A tunable LIC1-adaptor interaction modulates dynein activity in a cargo-specific manner

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Cytoplasmic dynein-1 (dynein) is the motor responsible for most retrograde transport of cargoes along microtubules in eukaryotic cells, including organelles, mRNA and viruses. Cargo selectivity and activation of processive motility depend on a group of so-called “activating adaptors” that link dynein to its general cofactor, dynactin, and cargoes. The mechanism by which these adaptors regulate dynein transport is poorly understood. Here, based on crystal structures, quantitative binding studies, and in vitro motility assays, we show that BICD2, CRACR2a, and HOOK3, representing three subfamilies of unrelated adaptors, interact with the same amphipathic helix of the dynein light intermediate chain-1 (LIC1).

While the hydrophobic character of the interaction is conserved, the three adaptor subfamilies use different folds (coiled-coil, EF-hand, HOOK domain) and different surface contacts to bind the LIC1 helix with affinities ranging from 1.5 to 15.0 μM. We propose that a tunable LIC1-adaptor interaction modulates dynein’s motility in a cargo-specific manner.

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In eukaryotic cells, a single motor, cytoplasmic dynein-1 (dynein), carries out the majority of microtubule-based retrograde transport of cargoes, including membranous organelles, protein complexes, mRNA, and virions. By itself, mammalian dynein adopts an autoinhibited so-called “Phi-particle” conformation that lowers its affinity for microtubules and inhibits processive motility. Dynein is activated for long-distance processive movement along microtubules through interactions with its general cofactor, dynactin, and one of many so-called “activating adaptors.” The activating adaptors (hereafter referred to as adaptors) fulfill multiple roles; they mediate key interactions that stabilize the dynein–dynactin complex, recruit cargoes, and regulate dynein’s activity and processive motility. Dynein is activated for long-distance processive movement along microtubules through interactions with its general cofactor, dynactin, and one of many so-called “activating adaptors.”

Results

LIC1 interaction with CC1-box-containing dynein–dynactin adaptors. A subfamily of dynein–dynactin adaptors, including BICD1–2 and BICDL1–2, Spindly, TRAK1–2 and HAP1, share a coiled-coil segment known as the CC1-box that has been directly implicated in LIC1 binding for some of these proteins and Spindly. We previously found that fragments encompassing the CC1-box of two of these proteins, BICD2–98 and Spindly1–142, were dimeric and bound two LIC1 helices (LIC1433–458) with dissociation constants in the low-micromolar range. Here, we set out to determine the structural basis of this interaction. A 2.4-Å resolution structure of human BICD2–98 in complex with human LIC1433–458 was determined ab initio using the single-wavelength anomalous dispersion method from crystals of selenomethionine-substituted BICD2–98 (Table 1). The electron density map reveals residues P4–A81 of one chain and L13–E80 of the other chain of BICD2, and residues E441–L452 of a single LIC1 helix (Fig. 1a, b). The uninterpretable coiled-coil segment of BICD2 comprises residues P19–L78, displaying the characteristic heptad repeat (Fig. 1c). Coiled-coil prediction programs also suggest a break of the coiled-coil structure around P19 and E80, consistent with the presence of typical helix-breaking amino acids at these two locations (P19 and G83). At the N-terminus, one of the BICD2 chains is disordered, whereas the other chain contains an additional helix (residues E6–E16) that runs antiparallel to the coiled-coil helices. This type of fold is conserved, multi-subunit complexes. The adaptors, in contrast, are generally unrelated, although they all contain long regions of coiled-coil structure and form parallel dimers. The sequence variability of the adaptors is important in at least two ways: (a) through their variable C-terminal regions, different adaptors appear to link dynein–dynactin to different cargoes; (b) through their variable N-terminal regions, the adaptors seem to mediate the speed, force and processivity of the dynein motor in different ways.

For some of the adaptors, including BICD2, BICDL1, and HOOK3, cryo-EM structures show that a ~350 Å coiled-coil segment runs parallel to the dynactin filament of actin-related subunits and provides part of the binding interface for the dynein tail. By itself, the otherwise unstructured and poorly conserved C-terminal region of coiled-coil, pairs of EF-hands or the HOOK domain. The fact that this interaction appears to be conserved on the LIC1 side but the LIC1 interaction was confirmed in the crystal structure, as well as in binding studies and in vitro motility assays. In contrast, other EF-hand containing adaptors (FIP3 and NIN) bind the LIC1 helix in a Ca2+–independent manner. Together, the results reveal a tunable LIC1–adapter interaction that may modulate the force, speed, and processivity of the dynein motor.

Table 1 Data collection and refinement statistics.

