait retrieved CYRI-B from multiple cDNA libraries **(Supplementary Fig. 2a)**. The core interacting sequence of CYRI-B encompasses amino acids 30-236 (hereafter the Rac Binding Domain - RBD), **(Supplementary Fig. 2b-c**). GFP-RBD expressed in CHL-1 human melanoma cells interacted selectively with GST-$Rac1^{Q61L}$ but not GST-$Rac1^{WT}$. Mutation of CYRI-B R160 or R161 (in GFP-RBD) to aspartic acid abrogated this interaction **(Fig. 2a-c and Supplementary Fig. 7).** GST-CYRI-B RBD and MBP-Rac1 also showed robust interaction **(Supplementary Fig. 2d-f and Supplementary Fig. 7)**. In this assay, CYRI does not co-precipitate with $Rac1^{T17N}$, $Rac1^{G12V}$, or Rac1 ^{WT}, likely due to the low affinity of CYRI-B for Rac1. However, the double mutant Rac1 $^{P29S/Q61L}$, recently shown to have a high affinity for the Scar/WAVE complex[21], displayed enhanced binding to CYRI-B RBD (~3-3.5-fold increase) over $Rac1^{Q61L}$ but no enhanced binding to Pak1-CRIB (**Supplementary Fig. 2 d-f and Supplementary Fig. 7**). Using surface plasmon resonance, immobilised CYRI-B RBD specifically interacted with Rac1 ^{Q61L} with a K_d of 27 μM and the reverse assay, with Rac1 ^{Q61L} immobilised returned a K_d of 22 μM **(Fig. 2g)**. As CYRI-RBD shows no homology to CRIB (Cdc42 and Rac interaction binding) motifs, we probed the specificity of the interaction of CYRI between Rac1, RhoA and Cdc42. Once again, CYRI-RBD interacted robustly with

Rac1^{Q61L} but not with constitutively active RhoA^{Q63L} or Cdc42^{Q61L} **(Fig. 2d, Supplementary Fig. 2g-h and Supplementary Fig. 7)**. Thus CYRI-B RBD interacts specifically with active Rac1. Two conserved basic residues in the DUF1394 (conserved in CYFIP) mediate this interaction. This suggests a signal-regulated interaction between active Rac1 and CYRI, similar to the Rac1-CYFIP interaction, defining DUF1394 as an active Rac1 interaction module.

CYRI interacts with active Rac1 in cells

We next explored the Rac1-CYRI interaction in cells. Proximity ligation[22] revealed an interaction between Rac1^{WT} and CYRI-B in COS-7 cells, as well as a stronger interaction between Rac1^{Q61L} **(Fig. 2h-i and Supplementary Figure 2i-l)**. Mutation of key arginines in CYRI-B$^{R160/161}$-HA abolished this interaction and dominant negative Rac1^{T17N} showed no interaction **(Fig. 2 h-I and Supplementary Fig. 2i-l)**. Targeting either CYRI or Rac1A to mitochondria[23] **(Figure 2j)** in *Dictyostelium*, revealed that CYRI^{WT}, but not CYRI mutated for the analogous R155/156D, strongly co-recruits active Rac1A $^{P29S/\ Q61L}$. The Pearson's coefficient of fluorescence correlation (PCC) for Rac1A-mCherry-mito and the GFP-fusions revealed a PCC 0.77 for CYRI^{WT}; and 0.05 for CYRI$^{R155/156D}$, where 1 = perfect, 0= no correlation and -1 = excluded. The PCC for Rac1A-mCherry and GFP-mito-fusions were: CYRI ^{WT} 0.44 and CYRI $^{R155/156D}$ -0.23. CYRI-GFP did not co-localise with a mitochondrial reporter (PCC = -0.06). Thus, CYRI interacts with activated Rac1, mediated by key conserved arginines, in both mammalian and *Dictyostelium* cells.

CYRI opposes recruitment of the Scar/WAVE complex to lamellipodia

Knockdown or knockout of *CYRI-B* by siRNA or CRISPR in COS-7 or CHL-1 cells did not affect proliferation, but promoted unusually large and broad lamellipodia highly enriched in WAVE2 **(Fig. 3a-b, Supplementary Fig. 3a-g and Supplementary Fig. 7)**. Cells spread over a larger area and adopted a "fried-egg" phenotype, correlating with an increase in circularity **(Fig. 3c-d, Supplementary Fig. 3e-g)**. Expression levels of Scar/WAVE complex subunits are not obviously altered in *cyri-b* knockout cells **(Supplementary Fig. 3h and Supplementary Fig. 7)**. Cell area and circularity were both rescued by re-expression of untagged CYRI-B^{WT}, but not the Rac1-nonbinding R160/161D mutant **(Fig. 3e-f, Supplementary Figure 3i-k and Supplementary Fig. 7)**. CYRI-B^{G2A} which cannot be N-myristoylated failed to rescue the phenotype **(Fig. 3g-h, Supplementary 3l-m and Supplementary Fig. 7),** reinforcing the importance of CYRI lipid modification. *cyri* knockout *Dictyostelium* cells also showed enhanced recruitment of the Scar/WAVE complex (GFP-HSPC300 reporter) to a much broader leading edge **(Supplementary Fig. 3n – yellow dotted line and Supplementary Movie 1)**. Moreover, Scar/WAVE patches in *cyri* knockout cells are ill-defined but longer-lived, suggesting CYRI's ability to suppress Scar/WAVE complex activity outside of active protrusions. (**Supplementary Fig. 3n,** heat map**).** We conclude that CYRI, via its interaction with active Rac1 and membrane targetting, opposes active Scar/WAVE complex at the plasma membrane and thus drives the formation of more focussed and sharper lamellipodial protrusions.

