Comparative analysis of two *Caenorhabditis elegans* kinesins KLP-6 and UNC-104 reveals common and distinct activation mechanisms in kinesin-3

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Abstract

Kinesin-3 is a family of microtubule-dependent motor proteins that transport various cargos within the cell. However, the mechanism underlying kinesin-3 activations remains elusive. In this study, we compared the biochemical properties of two *Caenorhabditis elegans* kinesin-3 family proteins, KLP-6 and UNC-104. Both KLP-6 and UNC-104 were predominantly monomeric in solution. As previously shown for UNC-104, non-processive KLP-6 monomer was converted to a processive motor when artificially dimerized. While it has long been thought that UNC-104 monomers do not have enough affinity to form homodimers, we found releasing the autoinhibition was sufficient to trigger dimerization of UNC-104 at nanomolar concentrations. In contrast, KLP-6 remained to be a non-processive monomer even when its autoinhibition was unlocked, suggesting a requirement of other factors for full activation. By examining the differences between KLP-6 and UNC-104, we identified a coiled-coil domain called CC2 that is required for the dimerization and processive movement of UNC-104. Our results suggest a common activation mechanism for kinesin-3 family members, while also highlighting their diversification.

Introduction

Cellular morphogenesis depends on intracellular transport. Kinesins, also known as kinesin superfamily proteins (KIFs), are microtubule-dependent molecular motors that are essential for intracellular transport and cell division (Hirokawa et al., 2009). Among kinesins, the kinesin-3 family is mainly involved in intracellular transport, including axonal transport of synaptic materials, lysosomal transport, mitochondrial transport and intraflagellar transport of mechanoreceptor complexes (Chiba et al., 2023; Guardia et al., 2016; Morsci and Barr, 2011; Nangaku et al., 1994; Okada et al., 1995) .

The functions and regulation of the kinesin-3 family have been elucidated through genetic studies in *Caenorhabditis elegans* (*C. elegans*) (Chiba et al., 2019; Cong et al., 2021; Hall and Hedgecock, 1991; Kumar et al., 2010; Niwa et al., 2016; Niwa et al., 2017; Otsuka et al., 1991; Wu et al., 2013; Zheng et al., 2014). *C. elegans* has three members of the kinesin-3 family: UNC-104, KLP-4 and KLP-6. UNC-104 transports synaptic vesicle precursors, mature synaptic vesicles and pre-synaptic membrane proteins in the axon (Hall and Hedgecock, 1991; Oliver et al., 2022; Otsuka et al., 1991). Its mammalian orthologs, KIF1A and KIF1Bβ, also play a role in the axonal transport of synaptic materials (Niwa et al., 2008; Okada et al., 1995; Zhao et al., 2001). Because mutations in KIF1A and KIF1Bβ have been associated with congenital disorders (Boyle et al., 2021; Budaitis et al., 2021; Klebe et al., 2012; Zhao et al., 2001), *C. elegans* has been used to study the molecular mechanisms of pathogenesis (Anazawa et al., 2022; Chiba et al., 2023; Lam et al., 2021). KLP-4, on the other hand, is responsible for the transport of glutamate receptor called GLR-1 in the dendrite. The transport of GLR-1 is significantly reduced in *klp-4* mutant worms (Monteiro et al., 2012). KLP-6 is an invertebrate specific kinesin-3 and transports a mechanosensory receptor complex in male cilia (Morsci and Barr, 2011; Peden and Barr, 2005). This mechanoreceptor complex consists of LOV-1 and PKD-2, orthologs of mammalian polycystin-1 and polycystin-2 (Barr et al., 2001) . In

human, mutations in *PKD-1* or *PKD-2* gene which encodes polycystin-1 or polycystin-2 cause autosomal dominant polycystic kidney disease (ADPKD) (Hughes et al., 1995; Mochizuki et al., 1996). In worms, mutations in *klp-6* gene lead to the reduction of LOV-1 and PKD-2 from the cilia and the male infertility because the mechanoreceptor complex is essential for male worms to locate the hermaphrodite vulva during mating behavior (Peden and Barr, 2005).

Among these kinesin-3 family members, extensive biochemical and structural analyses have been conducted on UNC-104 and KLP-6 (Al-Bassam et al., 2003; Klopfenstein et al., 2002; Tomishige et al., 2002; Wang et al., 2022). It has been proposed that these motors are regulated by a monomer-to-dimer conversion mechanism (Al-Bassam et al., 2003; Tomishige et al., 2002; Wang et al., 2022). UNC-104 dimers, which are stabilized by fusing with a coiled-coil domain of kinesin-1, exhibit plus-end directed movement on microtubules, whereas purified UNC-104 monomers show only one-dimensional diffusion (Soppina et al., 2014; Tomishige et al., 2002). UNC-104 requires a high concentration (at least the range of 1 to 7 μM) for dimerization (Tomishige et al., 2002). Furthermore, UNC-104 mini-motors, generated by deleting most of the stalk domains, have been shown to be enriched in phosphatidylinositol 4, 5- bisphosphate (PIP2) microdomains on artificial liposomes (Klopfenstein et al., 2002). As a result, the mini-motor can efficiently transport PIP2-carrying vesicles in vitro. These results collectively suggested that the non-processive UNC-104 monomer is accumulated to high concentration and converted into an active dimer at PIP2 microdomains on the cargo membrane (Klopfenstein et al., 2002; Tomishige et al., 2002). Cryo-electron microscopy (cryo-EM) analysis of UNC-104 and the X-ray structure of full length KLP-6 have revealed the autoinhibited state of kinesin-3 (Al-Bassam et al., 2003; Ren et al., 2018; Wang et al., 2022). These structures suggest that stalk domains, including coiled-coil 1(CC1), Forkhead-associated (FHA) and coiled-coil 2 (CC2) domains, bind to the motor domain (MD) and the neck coiled-coil (NC) domain and inhibit the microtubule-dependent motor activity of kinesin-3