| Data collection (Se-Met derivative) | BICD2–LIC1 complexa | CRACR2a–LIC1 complexa |
|-----------------------------------|---------------------|----------------------|
| Wavelength (Å)                    | 0.9775              | 0.979                |
| X-ray source                     | MacCHESS beamline F1| SSRL beamline 14-1   |
| Space group                      | P 21, 21, 21        | P 21, 21, 21         |
| Cell dimensions                  | a, b, c (Å)         | 54.77, 61.84, 81.68  |
|                                   | α, β, γ (°)         | 90.0, 90.0, 90.0     |
| Resolution (Å)                   | 49.04–2.40          | 43.35–2.66           |
| Rmax (%), Rref (%)                | (2.95–2.90)         | (2.75–2.95)          |
| Rfree (%)                        | 0.109 (0.785)       | 0.359 (0.953)        |
| Rmerge (%)                       | 0.033 (0.289)       | 0.114 (0.330)        |
| L(c) (Å)                         | 12.0 (1.8)          | 7.2 (3.0)            |
| No. of reflections               | 218993 (8766)       | 303250 (27481)       |
| No. of unique reflections        | 19602 (1396)        | 28181 (2798)         |
| Completeness (%)                 | 94.9 (67.1)         | 99.9 (100.0)         |
| Redundancy                       | 11.2 (6.4)          | 10.8 (9.6)           |
| Wilson B-factor (A2)             | 35.8                | 33.3                 |
| Rmerge (%)                       | 0.99 (0.76)         | 0.96 (0.44)          |
| Overall d_min (Å)                | 2.40                | 2.50                 |
| d_min along a*, b*, c* (Å)       | 2.88, 2.57, 2.47    | 2.52, 3.00, 2.50     |
| Refinement Resolution (Å)        | 34.08–2.40          | 43.35–2.66           |
| (2.49–2.40)                      | (2.73–2.66)         |
| No. of unique reflections        | 14,423 (907)        | 23,908 (980)         |
| Completeness (%)                 | 69.2 (23.0)         | 94.5 (50.6)          |
| Rmerge/Rfree (%)                 | 19.2 (26.8)/24.6 (32.5) | 19.7 (25.5)/23.2 (30.6) |
| No. of atoms                     | Protein             | 1309                 |
|                                  | Ca2+                | 8                    |
|                                  | Water               | 42                   |
| B-factors (A2)                   | Protein             | 49.8                 |
|                                  | Ca2+                | 49.5                 |
|                                  | Water               | 42.1                 |
| R.m.s. deviations               | Bond lengths (Å)    | 0.008                |
|                                  | Bond angles (°)     | 0.98                 |
|                                  | Ramachandran (%)    | 59.4                 |
|                                  | Favorited           | 96.0                 |
|                                  | Outliers            | 0.6                  |
|                                  | PDB code            | 6PSE                 |

*Anisotropy data correction with HKL2000 eliminated weak, unreliable reflections, while reducing the completeness of the data used in refinement.
**Fig. 1 LIC1 interaction with CC1-box-containing dynein-dynactin adaptors.** a Domain organization of human LIC1 and BICD2, highlighting the BICD21–98 fragment containing the CC1-box that was co-crystallized with LIC1433–458. b Ribbon diagram showing two perpendicular views of the structure of the complex of BICD21–98 (coiled-coil chains A and B are colored magenta and pink, respectively) with LIC1433–458 (blue). The side chains of LIC1433–458 are shown using a stick representation and colored by atom type. A symmetry-related BICD21–98 molecule (gray) occludes the second LIC1433–458 binding site. c Helical-wheel representation of the interaction of LIC1433–458 with the CC1-box motif of BICD2. d, e Close-up views showing the interactions of LIC1433–458 and a symmetry-related molecule on opposite sides of the BICD2 coiled-coil. The 2Fo–Fc electron density map (blue mesh) contoured at 1.0σ is shown around an all-atom representation of the LIC1 helix. Note that residues on the hydrophobic face of the LIC1 helix insert into a hydrophobic pocket formed at the interface of the two BICD2 chains. BICD2 residue Y46, which was mutated in this study, is shown in green. f ITC titrations of LIC1433–458 into the indicated heterodimers of WT and mutant Y46D BICD2 obtained by fusing a ZIP+/ZIP– leucine zipper C-terminally to the BICD2 constructs (see also Supplementary Fig. 1). ITC data shown are representative of n = 3 independent experiments, with similar results. Listed for each titration are the experimental conditions and fitting parameters, including the dissociation constant (K<sub>D</sub>) and binding stoichiometry (N) derived from fitting to a binding isotherm (black line). Open symbols correspond to a control titration into buffer, shown for the first eight injections of the first experiment, since all the proteins were dialyzed in parallel. The K<sub>D</sub> and N values are given as mean ± s.d. of the fits.
somewhat reminiscent of the Rab-interacting lysosomal protein (RILP) homology-1 (RH1) domain in the structure of RILP-L2 (RILP-like protein 2) bound to the globular tail domain of myosin-V21. Both structures consist of a central coiled-coil preceded by a helix that runs antiparallel to the coiled-coil. As its name indicates, RILP-L2 is related to RILP, which together with JIP1–4 (JNK-interacting proteins 1–4) forms part of a subfamily of RH1 domain-containing proteins22 that has been implicated in interactions with the C-terminal region of LIC117,23, although these proteins are not confirmed dynein–dynactin adaptors3.

The LIC1 helix binds near the middle of the BICD2 coiled-coil segment and is oriented diagonally with respect to the axis of the coiled-coil (Fig. 1b). Like in the structure of the LIC1 helix bound to the hook domain18, residues L44, F447, F448, and L451 on the hydrophobic face of this helix insert into a hydrophobic pocket formed at the interface between the two chains of BICD2 (Fig. 1c–e). Critical to the formation of this pocket is the presence of highly conserved alanine and glycine residues in the CC1-box that, based on the structure, can be more precisely defined as consisting of human BICD2 residues 339EKIQAAEVGCVQVLE353 (where, bold letters indicate residue conservation ≥ 50% among 100 CC1-box-containing sequences, Supplementary Fig. 1a). The sequence of the CC1-box is atypical for a coiled-coil structure. In their unusually small size for these positions, they help create a cavity for the binding of the LIC1 helix (Fig. 1c, e).