To determine the requirement for Rac1 for the phenotype of *cyri-b* knockout cells, we co-depleted Rac1 and CYRI-B from mouse tail skin fibroblasts with ROSA26-Cre::ER^{T2+};*p16Ink4a*$^{-/-}$, *Rac1*$^{fl/fl}$ genotype[24], treated with hydroxytamoxifen (OHT, to induce deletion of *Rac1*) and then with siRNA against *Cyri-b* (**Supplementary Fig. 3o and Supplementary Fig. 7**). Deletion of *Rac1* led to a spindle-shaped morphology and a loss of lamellipodia as previously described [25-27]. Loss of CYRI-B did not cause excessive lamellipodia or rescue circularity in Rac-deleted cells **(Fig. 3i-k).** Thus, Rac1 is absolutely required for CYRI-B driven actin reorganisation.

The increased circularity of *cyri-b* depleted cells is reminiscent of Rac1 hyperactivation phenotypes[28], suggesting that CYRI-B might buffer Rac1 activity. Indeed, a dark acceptor mTq2-sREACH Raichu FRET probe[29, 30] showed a consistent increase in Rac1 signaling activity in CYRI-B depleted cells, as measured by FRET efficiency in both COS-7 **(Fig. 3l-m)** and CHL-1 cells **(Supplementary Figure 3p-q),** which was confirmed by biochemical pulldown **(Fig. 3n-o and Supplementary Fig. 7)**. Together, these data indicate an increase in Rac1 signaling activity in CYRI-B depleted cells. Conversely, inducible overexpression of untagged CYRI-B **(Supplementary Fig. 4a-b and Supplementary Fig. 7)** resulted in fractal-like lamellipodia, decreasing WAVE2 recruitment, cell area and circularity **(Fig. 4a-d, Supplementary Fig. 4c-f –** Vehicle-treated controls**)**. In parallel, overexpression of CYRI-B also drove a decrease in the Rac1 activity signal of the Raichu FRET probe (**Fig. 4e-f)** which was fully reversed by an R160/R161 double mutation **(Fig. 4g)**. Thus, CYRI-B opposes Rac1-Scar/WAVE mediated expansion of lamellipodia protrusions. Adding a GFP-tag to either end of CYRI-B interfered with its function, precluding dynamic analysis, likewise, available antibodies to Fam49B did not give specific staining by immunofluorescence, but CYRI-B-FLAG showed significant co-enrichment with WAVE2 at leading pseudopods **(Fig. 4h-i)**. Thus CYRI co-accumulates with WAVE2 at lamellipodia protrusions. Overall, *cyri-b* knockout cells show broader Scar/WAVE driven lamellipodia and increased Rac1 activation, supporting a role for CYRI-B as a buffer of Rac1 and Scar/WAVE complex activation activity at the leading edges of cells.

CYRI regulates the duration and extent of protrusions

We next sought to determine the consequences of CYRI-B depletion for lamellipodial actin dynamics. First, we observed actin dynamics live using fast frame-rate videos in CHL-1 cells expressing GFP-Lifeact **(Fig. 5a –** Left panel and **Supplementary Movie 2).** We tracked the cell edge and used unwrapped (polar) kymographs (**Fig. 5a** middle panels) to visualise and measure the area of protrusion **(**yellow colour**)** versus retraction **(**purple colour**)** over time. Control cells showed small but rapid bursts of actin-based protrusion (yellow patches on kymograph), while *cyri-b* knockouts had longer-lived less dynamic responses **(Fig. 5a,b)**. If CYRI-B buffers Rac1 at the lamellipodium, we speculated that *cyri-b* knockout cells would struggle to restrain protrusion formation upon Rac1 activation. To investigate this, we used the Rac1-LOV optogenetic probe, which triggers activation of Rac1 with blue light [31]. Rac1 was activated with pulses of blue light in a discrete area on the cell periphery **(Fig. 5c-d and Supplementary Movie 3)**. C*yri-b* knockout cells showed a more sustained and extensive

protrusion response and increased peripheral propagation of lamellipodia **(Fig. 5e-g)**. Thus, CYRI-B limits Rac1- mediated activation of the Scar/WAVE complex and shortens the Rac1-activated protrusion.

CYRI focuses actin assembly in leading pseudopods to promote plasticity of migration

Plasticity of protrusion is important for directional migration, such as during chemotaxis. CHL-1 melanoma cells are normally nearly static when seeded at low density in 2D-culture, but *cyri-b* knockout cells migrated 1.5-2-fold faster (**Fig. 6a-b and Supplementary Movie 4**). C*yri-b* knockout cells frequently assumed a C-shape, with a broad spread lamellipodium at the front half of the cell and a convex rear which resembled the fast-moving goldfish keratocyte [32] **(Supplementary Fig. 5a** yellow arrows**, Supplementary Movie 4)**. C-shaped cells moved faster than the other common shapes **(Fig. 6c,d)** and C-shape correlated with faster migration **(Fig. 6e-f and Supplementary Fig. 5b-c)**. Lamellipodia need to be polarized and dynamic for efficient cell migration[27, 33], so when *cyri-b* knockout cells became polarized into a C-shape, they gained motility.