(Ren et al., 2018; Wang et al., 2022).

Based on the genetic screening and the crystal structure, point mutations that disrupt the autoinhibition of UNC-104 and KLP-6 have been identified. These prior studies have indirectly detected the activation of UNC-104 and KLP-6 by observing the localization of cargo vesicles, measuring ATPase activity or observing tip accumulation in neuronal cells (Cong et al., 2021; Niwa et al., 2016; Wagner et al., 2009; Wang et al., 2022; Wu et al., 2013). However, we still lack insights into oligomeric states of UNC-104 and KLP-6 at submicromolar. Without direct visualization of purified UNC-104 and KLP-6 itself at low nanomolar concentration, it is difficult to determine whether UNC-104 and KLP-6 are capable of dimerization on their own upon autoinhibition release or whether other cellular factors are required for their dimerization. Furthermore, confirming that UNC-104 and KLP-6 motors form dimers at physiological concentration is necessary, since the concentration of most cellular proteins is at the range of nanomolar (Wuhr et al., 2014).

In this study, we conducted mass photometry assays and single-molecule motility assays to investigate the oligomeric state and motility of two purified *C. elegans* kinesin-3 motors, KLP-6 and UNC-104. Our results demonstrate that UNC-104, but not KLP-6, can form a dimer on its own in solution at nanomolar concentrations when autoinhibition is released. We also found that a previously unexplored coiled-coil domain, called the CC2 domain, is essential for the formation of UNC-104 dimers at nanomolar concentrations. The CC2 domain was essential for the processive movement of UNC-104. Unlocking of the autoinhibition alone was sufficient to induce the dimerization and activation of UNC-104 without the binding with cargo vesicles.

Results

Full length KLP-6 is a monomeric motor

Although the full-length structure of KLP-6 has been solved previously (Wang et al., 2022),

its biochemical properties have not been thoroughly examined. Here, we have characterized the motile properties of a recombinant KLP-6 construct consisting of full length KLP-6 (aa 1-928) with a C-terminal green fluorescent protein (KLP-6FL) (Fig. 1A). In a size exclusion chromatography (SEC), KLP-6FL was eluted from a single peak (Fig. 1B), corresponding to the size of monomeric KLP-6FL, as in the previous study (Wang et al., 2022). Mass photometry confirmed that KLP-6FL was monomeric in solution (Fig. 1C). We found purified KLP-6FL was an active microtubule-dependent motor in a microtubule gliding assay. However, the velocity of the movement in the gliding assay (48.4 ± 42.0 nm/sec, mean ± Standard deviation (S.D.), n = 70 microtubules) was approximately 14 times lower than that of KLP-6::GFP in cilia (0.72 ± 0.18 μm/sec) (Morsci and Barr, 2011). We next examined KLP-6FL processivity by imaging single GFP-labeled molecules using a total internal reflection fluorescent (TIRF) microscope. Our results showed that KLP-6FL bound to microtubules and exhibited one-dimensional diffusion, but rarely showed directional movement (Fig. 1D).

KLP-6 has a second microtubule-binding domain in the tail

While we observed KLP-6FL bound to microtubules and showed diffusion on microtubules (Fig. 1D), the structure of KLP-6 shows that the stalk domains of KLP-6 cover its motor domain (MD) and prevent the MD from binding to microtubules (Wang et al., 2022). This raised a possibility that the tail domain of KLP-6 binds to microtubules in the autoinhibited state. To test this hypothesis, we purified deletion mutants of KLP-6 and examined their association with microtubules. We found that KLP-6(350-928), which lacks the MD, and KLP-6(580-928), which lacks the MD-NC-CC1-FHA domains, bound to microtubules (Fig. 2). Inversely, KLP-6(1-587), lacking the MBS and MATH domains, rarely bound to microtubules. However, KLP-6(1-390), which lacks the CC1-FHA-CC2 domains, was able to bind to microtubules. Note that none of the

KLP-6 proteins showed processive movement on microtubules (Fig. S1A-C). These suggests that KLP-6 has a second microtubule binding domain in the tail domains.