The presence of a single LIC1 helix bound to BICD2 in the structure is surprising (Fig. 1b, d), since previous analysis suggested two binding sites18. However, the second LIC1-binding site, predicted to be symmetrically positioned on the opposite side of the BICD2 coiled-coil from the one observed in the structure, is masked by a crystal packing contact (Fig. 1b, e). Therefore, to conclusively answer whether BICD2 can simultaneously bind two LIC1 helices we made BICD2 heterodimers, consisting of different combinations of a WT subunit and a subunit carrying the mutation Y46D in the middle of the CC1-box, which is predicted to disrupt LIC1 binding (Fig. 1e). To ensure homogeneous heterodimer formation during coexpression, we appended a heterodimerizing leucine zipper (ZIP+)–ZIP−24 C-terminally to the BICD2 constructs (Supplementary Fig. 1b). Heterodimer formation was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and light scattering analysis (Supplementary Fig. 1c, d). Three variants of this construct were made, each a half-functional heterodimer in which only one BICD2 chain was mutated (Y46DZIP+/-WTZIP−) and two controls, Y46DZIP+/-Y46DZIP− and WTZIP+/WTZIP−, which should not bind the LIC1 helix or bind two helices, respectively. Binding was quantitatively assessed using ITC. Consistent with our previous observations18, the titration of LIC1433–458 into WTZIP+/WTZIP− produced an exothermic reaction that fitted best to a two-site binding isotherm (N = 2.0) with Kd ~ 6.8 μM for each of the sites (Fig. 1f and Supplementary Fig. 1e). As anticipated, the titration of LIC1433–458 into Y46DZIP+/Y46DZIP− was indistinguishable from a control titration into buffer, i.e., no heat of exchange was observed, indicating lack of binding. In contrast, Y46DZIP+/-WTZIP− bound a single LIC1 helix (N = 1.1) with Kd ~ 6.2 μM (Fig. 1f). These results conclusively show that BICD2 and likely other CC1-box-containing adaptors have the ability to interact with two LIC1 helices and with similar low-micromolar affinities.

**LIC1 interaction with EF-hand-containing dynein–dynactin adaptors.** CRACR2a (calcium release-activated calcium channel regulator 2A), Rab45 (Ras-related protein Rab-45), FIP3 (Rab11 family-interacting protein 3), and NIN (Ninein) form part of a subfamily of dynein–dynactin adaptors characterized by the presence of EF-hand domains3,8,10 (Fig. 2a and Supplementary Table 1). In pull-down experiments, FIP3 and NIN have been shown to interact with the LIC1 helix19, although the interaction was not specifically mapped to the EF-hand domains. Yet, for at least one of these adaptors, CRACR2a, the ability to activate processive dynein–dynactin motility is regulated by Ca2++19, which indirectly implicates the EF-hand domains. The EF-hand domain is a protein–protein interaction module that almost invariably functions as pairs of EF-hand domains and is often, albeit not always, regulated by Ca2+ binding to either one or both EF-hands in a pair23. We set out to directly test whether the LIC1 helix binds to the EF-hand domains of CRACR2a, FIP3 and NIN, and whether Ca2+ regulates the interactions.

CRACR2a and FIP3 each contains a single EF-hand pair, whereas NIN contains two EF-hand pairs in addition to a single EF-hand domain that precedes the first coiled-coil segment (Fig. 2a). Therefore, in these experiments we used constructs comprising individual EF-hand pairs of the three proteins (MBP-CRACR2a47–122, FIP3206–270, and NIN180–350) encompassing the last three EF-hand domains of NIN. Binding of these adaptors to either full-length LIC1 (construct MBP-LIC1FL) or the LIC1 helix (LIC1433–458) was measured using ITC, both in the presence and the absence of CaCl2. CRACR2a47–122 has low UV-absorbance, and thus to facilitate the determination of accurate protein concentrations and stoichiometries for fitting of the ITC experiments the N-terminal maltose-binding protein (MBP) tag was not removed. To maximize the heat of injection signal of each titration and thus the accuracy of the fits, the temperature was set to either 20 or 30 °C.

In the presence of 5 mM CaCl2, MBP-CRACR2a47–122, FIP3206–270, and NIN1–87 bound MBP-LIC1FL with low-micromolar affinities (Kd ~ 3.6, 6.1, and 12.0 μM, respectively) and ~1:1 stoichiometry (Fig. 2b). In contrast, in the presence of 5 mM EGTA (a Ca2+–chelator) MBP-CRACR2a47–122 failed to bind MBP-LIC1FL, whereas the binding of FIP3206–270 and NIN1–87 was mostly unaffected (Fig. 2c). We separately confirmed that Ca2+ does not bind to MBP (Supplementary Fig. 2a), such that the Ca2+-dependence of the interaction between MBP-CRACR2a47–122 and MBP-LIC1FL must be contained within the EF-hands of CRACR2a47–122. We also established that FIP3206–270 and NIN1–87 are properly folded in the absence of Ca2++, displaying circular dichroism spectra characteristic of a-helical structures, with minima around 222 and 208 nm and no evidence of unfolding (Supplementary Fig. 2b). Finally, NIN180–350 did not bind MBP-LIC1FL, suggesting that the three EF-hand domains contained within this construct are not implicated in the LIC1 interaction (Supplementary Fig. 2c). While Ca2+ is present during expression/purification, neither CaCl2 nor EGTA were added in the latter experiment, since the EF-hands of NIN180–350 lack key residues necessary for Ca2+ binding (see below).

For other subfamilies of adaptors, we have found that the amphipathic helix LIC1433–458 fully accounts for the LIC1-adaptor interaction (Fig. 1f)18. Consistently the three EF-hand-containing adaptors analyzed here bound this helix with the same stoichiometry and somewhat higher affinity as full-length LIC1 (Fig. 2d). The LIC1 helix may be partially occluded within construct MBP-LIC1FL, which could explain why the affinity of the isolated helix is consistently higher for the three adaptors. The affinities of Ca2+–CRACR2a (Kd ~ 1.4 μM), FIP3 (Kd ~ 2.0 μM) and NIN (Kd ~ 5.4 μM) for the LIC1 helix fall within the same range as those previously measured by us for BICD2 (Kd ~ 1.5–7.5 μM), Spindly (Kd ~ 3.5–7.6 μM), HOOK1 (Kd ~ 15.7 μM), and HOOK3 (Kd ~ 6.3 μM)18.
Fig. 2 LIC1 interaction with EF-hand-containing dynein-dynactin adaptors.  

**a** Domain organization of EF-hand-containing adaptors, highlighting the EF-hand pair of CRACR2a that was co-crystallized with LIC1\textsubscript{433–458}.  