Since cells need to maintain plasticity of their lamellipodia to respond effectively to directional cues[34], we predicted that depletion of CYRI-B would affect chemotactic migration. CHL-1 cells are not chemotactic to serum, but WM852 melanoma cells are highly chemotactic[35]. Loss of CYRI-B (**Supplementary Fig. 5d-e and Supplementary Fig. 7**) severely affected chemotaxis of these cells towards serum with no effect on basal speed; Knockouts often migrated very long distances in the opposite direction to the chemoattractant gradient, having lost the plasticity to reorient toward the gradient **(Fig. 6g-i and Supplementary Movie 5)**. Thus, CYRI-B strongly impacts how cells polarize and remodel their lamellipodia and reorient during directed migration.

CYRI promotes pseudopod splitting and opposes persistent migration in *Dictyostelium*

We examined *Dictyostelium* cells (Ax3, *cyri* knockout and rescue - **Supplementary Fig. 5f and Supplementary Fig. 7**) migrating under agarose up self-generated gradients of the chemoattractant folate[36] **(Supplementary Fig. 5g**). Similar to CHL-1 cells, *cyri* knockout cells were rounder, with blunted pseudopods **(Fig. 6j-k, Supplementary Movies 6-7).** *Dictyostelium* cells primarily turn by splitting their leading pseudopod into differently-oriented daughters[8]; *cyri* knockouts generated fewer protrusions/min **(Fig. 6l)** and showed fewer splits (from ~5/min to ~2/min, **Fig. 6m)** and decreased speed **(Fig. 6n)**. Cells still oriented towards the folate gradient, but their less efficient turning was clearly reflected by a smaller angle of turn between steps (**Supplementary Fig. 5h**). Thus, CYRI promotes pseudopod splitting in *Dictyostelium* cells, which is dispensible for gradient sensing, but compromises the speed of migration and reorientation while steering.

We rescued *Dictyostelium cyri* knockouts with CYRI^{WT} or CYRI$^{R155/156D}$ as stable, single-copy transfectants[37] under an actin15 promoter (**Fig. 6 j-n, Supplementary Movies 6-7**). CYRI^{WT} expressing cells exhibited more

numerous fractal pseudopods as well as decreased circularity and enhanced frequency of protrusion generation and pseudopod splitting (**Fig. 6j-m**) even over WT cells. Rescue with CYRI^{WT} also restored cells' ability to turn during chemotaxis (**Supplementary Fig. 5h**).

Another widely- used chemotaxis assay involves a chemoattractant-filled microneedle introduced just next to *Dictyostelium* cells, inducing new pseudopods directly toward the needle, and consequently reorienting the cells. When cyclic-AMP (cAMP)-sensitive *cyri* knockout or rescue cells were challenged with cAMP in a needle assay, *cyri* knockouts were initially unable to form new pseudopods **(Fig. 6o)**, while CYRI^{WT} cells rapidly protruded pseudopods and reoriented toward the needle **(Figure 6o-p and Supplementary Movie 8)**. C*yri* knockouts eventually elongated and streamed toward the needle, but they maintained resistance to new pseudopod formation and rapid reorientation. Thus, cells that lack CYRI can still sense an attractant gradient, but their broad and unfocussed protrusions split rarely, and their diminished ability to generate new pseudopods cripples their response to changing gradients.

Modeling CYRI's role in pseudopod plasticity

Since CYRI affects plasticity of pseudopod dynamics, we likened its activity to the mathematical model of Meinhardt [38], where local inhibitors are recruited by an activation signal and limit the amount of cell edge devoted to pseudopods. Actin assembly pathways are not linear cascades, but rather feedback loops where positive stimulation is self-reinforcing and causes further activation until overcome by negative feedback[1, 10]. In models of migration based around positive feedback, a locally-acting inhibitor is also needed to destabilise existing pseudopods, so the cell can change direction. Without this, cells polarize, but cannot turn to migrate toward an attractant. We used a modified version of a published simulation[39] based on the Meinhardt model[38] to visualise the concentrations of the activator and the local inhibitor at the cell edge **(Supplementary Fig. 5i and Supplementary Movie 9)**, to illustrate the how CYRI-B regulates Rac1 and Scar/WAVE signaling. A peak in the activator (which represents active Rac1 and Scar/WAVE) results in the formation of a new pseudopod. The peak also causes an increase in the concentration of the local inhibitor, which is smaller and thus diffuses faster[38]. Initially, the inhibitor limits the lateral spread of the pseudopod (**Supplementary Fig. 5i**, panel 1); later, levels of inhibitor rise in the middle of the pseudopod, destabilizing it and causing a split (**Supplementary Fig. 5i**, panel 2). The weaker of the pseudopods then retracts and the stronger is reinforced until the cycle of inhibition catches up with it and re-starts the splitting cycle (**Supplementary Fig. 5i**, panels 3-4). The local inhibitor thus increases both the morphological complexity of the cell and the competition between pseudopods. This is supported by the lack of pseudopod splitting in *Dictyostelium* and our optogenetic data showing that protrusions in *cyri* knockout cells are more long-lived and spread laterally to a greater extent. Thus, Meinhardt's model offers insight into the role of CYRI proteins as local inhibitors, which enhance leading edge dynamics and add plasticity to the positive feedback loops driving migration.