KLP-6 is converted to a processive motor by dimerization

KLP-6(1-390) but not KLP-6(1-587), bound to microtubules (Fig. 2). This result is consistent with previous findings showing that the CC1-FHA-CC2 domains can inhibit the motor activity in kinesin-3 (Hammond et al., 2009; Niwa et al., 2016; Wang et al., 2022). Although KLP-6(1-390) did not show any processive runs on microtubules in the TIRF assay (Fig. S1), we found KLP-6(1-390) is an active microtubule-dependent motor in a microtubule gliding assay. The velocity of microtubule gliding was 137.8 ± 29.8 nm/sec (Mean ± S.D., n = 137 microtubules). For kinesin-3 motors, it has been proposed that dimerization is required to achieve processive runs on microtubules (Soppina et al., 2014). Therefore, we dimerized KLP-6(1-390) using the Leucine zipper (LZ) domain of GCN4 (Soppina et al., 2014) (Fig. 3, A and B). As a result, we found KLP-6(1-390)LZ moved on microtubules processively in the TIRF assay (Fig. 3, C-E). The velocity was 0.34 ± 0.24 µm/sec (Mean ± S.D., n = 255 molecules), which is within the same range of velocity as cellular KLP-6::GFP (Morsci and Barr, 2011). These results suggest that KLP-6 is an inactive monomer and requires dimerization to perform directional movement on microtubules.

KLP-6 remains to be a non-processive monomer when the autoinhibition is relieved

In a previous study, UNC-104(1-653) was shown to be a monomeric and inactive protein, also known as U653 (Tomishige et al., 2002). KLP-6(1-587) has a similar domain architecture to UNC-104(1-653) (Figs. 4A and S2) and was an inactive motor. To investigate the activation mechanisms of kinesin-3, we analyzed KLP-6(1-587) and UNC-104(1-653) more. Wang et al. identified mutations that disrupt the autoinhibition of KLP-6 based on its autoinhibited structure

(Wang et al., 2022). It was demonstrated that these mutations activate KLP-6 in cultured cells (Wang et al., 2022). Among mutations analyzed in their study, KLP-6(D458A), an FHA domain mutation, has the strongest effect on the activation of KLP-6 (Fig. 4A) (Wang et al., 2022). Therefore, we introduced the D458A mutation into KLP-6(1-587) to create a mutant protein, KLP-6(1-587)(D458A). KLP-6(1-587)(D458A) exhibited similar properties to wild-type KLP-6(1-587) in the SEC analysis (Fig. 4B). Subsequent mass photometry confirmed that both KLP-6(1-587) and KLP-6(1-587)(D458A) were predominantly monomeric in solution (Fig. 4C). In the TIRF assay, KLP-6(1-587)(D458A), but not wild type KLP-6(1-587), frequently bound to microtubules (Fig. 4, D-F). However, KLP-6(1-587)(D458A) only exhibited one-dimensional diffusion and did not show any processive runs on microtubules (Fig. 4D). Considering that KLP-6(1-390)LZ dimers, but not KLP-6(1-390) monomers, are processive, these observations indicate that the autoinhibition of KLP-6(1-587)(D458A) is relieved but that KLP-6(1-587)(D458A) still cannot form processive dimers.

UNC-104 is converted to a processive dimer when the autoinhibition is relieved

Our previous work has identified mutations in the CC1 domain of UNC-104, such as UNC-104(E412K), that activate axonal transport of synaptic vesicle precursors (Niwa et al., 2016). Autoinhibited KLP-6 and KIF13B structures suggest that the E412K mutation in the UNC-104 disrupts the autoinhibition of UNC-104, similarly to the KLP-6(D458A) mutation (Ren et al., 2018; Wang et al., 2022). Therefore, we introduced the E412K mutation into UNC-104 and analyzed biochemical properties (Fig. 5A). UNC-104(1-653) exhibited two peaks in the SEC analysis (Fig. 5B), corresponding to the size of dimers and monomers, respectively. UNC-104(1-653) predominantly eluted in the monomer peak (Fig. 5B, lower panel). Unlike KLP-6(1-587), UNC-104(1-653) exhibited a clear peak shift in the SEC analysis by introducing a mutation to

relieve the autoinhibition (Fig. 5B). UNC-104(1-653)(E412K) eluted in a single peak whose expected molecular weight was that of a dimer. To confirm that the peak shift was due to the conversion from monomer to dimer, we analyzed the main peak fractions from UNC-104(1-653) and UNC-104(1-653)(E412K) by mass photometry. Mass photometry revealed that wild-type UNC-104(1-653) was mostly monomeric (Fig. 5C, blue), with less than 5% of dimers detected. In contrast, UNC-104(1-653)(E412K) was a mixture of monomers and dimers (Fig. 5C, orange), with the ratio of monomers and dimers almost 1:1. It is likely that the difference between SEC and mass photometry can be attributed to the concentration disparity between the two techniques; SEC operates in the micromolar range, while mass photometry operates in the nanomolar range, as further discussed later. While the prior study showed that UNC-104(1-653) do not show any processive runs on microtubules (Tomishige et al., 2002), we found wild-type UNC-104(1-653) exhibited a trace number of processive runs (Fig. 5D). Furthermore, UNC-104(1-653)(E412K) very frequently exhibited processive movement on microtubules (Fig. 5, D-G). Although the velocity of moving particles did not differ significantly (Fig. 5F), the run length of UNC-104(1-653)(E412K) was much longer than that of wild type UNC-104(1-653) (Fig. 5G). These results suggest that the equilibrium between monomers and dimers is shifted towards the dimer form when the autoinhibition of UNC-104 is released. Finally, we confirmed that the difference between UNC-104 and KLP-6 is not due to the effect of different mutations because KLP-6(E409K) did not convert to dimers (Fig. S3).