**b–d** ITC titrations of EF-hand pairs from human CRACR2a, FIP3 and NIN into MBP-LIC1\textsubscript{TL} or LIC1\textsubscript{433–458} and in the presence of 5 mM CaCl\textsubscript{2} or 5 mM EGTA (as indicated). ITC data shown are representative of \( n = 2 \) independent experiments, with similar results. Listed for each titration are the experimental conditions and fitting parameters, including the dissociation constant (\( K_D \)) and binding stoichiometry (\( N \)) derived from fitting to a binding isotherm (black line). The \( K_D \) and \( N \) values are given as mean ± s.d. of the fits. Open symbols correspond to a control titration into buffer.
These results suggest that the EF-hand pair of CRACR2a is regulated by Ca\(^{2+}\), whereas those of FIP3 and NIN are not. To further explore the Ca\(^{2+}\)-dependence of the LIC1 interactions we assessed the ability of these EF-hand pairs to bind Ca\(^{2+}\) by ITC. The titration of Ca\(^{2+}\) into MBP-CRACR2a\(_{47-122}\) revealed a single, relatively low-affinity Ca\(^{2+}\)-binding site \(N \approx 0.90\); \(K_D \approx 14.7\) mM, whereas FIP3\(_{206-270}\) and NIN\(_{2-87}\) did not bind Ca\(^{2+}\) (Fig. 3a). To understand the source of these differences, we compared the sequences of the EF-hands of these three proteins to those of 3071 EF-hand domains from known Ca\(^{2+}\)-binding proteins (Fig. 3b). This analysis shows that amino acids crucial for Ca\(^{2+}\)-binding in these proteins (conservation score \(\geq 0.5\)) are conserved in the second EF-hand of CRACR2a and in both EF-hands of Rab45, but several are mutated in the first EF-hand of CRACR2a and in the EF-hands of FIP3 and NIN (Fig. 3b).

Crystal structure of the EF-hand pair of CRACR2a in complex with the LIC1 helix. A crystal structure of CRACR2a\(_{47-122}\) (MBP removed) in complex with LIC1\(_{433-458}\) was obtained at 2.66 Å resolution (Fig. 4a and Table 1). The structure was determined ab initio using the single-wavelength anomalous dispersion method from crystals of selenomethionine-substituted CRACR2a\(_{47-122}\). The monoclinic crystals, belonging to space group P2\(_1\), contain eight complexes in the asymmetric unit. Two of the complexes are poorly defined in the electron density map due to higher mobility, yet eightfold non-crystallographic symmetry averaging was used throughout the initial steps of the refinement, yielding well-defined electron density for CRACR2a residues G47–Q120 and LIC1 residues T439–K454 (Fig. 4b, c). The non-crystallographic symmetry restraints were then relaxed toward the end of the refinement, resulting in RMSDs of <0.4 Å for equivalent Ca atoms among the eight complexes in the asymmetric unit (Supplementary Fig. 3a).

The two EF-hands of CRACR2a display a canonical fold, consisting of an incoming helix (helices a1 and a3), loop and exiting helix (helices a2 and a4) (Figs. 3b and 4a). As indicated by the ITC titration (Fig. 3a), the structure reveals a single Ca\(^{2+}\)-binding site. Consistent with the sequence analysis (Fig. 3b), the Ca\(^{2+}\)-density is observed within the second EF-hand domain of all the complexes of the asymmetric unit (Fig. 4d), whereas none of the complexes displays Ca\(^{2+}\)-density within the first EF-hand (Supplementary Fig. 3b). The conformation and Ca\(^{2+}\)-coordination of the second EF-hand of CRACR2a is similar to those of troponin C and calmodulin (Fig. 4d). Ca\(^{2+}\) is most commonly coordinated by six ligands that form a sevenfold, pentagonal–bipyramidal ligation network around the ion, with bond distances ranging from 2.3 to 2.6 Å. In CRACR2a, Ca\(^{2+}\) is ligated by the side chains of D97, D99, N101, E108, the main-chain carbonyl oxygen of Y103 and a water molecule that is in turn coordinated by T105 (Fig. 4d). In the traditional Ca\(^{2+}\)-binding loop nomenclature, these amino acids occupy positions X (D97), Y (D99), Z (N101), −Y (Y103), −X (T105), and −Z (E108) (Fig. 3b). Like in other EF-hands that bind Ca\(^{2+}\), the amino acid at position −Z coordinates the Ca\(^{2+}\) in a bidentate fashion to complete the sevenfold ligation network. To understand why certain residues are allowed at specific positions of the Ca\(^{2+}\)-binding loop while others are not, we need to analyze the structures. Thus, positions Y and Z can be either D, N, or S because these positions contribute a single side-chain oxygen to the interaction with Ca\(^{2+}\) (Figs. 3b and 4d). Similarly, position −X contributes a main chain contact, and accordingly almost any amino acid is allowed at this position. In contrast, only E is allowed at position −Z, and not even D, which has a shorter side chain, can account for this bidentate interaction (see “Discussion”).

Like in other members of the EF-hand family, the target-binding site consists of a hydrophobic cleft formed between the incoming and exiting helices of the two EF-hand domains. The hydrophobic face of the LIC1 helix binds in this cleft (Fig. 4a–c), and the same conformation is observed in all eight complexes of the asymmetric unit (Supplementary Fig. 3a). Like in the complexes with the HOOK domain and BICD2 (Fig. 1e), highly conserved, hydrophobic residues of the LIC1 helix (L444, F447, F448, and L451) insert into the hydrophobic cleft formed by the EF-hand pair of CRACR2a (Fig. 4c). Because CRACR2a does not bind LIC1 in the absence of Ca\(^{2+}\) (Fig. 2c), it is likely that this cleft only opens in the Ca\(^{2+}\)-bound state, which is a common feature among EF-hand proteins. However, other EF-hands, including those in some myosin light chains, open in the presence of a target sequence, without the need for Ca\(^{2+}\) pre-activation, as appears to be the case for FIP3 and NIN. A sequence alignment of EF-hand pairs that either bind or do not bind the LIC1 helix fails to reveal specific amino acids that would be present in one group and not the other, which based on the limited information we have now precludes predicting potential interactions of LIC1 with other EF-hands.