CYRI-B regulates epithelial polarity via a Rac1-dependent mechanism

Finally, we tested a role for CYRI-B the polarized epithelial cyst [40, 41] where asymmetric Rac1 activation is also crucial. As cells form a cyst, they establish a lumen via selective membrane trafficking and polarized recruitment/activation of cytoskeletal components[42]. Specific spatial regulation is dependent on matrix and adhesions, but Rac1 activation also regulates lumen formation[43] and is specified by differential recruitment of the GEF TIAM1 across the cyst, leading to an apico-basal activation gradient[40]. We hypothesized that CYRI-B might help maintain the Rac1 activation gradient, allowing Scar/WAVE complex recruitment and activation to be spatially controlled during cyst formation. Indeed, knockdown of *CYRI-B* using shRNA in MDCK cells **(Supplementary Fig. 6a-b and Supplementary Fig. 7)** led to a multilumen phenotype during cyst formation, similar to deregulation of active Rac1 (**Figure 7a-b**, [42]). WAVE2 is normally prominently localized to the basolateral surfaces of the cysts, but mostly absent from the luminal surface, as marked by podocalyxin (PODXL) **(Fig. 7c)**. However, when CYRI-B was depleted, WAVE2 staining was increased at the luminal periphery coincident with PODXL staining **(Fig. 7c)**. Mislocalisation of the actin cytoskeleton machinery to cyst luminal surfaces results in aberrant orientation of the mitotic cleavage plane during polarized cell division, which occurred in *cyri-b* knockdown cysts **(Supplementary Fig. 6c-e)**. To test whether the multilumen phenotype was due to inappropriate Rac1 activation, we used moderate concentrations of either EHT1864 **(Fig. 7d-e)** or NSC23766 **(Supplementary Fig. 6f)** to dampen Rac1 activity; these both provided a substantial rescue. Thus, loss of CYRI-B destabilised epithelial polarity during the formation of epithelial cell cysts by allowing inappropriate Rac1-mediated recruitment of the actin machinery to the nascent luminal surface. CYRI-B thus maintains spatial regulation of activation of the Scar/WAVE complex by dynamic buffering of Rac1.

Discussion

CYRI is highly conserved and DUF1394 represents a Rac1 interaction module

CYRI proteins are highly conserved in eukaryotes and function as a Rac1 interaction module that directly limits Rac1-mediated lamellipodia extension. The DUF1394 domain of CYRI comprises the Rac1 binding site and is shared with CYFIP proteins of the Scar/WAVE complex. This interaction requires two highly conserved arginine/lysine residues, previously described on CYFIP1[3]. CYRI, like CYFIP1, is specific for activated Rac1 over RhoA and Cdc42. Myristoylation of glycine 2 of CYRI may allow recycling of CYRI between active pseudopods and the cytoplasm or membrane vesicles [44]. The Rac1-interacting formin FMNL2 is also myristoylated[45], implying potential common mechanisms for recruitment to actin protrusions. CYRI has no homology to GTPase activating proteins (GAPs), so it likely doesn't alter nucleotide hydrolysis by Rac1. Why would a cell need CYRI if it has Rac-GAPs? We propose CYRI could be a specific buffer for Scar/WAVE-driven lamellipodia plasticity, rather than a general protein to turn off Rac1.

CYRI opposes recruitment of active Scar/WAVE complex to leading edges and promotes plasticity

Modulating the levels of CYRI differently affected cell speed in the cell types we assayed. While this may seem paradoxical, the basal speeds of these cell types and modes by which they migrate are different. Furthermore, migration speed is multiparametric, being the result of a combination of protrusion, adhesion and directionality/persistence. Migration speed is thought to require optimal levels of Rac1 activation and can be slowed by too little/much active Rac1[33]. *Dictyostelium* are optimized by nature to be fast-moving and relatively non-adhesive, so nearly any change will result in slower migration. In contrast, the speed of adhesive slow-moving cancer cells may benefit from removing the brakes on Rac1 activity.

Negative regulators of Arp2/3 complex have been described [11, 12, 46], but thus far, CYRI is the only negative regulator of the Scar/WAVE complex. Importantly, it is widely conserved in evolution along with the Scar/WAVE complex, so is a universal negative regulator. CYRI and CYFIP likely resulted from an ancient gene duplication and retained the same Rac1 binding function, placing CYRI as a Meinhardt local inhibitor[38]. But a local inhibitor should be present at high enough concentration to compete with the activator. A recent quantitative mass spectrometry study estimated concentrations of CYRI-B to be 4-fold higher in protein copy number than Scar/WAVE complex [47] in 3 of 4 cell lines (A549 4-fold, HepG2 5-fold, PC3 4.4-fold and U87 0.53-fold, based on comparison with CYFIP1). Thus, there is likely enough CYRI-B in cells to compete with the Scar/WAVE complex for Rac1 binding.

CYRI provides spatiotemporal regulation of the connection between Rac1 and Scar/WAVE complex

Cell migration involves cycles of protrusion and retraction coupled with adhesion to produce forward locomotion [48]. Cells with wild-type levels of CYRI showed rapid protrusion-retraction dynamics indicative of transient activation of the Scar/WAVE complex (e.g. kymograph **Fig. 5a**). *cyri* knockouts showed broader and more sustained lamellipodia and increased Scar/WAVE recruitment, placing CYRI as a key part of the feedback loop controlling leading edge actin dynamics, in line with Arpin, a negative regulator of the Arp2/3 complex [12] and coronin, which positively regulates Rac1 activation [49, 50,1]. Breaking the feedback loop by deleting CYRI affected both Scar/WAVE recruitment and Rac1 signalling activity. Thus, the actin machinery feeds back to Rac1 dynamically. This dynamic feedback is necessary for cells to change direction and respond with plasticity to stimuli such as chemotactic gradients.

CYRI also regulates polarized function of Rac1-Scar/WAVE complex in epithelial cells in 3D. Epithelial cells establish a Rac1 gradient that maintains polarity by asymmetric distribution of β2-syntrophin and Par3 [40]. Par3, localized apically, inhibits the Rac-GEF TIAM1, while β2-syntrophin, localized basally, activates TIAM1. This gradient is required for proper luminogenesis. CYRI helps direct formation of a single polarized lumen by regulating the Rac1 gradient required

for proper spindle orientation. A role for CYRI in epithelia could have broad implications for development and cancer.