The CC2 domain of UNC-104 is essential for the dimerization and processive motility

The sequences of KLP-6(1-587) and UNC-104(1-653) are very similar, except that the CC2 domain of KLP-6 is much shorter than UNC-104 as depicted in Supplementary Figure S2. We analyzed KLP-6(1-587) and UNC-104(1-653) with a coiled-coil prediction algorism called Marcoil (Delorenzi and Speed, 2002), which is one of the most reliable prediction algorisms (Gruber et al.,

2006). Although the X-ray structure of monomeric KLP-6 has suggested a short α-helix domain is the CC2 domain of KLP-6 (Wang et al., 2022), the probability of coiled-coil formation expected from the Marcoil algorism is very low (Fig. 6A). On the other hand, the CC2 domain of UNC-104 is expected to be a conventional coiled coil with a high probability (Fig. 6A). This difference in the structure may contribute to the distinct biochemical properties observed between KLP-6 and UNC-104. To compare the properties of the CC2 domains of KLP-6 and UNC-104, we fused them with a fluorescent protein mScarlet (Fig. 6B). We analyzed the purified proteins by SEC (Fig. 6C). While the peak of KLP-6CC2-mScarlet was indistinguishable from mScarlet alone, UNC-104CC2-mScarlet exhibited a clear peak shift (Fig. 6C). The analysis indicates that UNC-104CC2, but not KLP-6CC2, is capable to induce dimerization.

To further explore the importance of the CC2 domain in the motility of UNC-104, we examined UNC-104(1-594) which consists of the MD, NC, CC1 and FHA domains, but not CC2 (Fig. 7A). In the SEC analysis, both UNC-104(1-594) and UNC-104(1-594)(E412K) showed almost identical profiles (Fig. 7B), which totally differed from the results that UNC-104(1-653)(E412K) shifted towards higher molecular weights compared to UNC-104(1-653) which presents two elution peaks (Fig. 5B). Mass photometry showed that both UNC-104(1-594) and UNC-104(1-594) (E412K) were predominantly monomeric in solution (Fig. 7C). TIRF microscopy analysis demonstrated that, UNC-104(1-594) rarely showed processive runs on microtubules at the nanomolar range. While UNC-104(1-594) (E412K) displayed some processive runs on microtubules, the run length and landing rate were much lower than those of UNC-104(1-653)(E412K) (Fig. 7, D-G). These results suggest that the CC2 domain is required for the stable dimer formation, activation and processive runs of UNC-104.

Discussion

Equilibrium between monomers and dimers in UNC-104

Our SEC analysis showed that UNC-104(1-653) was a mixture of dimers and monomers, with monomers predominating (Fig. 5B). A prior study has observed similar phenomena. UNC-104(1-653) can form active dimers at micromolar concentrations (Tomishige et al., 2002). In contrast, UNC-104(1-653)(E412K) showed a different profile in the SEC analysis, with most of the protein recovered from dimer fractions (Fig. 5B). However, in the mass photometry, UNC-104(1-653)(E412K) was a 1:1 mixture of dimers and monomers (Fig. 5C). This difference could be due to the different concentrations used in the two techniques, with the SEC analysis performed at micromolar concentrations and mass photometry at nanomolar concentrations. These results suggest that (1) UNC-104 exists in an equilibrium between monomers and dimers and (2) the release of the autoinhibition, such as by UNC-104(E412K) mutation, shifts the state of the equilibrium towards the formation of dimers. In contrast, we have not detected KLP-6 dimers in either SEC or mass photometry.

The CC2 domain is required for the processive movement

Our results indicate that the CC2 domain is essential for the formation of stable dimers (Fig. 7C). The CC2 domain has been demonstrated to be crucial for the autoinhibition of KIF1A and UNC-104 (Hammond et al., 2009; Lee et al., 2004). We have previously shown that the E612K mutation in the CC2 domain of UNC-104 disrupt its autoinhibition (Niwa et al., 2016). Moreover, crystal structure analysis revealed that the short CC2 domain of KLP-6 is also involved in autoinhibition. In addition to the role in the autoinhibition, CC2 domain is required for KIF1A to bind to cargos (Hummel and Hoogenraad, 2021). It has been suggested that CC2-mediated dimerization is essential for the cargo binding. We suggest here that, in addition to these functions, CC2 is required for the full activation and processivity of kinesin-3. Because KLP-6 does not have a