Disrupting the CRACR2a–LIC1 interaction impairs dynein motility in vitro. We disrupted the CRACR2a–LIC1 interaction through mutagenesis to assess its functional importance in CRACR2a-induced dynein–dynactin motility using a single-molecule assay. CRACR2a residue F58 in the hydrophobic cleft that binds the LIC1 helix was mutated to aspartic acid and the mutant’s inability to bind the LIC1 helix in the presence of Ca\(^{2+}\) was confirmed by ITC (Fig. 5a). We then used total internal reflection fluorescence (TIRF) microscopy to track the movement of CRACR2a–dynein–dynactin complexes from lysates of cells expressing either wild-type (WT) or mutant F58D TMR-labeled Halo-CRACR2a and in the presence of 2 μM free Ca\(^{2+}\) or 2 mM EGTA (Fig. 5b and Supplementary Fig. 4). Polarity-marked dynamic microtubules (HiLyte Fluor 488-labeled microtubules growing from HiLyte Fluor 647-labeled seeds) were used in these experiments to ensure only retrograde, dynein-driven runs (accounting for ~95% of all the runs) were analyzed (Supplementary Fig. 4c). For WT CRACR2a, the number of processive motile events, defined as runs of ≥1.0 μm, was significantly higher with than without calcium, and the F58D mutation reduced the number of runs to that seen with WT CRACR2a in the absence of calcium (Fig. 5c). No runs were observed when HaloTag was expressed alone as a negative control (Supplementary Fig. 4d). In the presence of 2 μM free Ca\(^{2+}\), motile events with WT CRACR2a had a mean velocity of 2 μm s\(^{-1}\) (Fig. 5d), consistent with in vivo measurements of dynein velocity. The limited number of runs observed with the mutant in the presence of calcium displayed a similar mean velocity (Fig. 5d). However, motile events were significantly shorter with the mutant than with WT CRACR2a (Fig. 5e). These results are generally consistent with a recent study and confirm the importance of Ca\(^{2+}\)-dependence of the CRACR2a–LIC1 interaction for dynein–dynactin processive motility.

Discussion
Contrary to kinesins that form a large superfamily with specialized functions and dedicated cargoes, long-range retrograde transport of most cargoes along microtubules, including RNAs, cellular organelles, and viruses, is driven by a single motor, dynein. For dynein, regulation of the motor’s activity depends on numerous intramolecular and intermolecular factors, including posttranslational modification of the microtubule track.
microtubule-associated proteins, cargo binding, auto-inhibitory interactions, and dynein-binding proteins such as LIS1 and NudE/NudEL. In addition, a large family of activating adaptors provides a physical link with specific cargoes and dynein's dedicated cofactor, dynein, while also contributing to activation of processive motility (Fig. 6a–c and Supplementary Table 1). Although these adaptors invariably contain long regions of coiled-coil structure, and thus form parallel
Dimers, they are generally unrelated at the sequence level. Because folds and mechanisms are typically more highly conserved than sequences, the current study of BICD2 and CRACR2a, together with our previous analysis of HOOK-family members and cryo-EM investigations of adaptor–dynein–dynactin complexes, allow us to draw some general conclusions about the mechanism of action of the adaptor family.

It is informative to use the interaction with dynactin as a reference to compare the various adaptors. Dynactin consists of a central actin-like filament, containing eight actin-related proteins (Arp1) subunits and one β-actin subunit, and capped at the barbed end by a CapZβ heterodimer and at the pointed end by a complex of four subunits (Arp11, p62, p25, and p27). Three other subunits (p150Glue/p135, p50 and p24) form the so-called shoulder domain, with their C-termini directed toward the pointed end of the dynactin filament. This structure is often used as a reference to map their interactions with dynactin. Less is known about the sequences C-terminal to the Spindly motif, which diverge the most among the adaptors and are thought to link dynein-dynactin directly or indirectly to specific cargoes. Some adaptors contain a short sequence known as the Spindly motif, which is conserved among most (possibly all) the adaptors have a short sequence known as the Spindly motif, which contacts subunits p25/p27 at the pointed end of the dynactin complex. The Spindly motif appears to be the sole element of the sequence conserved among most (possibly all) the adaptors (Fig. 6a–d and Supplementary Fig. 5), providing a useful reference to map their interactions with dynactin. Less is known about the sequence C-terminal to the Spindly motif, which diverge the most among the adaptors and are thought to link dynein-dynactin directly or indirectly to specific cargoes (Supplementary Table 1), somewhat analogous to the variable tail domains of the kinesin family.

Dynein–dynactin interactions mediated by the Spindly motif and the coiled-coil segment of the adaptors are insufficient to promote the activation of dynein’s motility, i.e., the transition from the auto-inhibited Phi-particle conformation to the open and parallel conformations that have higher affinity for both microtubules and dynactin. An additional layer of regulation involves the interaction between a conserved amphipathic helix within the low-complexity C-terminal region of LIC1 and structurally diverse domains N-terminal to the dynactin-binding coiled-coil segment of the adaptors. Here, based on structural and biochemical analysis of this interaction, we classify the adaptors into three subfamilies: CCI-box-, EF-hand-, and HOOK domain-containing adaptors (Fig. 6a–c and Supplementary Table 1).

Interestingly, while the LIC1–adapter interaction has a conserved hydrophobic character and is conserved on the LIC1 side, it is notably variable on the side of the adaptors (Supplementary Fig. 5).