Cell migration is the outcome of feedback loops that control the dynamics of cell shapes [10, 38, 51-53]. Travelling and spreading wave patterns (for example [10, 51]) manifest in actin-based protrusions, implying positive feedback loops. However, negative feedback is also required[38] to prevent uniform activation. Actin and actin-binding proteins can comprise an excitable system [10, 52] also modulated by systems involving small GTPases, kinases and signaling lipids e.g.[9]. Our data imply that CYRI acts at the interface; by competing with Scar/WAVE (an actin-nucleating complex) for Rac1 (a small GTPase) it connects signaling with actin polymerization, moderating excitable behaviours.

In conclusion, we propose that CYRI is a highly conserved regulator of the dynamics of the Rac1 – Scar/WAVE pathway, providing plasticity and adding complexity to leading edge dynamics.

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Author contributions

R.H.I. and J.B. conceived and carried out the initial screen and recognized the similarity of CYRI to CYFIP. L.F. designed and carried out the majority of the experiments on mammalian CYRI-B. L.M.M., R.H.I. and L.F. conceived the study and wrote the paper. P.A.T. designed and constructed the mitochondrial relocalisation tools and carried out the *Dictyostelium* experiments in Figs 2 and 6. K.M. and K.I.A. designed the Raichu FRET probe and with L.F. carried out the FRET experiments. P.B. and L.F. carried out the surface plasmon resonance experiments. J.G., N.C.O.T., and L.C. synthesized probes for, advised on and carried out the myristoylation experiments with L.F. S.L. and S.Z. carried out and analysed the mass spectrometry with L.F. and J.B. P.A.T., G.S.M., J.A.W., H.J.S., L.T and S.I. provided essential advice, carried out experiments and analysis of data. M.N. and R.H.I. constructed the model and advised on its use.

Figure Legends

Figure 1 - CYRI (Fam49) proteins show homology to CYFIP and contain a putative Rac1 interaction motif

a – Volcano plot illustrating pooled results from four LC-MS/MS experiments showing comparison of formaldehyde crosslinked proteins co-immunoprecipitating with GFP or GFP-NAP1 in *Dictyostelium napA* knockout cells. Color-coding based on two-tailed Welch's t test difference. Curved line is 5% false discovery rate. Identified interactors are labeled with gene symbols and presented in **Supplementary Table 2**. (n=4 independent experiments).

b – Schematic of human CYFIP1/2 and CYRI-A/B showing amino acid numbers and domains. Common DUF1394 domain (Pfam PF07159) in red and CYFIP1/2 C-terminal cytoplasmic Fragile X Mental Retardation FMR1-interacting domain (FragX-IP, Pfam PF05994) in light green.

c - Two views of ribbon crystal structure of the Scar/WAVE complex (PDB 3P8C)[2]. NCKAP1 in lilac, CYFIP1 in light green and red, Scar/WAVE in peach, HSPC300 in yellow and ABI1 in orange. DUF1394 is red, with putative Rac1 interaction residues in blue and highlighted by arrows.

d – Phyre prediction of structure of the DUF1394 domain of CYRI-B. The putative Rac1-binding domain of CYRI is blue with Arg160 and Arg161 indicated as a stick representation.

e - Sequence alignment of the putative Rac1-binding domain of CYRI in different organisms. The CYFIP Lys189 and Arg190 equivalent residues are well conserved in CYRI (Arg160 and Arg161) and are highlighted in red.

f - Sequence alignment covering the N-terminal region of CYRI from representative evolutionarily diverse eukaryotes. UniProt accession numbers are reported. Color code represents the number of entries with an identical amino acid at this position. The glycine in the 2nd position (highlighted red) is a putative myristoylation site.

g-h - CLICK chemistry analysis of the glycine 2 of CYRI-B. Myristoylation was labeled in HEK293T cells and measured by incorporation of myristate-azide (green) in GFP, CYRI-B^{WT}-GFP or CYRI-B^{G2A}-GFP mutant transfected cells (magenta), following GFP immunoprecipitation. Molecular markers shown left (**g**) See also **Supplementary Fig. 7 and Supplementary Table 6**. Relative incorporation was quantified by densitometry and reported in (**h**). One way ANOVA with Tukey post-test was applied. *** p<0.001. (n=3 independent assays). Bar graph represents mean and S.E.M.

Figure 2 - CYRI proteins interact with active Rac1

a-c - Western blot pulldown of GST control, GST-Rac1^{WT} or GST-Rac1^{Q61L} beads, mixed with lysates expressing GFP alone, positive control PAK1 eCFP-CRIB-PBD, GFP-RBD^{WT}, GFP-RBD^{R160D} or GFP-RBD^{R161D} (**a**). Densitometry is relative to GST (**b-c**). (n=3-4 independent experiments, see also **Supplementary Fig. 7**).

d-f - Western blot pulldown of GST control, GST-Rac1^{P29S} or GST-Rac1^{Q61L} or double mutant GST-Rac1$^{P29S/Q61L}$ beads, with cell lysate expressing either GFP alone, eCFP-CRIB-PBD, GFP-RBD^{WT} (**d**). Densitometry is relative to GST (**e-f**). (n=3 independent experiments, see also **Supplementary Fig. 7**).