conserved CC2 domain, KLP-6 cannot form stable dimers (Fig. 4, B and C), although KLP-6 needs to be dimerized to obtain processivity (Fig. 3, B-E). A prior work has shown that a deletion mutant of KIF13B composed of MD, NC and CC1 can form a dimer when the autoinhibition is unlocked by mutations, but the major population remains monomeric in the ultracentrifugation analysis (Ren et al., 2018). This would be because CC2 is not included in their assays. Indeed, the monomer to dimer conversion was more clearly observed upon release of the autoinhibition in the case of the full-length of KIF13B which has the CC2 domain (Fan, 2022). The CC2 domain is conserved in most of the kinesin-3 family proteins including KIF1Bα, KIF1C, KIF13A, KIF16A and KIF16B (Hirokawa et al., 2009). Taken together, we suggest that the CC2 domain is fundamental to the stable dimer formation in kinesin-3. Moreover, our data suggest that the CC2 domain is required for UNC-104 to move long distances on microtubules (Fig. 7, D-G). Without the CC2 domain, UNC-104 shows only very short runs even if they are activated for microtubule binding. Therefore, the CC2 domain would be essential for long-distance transport, such as axonal transport. Consistent with this idea, forward genetic screening in *C. elegans* has identified a CC2 domain mutation, UNC-104(L640F), as a weak loss-of-function mutation (Cong et al., 2021). It is possible that UNC-104(L640F) reduces the efficiency of dimerization while the effect of UNC-104(L640F) mutation on the motility of UNC-104 remains to be elusive.

Activation of UNC-104 and KLP-6

While Tomishige at al. showed that wild-type UNC-104(1-653) does not show any processive runs on microtubules (Tomishige et al., 2002), we find even wild-type UNC-104(1-653) shows some processive runs on microtubules in the single molecule motility assays. This difference in results may be attributed to the differences in the microtubules used in the two studies. While we observed the motility of UNC-104 on purified porcine microtubules, Tomishige et al. observed the

motility on *Chlamydomonas* axonemes. Axonemes are heavily modified by both post-translational modification and microtubule associated proteins, which could potentially act as inhibitors of the UNC-104 motility. Moreover, our study provides compelling evidence that the release of autoinhibition is sufficient for monomeric UNC-104 to dimerize and move processively on microtubules at low nanomolar concentrations. This result revisits previous notion that UNC-104 cannot form dimers by its own and needs to be enriched to PIP2 microdomains on cargo vesicles to dimerize and move processively (Klopfenstein et al., 2002; Tomishige et al., 2002). A recent study has shown that KIF1A, a mammalian ortholog of UNC-104, needs to form a homodimer to bind to cargo vesicles, indicating that KIF1A dimerizes before interacting with cargo vesicles, rather than on cargo vesicles (Hummel and Hoogenraad, 2021). Based on these findings, we propose that UNC-104 forms dimers in cytosol upon release of autoinhibition, rather than on cargo vesicles, as a prerequisite for motor activation and cargo binding. Indeed, the number of synaptic vesicles transported by UNC-104 is increased by the UNC-104(E412K) mutation (Niwa et al., 2016). It is possible that PIP2 microdomains may play a role in stabilizing UNC-104 dimers to ensure long-distance axonal transport because UNC-104 dimers are more stable at higher concentration even when the autoinhibition is unlocked (Fig. 5, B and C).

Unlike UNC-104, autoinhibition release is insufficient to induce dimerization of KLP-6. KLP-6 is monomeric in solution even when autoinhibition is unlocked (Fig. 4). Therefore, other unidentified factors, such as post-translational modifications and binding proteins, may be required for KLP-6 dimerization. This hypothesis is supported by the finding that KLP-6 can be activated in cultured cells when autoinhibition is unlocked (Wang et al., 2022). Further investigations are needed to fully unravel the molecular mechanisms underlying the dimerization and activation of these motors by identifying factors that can unlock the autoinhibition of UNC-104 and KLP-6 in vitro, as well as factors that induce the dimerization of KLP-6.

Methods

Preparation of klp-6 and unc-104 cDNA

Wild type N2 strain was obtained from *C. elegans* genetic center (Minnesota, USA). Total RNA was purified from N2 using Nucleospin RNA®(Takara Bio Inc., Kusatsu, Japan) as described in manufacture's procedure. From the total RNA, cDNA was obtained using Superscript IV reverse transcriptase in combination with Oligo dT primer (Thermo Fisher Scientific Japan, Tokyo, Japan). *klp-6* cDNA was amplified by polymerase chain reaction (PCR) using KOD FX neo DNA polymerase (TOYOBO, Tokyo, Japan). Following primers were used:

*klp-6*_F, ATGGGAAAGGGTGACTCCATAATCG

*klp-6*_R, CCCTTATTTCGCCTTTGGTTTCTTCG

unc-104 cDNA was codon optimized for *Spodoptera frugiperda* and synthesized by GeneArt (Thermo Fisher Scientific) because we could not express enough amount of UNC-104 using the original *unc-104* cDNA. *klp-6* and *unc-104* cDNA fragments were amplified by PCR and cloned into the pAcebac1-sfGFP vector (Chiba et al., 2019). KLP-6(1-390)::LZ::mScarlet-I::Strep-tag II was generated by Gibson assembly (Gibson et al., 2009) based on the KIF1A(1-393)::LZ::mScarlet-I::Strep-tag II vector (Anazawa et al., 2022).