**Fig. 4 Crystal structure of the EF-hand pair of CRACR2a in complex with LIC1433-458.** a Ribbon diagram and surface representation of the structure of the complex of human CRACR2a (green) with LIC1433-458 (blue). The side chains of LIC1433-458 are shown using a stick representation and colored by atom type. b, c Close-up views showing the binding of LIC1433-458 in a hydrophobic cleft formed between the incoming (α1 and α2) and exiting (α3) helices of the two EF-hands. The 2Fo −Fc electron density map (blue mesh) contoured at 1.0σ is shown around an all-atom representation of the LIC1 helix. Note that residues on the hydrophobic face of the LIC1 helix insert into the hydrophobic cleft formed by the EF-hand pair (side chains colored gray). d Close-up view of the Ca2+-binding loop of human CRACR2a (left) compared to the third Ca2+-binding loop of human troponin C (middle, PDB code: 1J1D) and the first Ca2+-binding loop of human calmodulin (right, PDB code: 1CLL). The 2Fo −Fc electron density map (blue mesh) contoured at 2.5σ is shown around the atoms of CRACR2a and a Fo −Fc difference map (green mesh) contoured at 5.0σ is shown around the bound Ca2+ ion.
Fig. 5 Disrupting the CRACR2a–LIC1 interaction impairs dynein motility. a Mutating residues F58 (red) in the hydrophobic cleft of CRACR2a (orange) that bids the LIC1 helix (blue) to aspartic acid inhibits this interaction, as revealed by the ITC titration of CRACR2a(47–122) F58D into MBP-LIC1FL (experimental conditions given in the figure). The lack of change in the heat of injection precludes fitting to a binding isotherm. b Representative kymographs of the dynein–dynactin driven motility of single Halo-CRACR2a-positive particles (WT and mutant F58D CRACR2a) analyzed by TIRF microscopy with either 2 mM free Ca²⁺ (left) or 2 mM EGTA (right). Kymographs are oriented with the + end of the microtubule (MT) on the left (scale bars: horizontal, 3 μm; vertical, 5 s). c Quantitative analysis of the number of processive motile events, determined using the Kruskal–Wallis test, followed by a Dunn’s multiple comparisons test. Bars represent the median and 95% confidence interval. d Velocities of WT or mutant F58D CRACR2a with 2 mM free Ca²⁺, statistically analyzed using an unpaired t test and two-tailed p values were calculated. Data is pooled from three replicates (WT n = 326; F58D n = 95). Bars represent the median and 95% confidence interval; p = 0.0579. e Histogram of run lengths for WT and mutant F58D Halo-CRACR2a-positive particles with 2 mM free Ca²⁺. The data were analyzed using a Mann–Whitney test and two-tailed p values were calculated (**p < 0.0001; n.s., nonsignificant or p > 0.05). The data were pooled from three replicates (WT n = 317; F58D n = 87). Source data for panels b-e are provided in the Source Data file.

Fig. 6). Thus, on the LIC1 side, the interaction always involves the same amphipathic helix (human LIC1 residues 433–458), implying that the interactions of different adaptors are mutually exclusive. Four highly conserved amino acids (L444, F447, F448, and L451) on the hydrophobic face of the LIC1 helix insert into hydrophobic clefts in the adaptors (Fig. 6a–c). On the adaptor-side, however, the interaction not only involves different folds but also substantially different protein-protein contacts (Supplementary Fig. 6). Despite these structural differences, quantitative analysis of the interactions of seven adaptors, representing the three subfamilies, reveals a relatively narrow range of binding affinities for the LIC1 helix, with K_{D,i} ranging from 1.5 to 15.0 μM (Figs. 1f and 2d) [18]. Note, however, that these affinities are those of monomers. Because all the adaptors form coiled coiled dimers, the actual affinities within the context of adaptor–dynein–dynactin complexes are likely enhanced through avidity.

While all the adaptors have the potential to bind two LIC1 helices, it is unclear how adaptors that recruit two dynein dimers use the dimeric character of the LIC1 interaction for activation. Indeed, there is no direct overlap between the crystal structures of adaptor–LIC1 helix complexes and the cryo-EM structures of adaptor–dynein–dynactin complexes [9, 10]. Yet, the cryo-EM structure of BICDL1–dynein–dynactin was determined at higher resolution (3.5 Å), and reveals side chains for a portion of mouse BICDL1 (residues R132–N209), which partially overlaps with the
structure of BICD2 determined here. By aligning the sequences of human BICD2 and mouse BICDL1 for the region N-terminal to the Spindly motif, which share only 16% sequence identity, we were able to superimpose the crystal structure of BICD2-LIC1 onto the cryo-EM structure of BICDL1–dynein–dynactin (Supplementary Fig. 7). The superimposition shows that for CC1-box-containing adaptors the interaction with the LIC1 helix occurs at the level of CapZαβ. Given the distance between the binding site of the LIC1 helix and the Ras GTPase-like domains of the four LIC1 chains bound to the two dynein dimers, the most
likely scenario is that each dynein dimer contributes one LIC1 helix to the interaction. In this way, a single adaptor can simultaneously activate two dynein dimers. Because the separation between the HOOK domain and the Spindly motif is also ~270 amino acids, a very similar scenario can be proposed for most of the HOOK-domain-containing adaptors (note that the location of the Spindly motif in CCDC88B is unclear) (Fig. 6c). The mechanism of activation for EF-hand-containing adaptors is less clear, since there is no apparent consistency in the location of the LIC1-binding EF-hand pair with respect to the Spindly motif among members of this subfamily (Fig. 6b).

Is the adaptor–LIC1 interaction regulated? Because the interaction with the LIC1 helix is primarily hydrophobic, regulation through phosphorylation of residues at the binding interface appears unlikely. Conformational changes in the adaptors appears more likely, and could be triggered by cargo binding at the C-terminus, as reported for BICD258, and analogous to the activation of several myosin and kinesin motors. Only CRACR2a is known to be activated by Ca\(^{2+}\), and to promote processive dynein–dynein motility in a Ca\(^{2+}\)-dependent manner.\(^{10}\) We have shown here that CRACR2a is regulated by Ca\(^{2+}\) binding to a single site within the EF-hand pair, which in turn triggers the binding of the LIC1 helix (Figs. 2–4). Disrupting this interaction through mutagenesis inhibits processive motility (Fig. 5).