g – Steady state surface plasmon resonance (SPR) binding curves between Rac1^{Q61L} and CYRI-B-RBD. Left: GST-CYRI-B was immobilized vs increasing concentrations of Rac1^{Q61L}. Right: His-Rac1 immobilised vs increasing concentrations of CYRI-B RBD. Simple 1:1 binding model. K_d = equilibrium dissociation constant, A.U. = arbitrary units.

h-**i** Proximity ligation assay with COS-7 cells on laminin and co-expressing either CYRI and Rac1 constructs as indicated. PLA signal (yellow), F-actin (magenta) and nuclei (blue). See **Supplementary Fig. 2** for negative controls. Quantification of 4 independent experiments in **(i)**. One-way ANOVA with Dunn's post-test between CYRI-B^{WT} and MYC-Rac1 constructs. Two-tailed Mann Whitney test between CYRI-B^{WT} and CYRI-B$^{R160/161D}$ for each MYC-Rac1 construct. n.s. $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (anti-HA n=55; anti-Myc n=54; Myc-WT/WT-HA n=55; Myc-WT/R160-161D-HA n=55; Myc-T17N/WT-HA n=63; Myc-T17N/ R160-161D-HA n=84; Myc-Q61L/WT-HA n=69; Myc-Q61L/ R160-161D-HA n=65, where n=cells)
Scale bar = 50 μm.

j - Still pictures from mitochondrial recruitment of CYRI (Forward) or Rac1A$^{P29S/Q61L}$ (Reverse) in Ax3 *D. discoideum*. Quantification (Imaris) showed Pearson's coefficient of correlation (PCC) for co-localisation (standard deviation SD and n number of cell) at the mitochondria for Rac1A-mCherry-mito and the GFP-fusions: CRIB-PBD 0.80 (SD: 0.20 – n=6 cells) ; CYRI^{WT} 0.77 (SD: 0.21 – n=8 cells) ; CYRI$^{R155/156D}$ 0.05 (SD: 0.12 – n=14 cells). The correlation coefficients for Rac1A-mCherry and the GFP-CYRI-mito fusions were: CRIB-PBD 0.33 (SD: 0.12 – n=6 cells) ; CYRI^{WT} 0.44 (SD: 0.19 – n=12 cells) ; CYRI$^{R155/156D}$ -0.23 (SD: 0.05 – n=6). Cells co-expressing a mitochondrial reporter (mCherry-gemA tail) and CYRI-GFP were also imaged (right panel), confirming the absence of mitochondrial localisation of CYRI. The correlation coefficient was -0.06 (SD: 0.15 – n=6 cells).
(For all experiments, >300 mitochondria/cell). Scale bar = 5 μm
(See also **Supplementary Fig. 7**)

Data in a-j represent at least 3 biologically independent experiments. Bar and scatter plots show data points with mean and S.E.M. unless indicated. Source data in **Supplementary Table 6**.

Figure 3 - Loss of CYRI-B increases Rac1-mediated Scar/WAVE localisation to lamellipodia

a-d - Immunofluorescence of control (Ctr) or *cyri-b* knockdown (siRNA #1 and 2) COS-7 showing WAVE2 (green), nuclei (blue) and F-actin (magenta). Scale bar = 50 μm. Box insets zoom, scale bar = 10 μm.
Ratio of WAVE2 (yellow dotted line) *vs* total cell perimeter (**b**). Cell area in (**c**) and circularity (**d**). One-way ANOVA with Dunn's post-test n.s. $p > 0.05$, *** $p \leq 0.001$. (**a-c:** Scramble n=111; #1 n=95; #2 n=96 – **d**: Scramble n=115; #1 n=92; #2 n=98) n represents cells in a-o.

e-f –COS-7 with *cyri-b* knockdown and rescued with pLIX-mVenus si-resistant CYRI-B (WT or R160/161D) or empty vector (EV). (see **Supplementary Fig. 3l**). Cell area (**e**) and circularity (**f**). One-way ANOVA with Dunn's post-test n.s. $p > 0.05$, *** $p \leq 0.001$. (Scramble/EV n=78; Scramble/WT n=58; Scramble/R160-161D n=66; #1/EV n=66; #1/WT n=64; #1/R160-161D n=60).

g-h – Control or *cyri-b* knockdown COS-7 with pLIX-mVenus and si-resistant CYRI-B (WT or G2A mutant) or EV. Cell area (**g**) and circularity (**h**). One-way ANOVA with Dunn's post-test n.s. $p > 0.05$, *** $p \leq 0.001$. (Scramble/EV n=70; Scramble/WT n=52; Scramble/G2A n=46; #1/EV n=63; #1/WT n=64; #1/G2A n=65)

i-k - Control (DMSO) or *rac1* knockout (OHT) mouse tail fibroblasts with Scramble (siCtr) or *Cyri-B* siRNA, showing WAVE2 (**i**). Scale bar = 50 μm. Lamellipodial WAVE2 (**j**) and circularity (**k**). One-way ANOVA with Dunn's post-test *** $p \leq 0.001$. two-tailed Mann Whitney test between OHT and control. ### $p \leq 0.001$. (n=30 cells/condition).

l-m - FLIM/FRET of mTq2-sREACH in control (siCtr) or *cyri-b* knockdown (siCYRI-B #1 and #2) COS-7. Jet2 color code (left) average lifetime, 1-4 ns blue to red. (**l**). FRET efficiency (**m**). One-way ANOVA with Dunn's post-test. *** $p \leq 0.001$. (Scramble n=61; #1 n=61, #2 n=63)
Scale bar = 50 μm

n-o - Active Rac1 pulldown comparing control CrispR ($Vector^{Ctr}$) or independent *cyri-b* CrispR (#1 and #2) COS-7 lines. See also Supplementary Fig. 7.