Expression of KLP-6 and UNC-104 in Sf9 cells

Sf9 cells (Thermo Fisher Scientific) were maintained in Sf900™ II SFM (Thermo Fisher Scientific) at 27°C. DH10Bac (Thermo Fisher Scientific) were transformed to generate bacmid. To prepare baculovirus, 1×10^6 cells of Sf9 cells were transferred to each well of a tissue-culture treated 6 well plate. After the cells attached to the bottom of the dishes, about ~5 μg of bacmid were transfected using 5 μL of TransIT®-Insect transfection reagent (Takara Bio Inc.). 5 days after initial

transfection, the culture media were collected and spun at 3,000 × g for 3 min to obtain the supernatant (P1). For protein expression, 400 mL of Sf9 cells (2×10^6 cells/mL) were infected with 200 µL of P1 virus and cultured for 65 h at 27°C. Cells were harvested and stocked at -80°C.

Expression of KLP-6 in E. coli

To express KLP-6(1-390)::LZ::mScarlet-I::Strep-tag II, LOBSTR(DE3) (Andersen et al., 2013) was transformed and selected on an LB agar plate supplemented with kanamycin at 37°C overnight. Colonies were picked and cultured in 10 ml LB medium supplemented with kanamycin overnight. Next morning, 5 ml of the medium was transferred to 500 ml 2.5× YT (20 g/L Tryptone, 12.5 g/L Yeast Extract, 6.5 g/L NaCl) supplemented with 10 mM phosphate buffer (pH 7.4) and 50 µg/ml kanamycin in a 2 L flask and shaken at 37°C. When OD_{600} reached 0.6, flasks were cooled in ice-cold water for 30 min. Then, 23.8 mg IPTG was added to each flask. Final concentration of IPTG was 0.2 mM. Flasks were shaken at 18°C overnight. Next day, bacteria expressing recombinant proteins were pelleted by centrifugation (3,000 × g, 10 min, 4°C), resuspended in PBS and centrifuged again (3,000 × g, 10 min, 4°C). Pellets were resuspended in protein buffer (50 mM HEPES-KOH, pH 8.0, 150 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol) supplemented with Phenylmethylsulfonyl fluoride (PMSF).

Purification of recombinant proteins

Sf9 cells were resuspended in 25 mL of lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM KCH3COO, 2 mM MgSO4, 1 mM EGTA, 10% glycerol) along with 1 mM DTT, 1 mM PMSF, 0.1 mM ATP and 0.5% Triton X-100. After incubating on ice for 10 min, lysates were cleared by centrifugation (15,000 × g, 20 min, 4°C) and subjected to affinity chromatography described below.

Bacteria were lysed using a French Press G-M (Glen Mills, NJ, USA) as described by the

manufacturer. Lysates were cleared by centrifugation (75,000 g, 20 min, 4°C) and subjected to affinity chromatography described below.

Lysate was loaded on Streptactin-XT resin (IBA Lifesciences, Göttingen, Germany) (bead volume: 2 ml). The resin was washed with 40 ml Strep wash buffer (50 mM HEPES-KOH, pH 8.0, 450 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol). Protein was eluted with 40 ml Strep elution buffer (50 mM HEPES-KOH, pH 8.0, 150 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol, 300 mM biotin). Eluted solution was concentrated using an Amicon Ultra 15 (Merck) and then separated on an NGC chromatography system (Bio-Rad) equipped with a Superdex 200 Increase 10/300 GL column (Cytiva). Peak fractions were collected and concentrated using an Amicon Ultra 4 (Merck). Proteins were analyzed by SDS-PAGE followed by CBB staining (Fig. S4). Concentrated proteins were aliquoted and snap-frozen in liquid nitrogen.

Mass photometry

Proteins obtained from the peak fractions in the SEC analysis were pooled, snap-frozen and stored until measurement. Prior to measurement, the proteins were thawed and diluted to a final concentration 5 - 20 nM in protein buffer without glycerol. Mass photometry was performed using a Refeyn OneMP mass photometer (Refeyn Ltd, Oxford, UK) and Refeyn AcquireMP version 2.3 software, with default parameters set by Refeyn AcquireMP. Bovine serum albumin (BSA) was used a control to determine the molecular weight. The results were subsequently analyzed using Refeyn DiscoverMP version 2.3, and graphs were prepared to visualize the data.