Combined these observations conclusively show that the LIC1–adaptor interaction is critical for activation of processive dynein motility, although not always necessary for the formation of a ternary complex with dynein–dyneinactin.\(^{10}\) The Ca\(^{2+}\)-binding affinity of the site in CRACR2a (\(K_D = 14.7 \mu M\)) is comparable to that of the low-affinity sites of the classical calcium sensors calmodulin and skeletal-muscle troponin C. These two proteins contain four Ca\(^{2+}\)-binding EF-hands, divided into two high-affinity sites with dissociation constants in the nanomolar range and two low-affinity sites with dissociation constants in the micromolar range.\(^{25,59,40}\) The high-affinity sites are thought to be filled with Ca\(^{2+}\) (or Mg\(^{2+}\)) under physiological conditions, whereas the low-affinity sites have high specificity for Ca\(^{2+}\) and respond to fluctuations in [Ca\(^{2+}\)] within the cellular milieu. The Ca\(^{2+}\)-binding site of CRACR2a seems to fall into the latter category, and thus may act as a regulatory site in cells. By analogy with troponin C,\(^{39}\) the Ca\(^{2+}\)-binding affinity of the CRACR2a site is expected to increase in the presence of a binding partner, i.e., the LIC1 helix. Our analysis further shows that two other EF-hand-containing adaptors, FIP3 and NIN, are not regulated by Ca\(^{2+}\) and this is due to substitutions of key amino acids within the Ca\(^{2+}\)-binding loop (Fig. 3b). Of particular importance are the amino acids at positions 1 (or X) and 12 (or Z) of the Ca\(^{2+}\)-binding loop, which in EF-hands that bind Ca\(^{2+}\) are almost invariably aspartic acid and glutamic acid. In the rare occasions when the amino acid at position 12 is substituted by asparagine, the cation specificity of the loop can switch from Ca\(^{2+}\) to Mg\(^{2+}\).\(^{26}\) Interestingly, the first EF-hand of FIP3 (FIP3_EFa, residues 202–237) is canonical, except for the presence of aspartic acid at position 12 (Fig. 3b), and thus Mg\(^{2+}\) could possibly regulate FIP3 in cells.

Two other proteins, JIP3 and RILP, contain regions of coiled-coil structure, and RILP has been shown to bind the C-terminal region of LIC1.\(^{17}\) Based on structural similarity, the N-terminal RH1 domain of these proteins may bind the LIC1 helix somewhat analogous to CC1-box-containing adaptors, which could possibly activate dynein. However, JIP3 and RILP seem to lack the Spindly motif and their coiled-coil regions are short, such that they are unlikely to function as bona fide dynein–dyneinactin adaptors. In summary, the structural and functional analysis of dynein–dyneinactin adaptors supports the notion that a tunable LIC1–adaptor interaction may modulate dynein’s motility in a cargo-specific manner.

### Methods

**Proteins.** The cDNAs coding for mouse BICD2 (UniProt ID: Q921C5-1), human FIP3 (UniProt: O73154-1), and human NIN (UniProt ID: Q8N4C6-1) were purchased from Addgene. The cDNAs coding for crystallizable CRACR2a (UniProt ID: Q9BSW2-1) was a gift from Yousang Gwack (UCLA). Primers used in cloning are listed in Supplementary Table 2. Constructs BICD2\(^{1-98}\), CRACR2a\(^{458-722}\), FIP3\(^{206-720}\), NIN\(^{150-356}\), and NIN\(^{112-167}\) were cloned into vector pMAL-c2X (New England Biolabs, Ipswich, MA), using the two different affinity tags (MBP- and His-tag) for equimolar purification of heterodimeric species.

The heterodimeric leucine zipper-BICD2 constructs were expressed in *E. coli* BL21 (DE3) cells (Invitrogen, Carlsbad, CA) and grown in Terrific Broth medium at 37 °C until the OD\(_{600}\) reached a value of 1.5–2, followed by 16 h at 19 °C in the presence of 0.25 mM isopropyl-\(\beta\)-D-thiogalactoside. The cells were collected by centrifugation, resuspended in 20 mM Tris (pH 7.0), 100 mM NaCl, 4 mM benzamidine hydrochloride, 1 mM PMSF, and 1 mM DTT and lysed using a Microfluidizer (Microfluidics, Newton, MA). The proteins were purified through an amylase-affinity purification column according to the manufacturer’s protocol (NEB). The MBP tag was removed by incubation with TEV protease overnight at 4 °C. The proteins were then loaded on a Ni-NTA-affinity column equilibrated with 50 mM Tris (pH 8.0), 500 mM NaCl, 4 mM benzamidine hydrochloride, 1 mM PMSF, and 1 mM TCEP (Buffer A). Proteins were eluted with Buffer A, supplemented with 300 mM Imidazole, and then additionally purified by gel filtration on a Superose 26/60 column (GE Healthcare, Chicago, IL) in 20 mM Tris (pH 7.0), 100 mM NaCl, and 1 mM DTT. For CRACR2a\(^{458-722}\) samples used in crystallization, 1 mM CaCl\(_2\) was added to the buffer.

**Isothermal titration calorimetry.** ITC measurements were carried out on either a VP-ITC instrument (MicroCal, Northampton, MA) or an Amino ITC instrument (TA Instruments, New Castle, DE). Protein samples were dialyzed for 3 d against 20 mM HEPES (pH 7.5), 100 mM NaCl, 0.5 mM TCEP (ITC buffer) and either 5 mM CaCl\(_2\) or 5 mM EGTA. The LIC1\(^{433-87}\) peptide was subjected to three cycles of solubilization/lyophilization in methanol 30% (v/v) to remove any trifluoroacetic acid and acetonitrile remaining after reverse-phase purification, followed by final solubilization in ITC buffer. The concentration of the peptide was determined by fluorescence with fluorescamine-labeled LIC1\(^{433-458}\). The proteins (or the LIC1\(^{433-458}\) peptide) in the syringe were titrated at a concentration 10 to 20 fold higher than that of the proteins in the ITC cell of total volume 1.44 ml (VP-ITC) or 0.94 ml (Amino ITC), as indicated in the figures. Titrations consisted of 10 µl injections, lasting for 10 s, with an interval of 300–400 s between injections. The heat of binding was corrected for the heat of injection, determined by injecting buffer proteins into buffer. Data were analyzed using the programs Origin (OriginLab, Northampton, MA) or NanoAnalyzer (TA Instruments, New Castle, DE). The temperature and parameters of the fit (stoichiometry and affinity) of each experiment are given in the figures.