Data in a-o represent N= 3 biologically independent experiments. All cells plated on laminin. See also Supplementary Table 6.
Bar and scatter plots show data points with mean and S.E.M.
Whisker plots show 10-90 percentile, median (bar) and mean (cross).

Figure 4 - Overexpression of CYRI-B opposes Rac1-mediated Scar/WAVE recruitment to the leading edge

a-d - Immunofluorescence of doxycycline-induced control empty vector (EV) or CYRI-B overexpression in COS-7 cells and fixed after 4h showing WAVE2 (magenta), nuclei (blue) and GFP (green). Scale bar = 50 μm. Insets show zoom of white dashed field. Scale bar = 10 μm (**a**). WAVE2 ratio and circularity in (**b**) and (**c**) respectively. Cell area quantification was based on phalloidin staining (**d**). Two-tailed Mann-Whitney test *** $p \leq 0.001$. (Dox/EV n=73; Dox/CYRI-B n=93) n represents cells in a-i

e-f - FLIM/FRET experiment with mTq2-sREACH Raichu Rac1 showing vehicle or doxycycline-treated COS-7 cells expressing a control empty vector (EV) or CYRI-B. The jet2 color code (bar at top) shows the average lifetime of the probe, spanning 1-4 ns (blue to red) (**e**). Quantification of the FRET efficiency (**f**). Two-tailed Mann-Whitney test n.s. $p > 0.05$, *** $p \leq 0.001$. (Veh/EV n=47; Veh/CYRI-B n=46; Dox/EV n=62; Dox/CYRI-B n=62)
Scale bar = 50 μm.

g - FRET efficiency obtained from control (EV) or COS-7 cells overexpressing $CYRI^{WT}$ or $CYRI\text{-}B^{R160/161D}$ after doxycycline induction. One-way ANOVA with Dunn's post-test was performed. n.s. $p > 0.05$, *** $p \leq 0.001$. (EV n=59; WT n=62; R160/161D n=63).

h-i - Immunofluorescence of COS-7 cells transfected with CYRI-B-FLAG and stained for FLAG-tag (green), F-actin (top row) or WAVE2 (bottom row) (magenta) and nuclei (blue). Scale bar = 50 μm (**h**). FLAG-staining is quantified by normalizing the fluorescence intensity running across 17 representative cells and ending at the protrusive end (normalized distance: 1=protrusive end and 0=opposite end). FLAG-tag and F-actin staining intensity are shown in green and magenta respectively (**i**) (n=17).

Data in a-i represent N=3 biologically independent experiments.
See also Supplementary Table 6.
Bar and scatter plots show data points with mean and S.E.M.
Whisker plots show 10-90 percentile, median (bar) and mean (cross).

Figure 5 - CYRI-B controls the duration and extent of Rac1-mediated protrusions

a - Control (Vector ^{Ctr}) and *cyri-b* CrispR knockout CHL-1 cells on laminin expressing GFP-LifeAct, recorded for 3 minutes at 1 frame/sec. The cell periphery (magenta) is tracked using the GFP-LifeAct signal (green) (Left panel). The membrane is unravelled from the orange arrow and a representative polar kymograph of the changes in membrane dynamics over time between control (Vector ^{Ctr} - Top) and *cyri-b* CrispR knockout (Bottom) CHL-1 cells is shown. Membrane extensions (positive values) are visualised in yellow through to orange, while retractions (negative values) are purple-blue (Middle panel). Thresholding of the kymograph to remove noise (values ≥ + 0.6) reveals protrusions over time (white signal – Right panel)
Still from movie S2. Scale bar = 25 μm.

b - Box plot representing the distribution of the average protrusion lifetime for each individual cell. Whisker plots represent mean and S.D. Two-tailed Mann Whitney test. *** $p \leq 0.001$. (n= 20 cells/condition)

c - Schematic representation showing protruding (blue) and retracting (magenta) area following photoactivation of Rac1-LOV probe. Photo activation area (green circle) was used as the origin to measure the maximal protrusion distance (outward - black line) and the longest uninterrupted lateral spread of the protrusion (red dotted line)

d - Still pictures from videos of photoactivation time course showing selected cells from DMSO (Control) or OHT-treated (knockout) immortalized CRE-ER^{T2+} *Cyri-B*$^{fl/fl}$ MEFs on fibronectin. Endpoint overlay as from schematic (**c**). Scale bar = 25 μm.

e-f - Quantification of the protrusion distance (**e**) and the spread of activation (**f**) between control (DMSO) or *cyri-b* knockout (OHT) MEFs.
Error bars represent 95% CI. Unpaired two-tailed t-test (**e**) and two-tailed Mann-Whitney test (**f**). *** $p \leq 0.001$, **** $p \leq 0.0001$. (DMSO n=29 cells; OHT n=30 cells).

g - Kymograph representation before and after photo activation. Membrane extensions are visualised in yellow through to orange, while retractions are observed in purple-blue. Time of photoactivation is highlighted by a white dotted line.

Data in a-g represent N=3 biologically independent experiments.
See also Supplementary Table 6.