TIRF single-molecule motility assays

TIRF assays were performed as described. Tubulin was purified from porcine brain as described. Tubulin was labeled with Biotin-PEG_2-NHS ester (Tokyo Chemical Industry, Tokyo,

Japan) and AZDye647 NHS ester (Fluoroprobes, Scottsdale, AZ, USA) as described. To polymerize Taxol-stabilized microtubules labeled with biotin and AZDye647, 30 μM unlabeled tubulin, 1.5 μM biotin-labeled tubulin and 1.5 μM AZDye647-labeled tubulin were mixed in BRB80 buffer supplemented with 1 mM GTP and incubated for 15 min at 37°C. Then, an equal amount of BRB80 supplemented with 40 μM taxol was added and further incubated for more than 15 min. The solution was loaded on BRB80 supplemented with 300 mM sucrose and 20 μM taxol and ultracentrifuged at 100,000 g for 5 min at 30°C. The pellet was resuspended in BRB80 supplemented with 20 μM taxol. Glass chambers were prepared by acid washing as previously described. Glass chambers were coated with PLL-PEG-biotin (SuSoS, Dübendorf, Switzerland). Polymerized microtubules were flowed into streptavidin adsorbed flow chambers and allowed to adhere for 5–10 min. Unbound microtubules were washed away using assay buffer (90 mM HEPES-KOH pH 7.4, 50 mM KCH_3COO, 2 mM $Mg(CH_3COO)_2$, 1 mM EGTA, 10% glycerol, 0.1 mg/ml biotin–BSA, 0.2 mg/ml kappa-casein, 0.5% Pluronic F127, 2 mM ATP, and an oxygen scavenging system composed of PCA/PCD/Trolox). Purified motor protein was diluted to indicated concentrations in the assay buffer. Then, the solution was flowed into the glass chamber. An ECLIPSE Ti2-E microscope equipped with a CFI Apochromat TIRF 100XC Oil objective lens (1.49 NA), an Andor iXion life 897 camera and a Ti2-LAPP illumination system (Nikon, Tokyo, Japan) was used to observe single molecule motility. NIS-Elements AR software ver. 5.2 (Nikon) was used to control the system.

Microtubule gliding assays

Tubulin was labeled with AZDye647, and the labeled microtubules were prepared as described in *TIRF single-molecule motility assays*. Microtubule gliding assays were performed as described with slight modifications. Glass chambers were coated with PLL-PEG-biotin (SuSoS). Streptavidin is adsorbed in flow chambers. Biotin-labelled anti-GFP antibodies (MBL Life Science,

Tokyo, Japan) diluted in BRB80 buffer were flowed into the chamber. Chamber was washed by assay buffer. sfGFP-tagged motors were diluted in assay buffer and flowed into the chamber. Chamber was washed by assay buffer again. Microtubules diluted by assay buffer was flowed into the chamber and analyzed by the ECLIPSE Ti2-E microscope equipped with a CFI Apochromat TIRF 100XC Oil objective lens (1.49 NA), an Andor iXion life 897 camera and a Ti2-LAPP illumination system (Nikon, Tokyo, Japan).

Statistical analyses and graph preparation

Statistical analyses were performed using Graph Pad Prism version 9. Statistical methods are described in the figure legends. Graphs were prepared using Graph Pad Prism version 9, exported in the pdf format and aligned by Adobe Illustrator 2021.

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Conflicts of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

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Figure legends

Figure 1 Full length KLP-6 shows diffusive motion on microtubules

(A) Schematic drawing of the domain organization of KLP-6 motor protein and the full length of KLP-6 fused with sfGFP (KLP-6FL). NC, Neck coiled-coil domain. CC1, Coiled-coil 1 domain. FHA, Forkhead-associated domain. CC2, Coiled-coil 2 domain. MBS, Membrane-associated guanylate kinase homolog (MAGUK)-binding stalk domain. MATH, Meprin and TRAF homology domain. Calculated molecular weight (M.W.) is shown at the right side.

(B) Size exclusion chromatography of KLP-6FL. The SDS-PAGE of the elution fractions is shown beneath the profile. Asterisks indicate fractions used for mass photometry and single molecule assays. The void volume of the column is indicated. Asterisks indicate fractions that are used for mass photometry and TIRF assays. Number shown at the left side indicates a molecular weight standard.

(C) Mass photometry of KLP-6FL. Histogram shows the particle count of KLP-6FL at 20 nM. The line shows a Gaussian fit (mean ± standard deviation (S.D.): 124 ± 15.5 kDa).

(D) Representative kymographs showing the motility of 5 pM KLP-6FL in the presence of 2 mM ATP. Note that KLP-6FL shows only one-dimensional diffusion on microtubules but does not show any processive runs. Horizontal and vertical bars show 10 µm and 10 seconds, respectively.

Figure 2 KLP-6 has a second microtubule binding domain

Schematic drawing of the domain organization of KLP-6 analyzed and representative TIRF images showing the bindings of GFP fused KLP-6 deletion mutant proteins (GFP, green) to microtubules (MT, cyan). The tail domain alone binds to microtubules. Bar, 10 µm.

Figure 3 KLP-6 is converted to a processive motor upon dimerization

(A) Schematic drawing of the domain organization of KLP-6(1-390) and KLP-6(1-390)LZ.

(B) Size exclusion chromatography of KLP-6(1-390) (orange) and KLP-6(1-390)LZ (cyan). The SDS-PAGE of the elution fractions is shown beneath the profiles. The number shown at the left side indicates molecular weight standard. Orange and cyan asterisks indicate fractions used for single molecule assays. Note that the fraction indicated by an orange arrow does not contain KLP-6(1-390) protein.