**Circular dichroism spectroscopy.** CD spectra were recorded from proteins and buffer only on a Chirascan-Plus CD Spectropolarimeter (Applied Photophysics, Leatherhead, UK) in 1 nm steps from 260 to 190 nm, using a 0.1 cm path-length quartz cuvette and a scan rate of 20 nm min\(^{-1}\). The proteins (NIN\(^{112-167}\) and FIP3\(^{206-720}\)) were concentrated to 100 µM in 20 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM TCEP. Reported spectra correspond to merged spectra after buffer subtraction.

**Crystallization and structure determination.** BICD2\(^{1-98}\) at ~10 mg ml\(^{-1}\) in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.5 mM DTT was mixed with a 1:5 molar excess of LIC1\(^{433-87}\) at 4 °C for 1 h. Crystals were obtained at 16 °C using the hanging-drop vapor diffusion method. The crystallization drop consisted of 0.5 µl (ITC buffer) and 1 µl of protein solution (TA Instruments, New Castle, DE). Protein samples were dialyzed for 3 d against 20 mM HEPES (pH 7.5), 100 mM NaCl, and 1 mM TCEP. Reported spectra correspond to merged spectra after buffer subtraction.
100 mM NaCl, 0.5 mM DTT, and 1 mM CaCl₂ was mixed with a 1.5 molar excess of LIC123–456 at 4 °C for 1 h. Crystal were obtained at 16 °C using the hanging-drop vapor diffusion method. The crystallization drop consisted of a 1:1 v/v mixture of protein solution and well solution containing 16% (w/v) polyethylene glycol 10000, 0.5 M Bis-Tris (pH 6.5), and 10 mM CoCl₂. The quality of the crystals was further improved by microseeding. Crystals with selenium-methionine-substituted BICD2–aa and CRACR2a–aa were obtained under identical conditions as those used for the WT proteins. Crystals of both complexes were flash-frozen in liquid nitrogen using a cryo-solution consisting of crystallization buffer supplemented with 30–35% (v/v) glycerol. X-ray datasets were collected from selenium-methionine-substituted crystals at the Cornell High Energy Synchrotron Source (CHESS) beamline P1 (CRACR2a) and the Stanford Synchrotron Radiation Lightsource beamline 14-1 (CRACR2a–aa–LIC1–LIC2–aa Complex). The diffraction datasets were processed using HKL200041. Anisotropy data correction with HKL2000 eliminated weak, unreliable reflections, which improved the quality of data used in structure determination and refinement, but reduced completeness. The structures were determined ab initio using the single-wavelength anomalous dispersion method. The program SnB42 was used to find the coordinates of the Se atoms in the structures. Model building was carried out with the program Coot43. The structures of BICD2–LIC1 and CRACR2a–LIC1 were refined with the programs Phenix44 and Refmac545, respectively. Data collection and refinement statistics are detailed in Table 1. Figures were generated with the program PyMOL (Schrödinger, New York, NY). Sequence alignments were generated with the program MAFFT46, and illustrated using either Jalview47 or ESPript48. The conservation scores of amino acids in sequence alignments were determined with the program Sorecon49.

**Multi-angle light scattering.** Samples were separated by size exclusion chroma- tography on a Superose 6 10/300 GL column (GE Healthcare) equilibrated with 20 mM Tris (pH 7.5), 100 mM NaCl, and 1 mM DTT, using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA). Light scattering was measured in-line, using a DAWN-HELOS multi-angle light scattering detector and an Optilab rEX refractive index detector. The scattering data were analyzed with the program ASTRA (Wyatt Technology, Santa Barbara, CA).

**Single-molecule motility assays.** The movement of CRACR2a-containing complexes from cell extracts was tracked using TIRF microscopy. Motility assays were performed in flow chambers constructed with a glass slide and a coverslip silanized with PlusOne Repel-Silane ES (GE Healthcare), held together with vacuum grease to form a 10–μm chamber. Rigor kinase-1εL (0.5 mM) was non-specifically absorbed to the coverslip and the chamber was then blocked with 5% pluronic L-127 (Sigma-Aldrich). GTP (10 μM), labeled at a 1:40 ratio with HiLyte Fluor 647 (Cytochrome, Denver, CO), were added with the lysate to grow dynamic microtubules from the seeds. HeLa cells grown in 10 cm plates to 70–80% confluence and expressing full-length Halo-tagged CRACR2a WT, CRA- CR2a mutant F58D, or HaloTag alone were labeled with TMR-HaloTag ligand (Organix, La Jolla, CA). A two-tailed t test was used for run-length analysis, and a Kruskal–Wallis test followed by a Dunn’s multiple comparisons test was used for analysis of the number of events.

**Data availability** Atomic coordinates and structure factor amplitudes for the structures of BICD2–LIC1 and CRACR2a–LIC1 were deposited with the Protein Data Bank (PDB) under accession codes 6PSE and 6PSD, respectively. Other data and materials are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
I.-G.L. and R.D. designed and performed the biochemical and structural experiments. E.L.F.H. and S.E.C. designed and performed the in vitro motility experiments. S.S.A. participated in the protein preparation. I.-G.L. and R.D. wrote the paper. All the authors reviewed the figures and paper and approved its final version.

Competing interests
The authors declare no competing interests.

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