Figure 6 - CYRI proteins mediate plasticity of protrusions needed for directional migration

a-c - Spider plots of control (Vector ^{Ctr}) or *cyri-b* CrispR knockout (#1 and #2) CHL-1 cells on collagen-I for 17h (**a**) (See **movie S4)**. Black and red lines = distance > or <100 µm respectively. Average speed (**b)**. Whisker plots 10-90 percentile and mean (cross). **(c**)– Duration as C-shape (Vector ^{Ctr}) and *cyri-b* CrispR knockout (#1 and #2) CHL-1 cells. **(b,c)** One-way ANOVA with Dunn's post-test *** $p \leq 0.001$. **(b)** (Ctr n=161; #1 n=228; #2 n=178). **(c)** (Ctr n=45; #1 n=53; #2 n=42). n represents cells in a-p.

d - Speed of control (Vector ^{Ctr}) or *cyri-b* CrispR knockout (#1 and #2) CHL-1 in shapes as indicated. One-way ANOVA with Dunn's post-test n.s. $p>0.05$, * $p \leq 0.05$, *** $p \leq 0.001$. (Ctr n=45; #1 n=53; #2 n=42)

e-f - Immunofluorescence of control (Vector ^{Ctr}) or *cyri-b* CrispR knockout (#1 and #2) CHL-1 cells on collagen. F-actin (magenta) and nuclei (blue) (**e**). Scale bar = 50 µm. Shape categories: Fried-egg, C-shape, Random (**f**). Two-tailed Chi-square test (95% confidence). *** $p \leq 0.001$. (Ctr n=276; #1 n=216; #2 n=210)

g-i - Spider and Rose plots of control (Vector ^{Ctr}) or *cyri-b* knockout WM852 cells migrating toward 10% FBS (**g**) (see **movie S5**). Red-dashed lines 95% confidence interval for mean direction. Cosθ (chemotactic index) (**h**) average speed (**i**). Two-tailed unpaired t-test. n.s. $p>0.05$, ** $p \leq 0.01$. (Ctr n=129; #1 n=132; #2 n=151).

j-n - DIC pictures from *Dictyostelium* under-agarose chemotaxis (**j**) (see **movie S6**). Scale bar = 10 µm. Automatic cell segmentation and tracking reveals circularity (**k**), protrusions (**l**), split frequency (**m**), and speed (**n**). Whisker plots show 10-90 percentile (**k-m**) and 1-99 percentile (**n**) with median (bar) and mean (cross). One-way ANOVA with Dunn's post-test. n.s. $p>0.05$, * $p \leq 0.05$, *** $p \leq 0.001$. (**k**: WT n=360; *cyri* KO n=352; *cyri* KO + CYRI ^{WT} n=480; *cyri* KO + CYRI $^{R155/156}$ n=240 - **l**: WT n=45; *cyri* KO n=57; *cyri* KO + CYRI ^{WT} n=53; *cyri* KO + CYRI $^{R155/156}$ n=31 - **m**: WT n=42; *cyri* KO n=62; *cyri* KO + CYRI ^{WT} n=46; *cyri* KO + CYRI $^{R155/156}$ n=33 - **n**: WT n=2389; *cyri* KO n=2460; *cyri* KO + CYRI ^{WT} n=3024; *cyri* KO + CYRI $^{R155/156}$ n=1169)

o-p - Needle assay using WT or *cyri* knockout Ax3 cells migrating toward cAMP (see also **movie S8**) (yellow start). Scale bar = 25 µm (**o**). Spider plots during 0-100 seconds following needle re-orientation (**p**). (WT n=86; *cyri* KO n=79)

Data in a-p represent N=3 biologically independent experiments.
Bar and scatter plots show data points with mean and S.E.M unless indicated.
See also Supplementary Table 6.

Figure 7 - CYRI-B regulates Rac1-dependent recruitment of Scar/WAVE complex during epithelial cystogenesis

a-b – Immunofluorescence of control (Vector ^{Ctr}) or *cyri-b* shRNA knockdown (#1 and #2) MDCK cysts fixed after 5 days of culture and stained for Podocalyxin (PODXL) (green), F-actin (red) and nuclei (blue). Top row is a confocal section and bottom row represents Z-maximal projection intensity of PODXL staining. Scale bar = 50 µm (**a**). Quantification of lumens in (**b**). One-way ANOVA with Dunn's post-test. *** $p<0.001$. (Ctr n=1000 cysts, #1 n=1000 cysts, #2 n=800 cysts).

c - Immunofluorescence of control (Vector ^{Ctr}) or *cyri-b* shRNA knockdown (#1 and #2) MDCK cysts stained for WAVE2 (green) and Podocalyxin (PODXL) (red) after 5 days of culture. Inverted LUT images, merge and representative surface profile plots shown. PODXL (red) and WAVE2 (green) staining intensity was measured along the blue line. Scale bar = 9 µm. Insets provide a magnified view of the dotted square area. Scale bar = 5 µm.

d-e – Immunofluorescence of control (Vector ^{Ctr}) or *cyri-b* shRNA knockdown (#1 and #2) MDCK cysts grown during 5 days, treated or not with 50 nM EHT1864 and stained for Podocalyxin (PODXL). Pictures represent the Z-maximal projection intensity from a representative z-stack running across the entire cyst volume. Scale bar = 50 µm (**d**). Number of lumens per cyst was quantified for vehicle or EHT1864-treated cysts and plotted in (**e**). One-way ANOVA with Dunn's post-test between control (Vector ^{Ctr}), shCYRI-B #1 and shCYRI-B #2 whereas unpaired two-tailed t-test between vehicle and drug-treated cyst. n.s. $p>0.05$, ** $p\leq0.01$ *** $p\leq0.001$. (250 cysts/condition)

Data in a-e represent N=3 biologically independent experiments.
Bar and scatter plots show data points with mean and S.E.M.
Whisker plots show 10-90 percentile, median (bar) and mean (cross).
See also Supplementary Table 6.

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