(C) Representative kymographs showing the motility of 5 pM KLP-6(1-390) and KLP-6(1-390)LZ in the presence of 2 mM ATP. Horizontal and vertical bars show 5 seconds and 5 µm, respectively.

(D) Dot plots showing the velocity of KLP-6(1-390) and KLP-6(1-390)LZ. Each dot shows a single datum point. Green bars represent mean ± S.D.. n = 255 for KLP-6(1-390)LZ. nd, no directional movement was detected in KLP-6(1-390).

(E) Dot plots showing the run length of KLP-6(1-390) and KLP-6(1-390)LZ. Each dot shows a single datum point. Green bars represent median value and interquartile range. n = 255 for KLP-6(1-390)LZ.

n.d., no directional movement was detected in KLP-6(1-390).

Figure 4 KLP-6 is a constitutive monomer

(A) Schematic drawing of the domain organization of KLP-6(1-587) and UNC-104(1-653). Calculated molecular weight is written at the right side.

(B) Size exclusion chromatography of KLP-6(1-587) (plum) and KLP-6(1-587)(D458A) (purple). The SDS-PAGE of the elution fractions is shown beneath the profiles. Asterisks indicate fractions used for mass photometry and single molecule assays. The number shown at the left side indicates molecular weight standard.

(C) Mass photometry of KLP-6(1-587) and KLP-6(1-587)(D458A). Histograms show the normalized particle count of KLP-6(1-587) (blue) and KLP-6(1-587)(D458A) (orange) at 40 nM. Lines show Gaussian fits (mean ± S.D.: 94 ± 44 kDa and 88 ± 35 kDa for KLP-6(1-587) and KLP-6(1-587)(D458A), respectively).

(D) Representative kymographs showing the motility of 5 pM KLP-6(1-587) and KLP-6(1-587)(D458A) in the presence of 2 mM ATP. Note that no directional movement was detected in either case. Horizontal and vertical bars show 10 µm and 10 seconds, respectively.

(E) Dot plots showing the landing rate of KLP-6(1-587) and KLP-6(1-587)(D458A). Each dot shows a single datum point. Green bars represent median value. n = 33 microtubules. Mann-Whitney U test. ****, $p < 0.0001$.

(F) Dot plots showing the dwell time of KLP-6(1-587) and KLP-6(1-587)(D458A) on microtubules. Each dot shows a single datum point. Green bars represent median value and interquartile range. n = 338 and 351 particles for KLP-6(1-587) and KLP-6(1-587)(D458A), respectively. Mann-Whitney U test. ****, $p < 0.0001$.

Figure 5 UNC-104 is converted to a processive dimer upon autoinhibition release

(A) Schematic drawing of the domain organization of UNC-104(1-653). Calculated molecular weight is written at the right side.

(B) Size exclusion chromatography of UNC-104(1-653) (blue) and UNC-104(1-653)(E412K) (orange). The SDS-PAGE of the elution fractions is shown beneath the profiles. Blue and orange asterisks indicate fractions used for mass photometry and single molecule assays. The number shown at the left side indicates molecular weight standard. Blue arrows indicate expected dimer fraction of wild-type UNC-104(1-653).

(C) Mass photometry of UNC-104(1-653) and UNC-104(1-653)(E412K). Histograms show the normalized particle count of UNC-104(1-653) (blue) and UNC-104(1-653)(E412K) (orange) at 10 nM. Lines show Gaussian fits. UNC-104(1-653) is distributed within 100 ± 15.3 kDa range (mean ± S.D.). UNC-104(1-653)(E412K) shows two peaks consisting of 32% and 27% of particles which are distributed within 100 ± 15.3 kDa and 192 ± 31 kDa range, respectively (mean ± S.D.). Note that wild-type UNC-104(1-653) also has a very small peak around 200 kDa, but the number of datum point is too little for Gaussian fitting.

(D) Representative kymographs showing the motility of 2 nM UNC-104(1-653) and UNC-104(1-653)(E412K) in the presence of 2 mM ATP. Horizontal and vertical bars show 10 µm and 10 seconds, respectively.

(E) Dot plots showing the landing rate of UNC-104(1-653) and UNC-104(1-653)(E412K). Each dot shows a single datum point. Green bars represent median value n = 31 and 30 microtubules for UNC-104(1-653) and UNC-104(1-653)(E412K), respectively. Mann-Whitney U test. ****, $p < 0.0001$.

(F) Dot plots showing the velocity of UNC-104(1-653) and UNC-104(1-653)(E412K). Each dot shows a single datum point. Green bars represent mean ± S.D.. n = 603 and 624 particles for UNC-104(1-653) and UNC-104(1-653)(E412K). Student t-test, ns, $p = 0.7$ and statistically not significant.

(G) Dot plots showing the run length of UNC-104(1-653) and UNC-104(1-653)(E412K). Each dot shows a single datum point. Green bars represent median value and interquartile range. n = 603 and 624 particles for UNC-104(1-653) and UNC-104(1-653)(E412K). Mann-Whitney U test. ****, $p < 0.0001$.

Figure 6 CC2 domain of UNC-104, but not KLP-6, is capable to form a dimer

(A) Coiled coil prediction of KLP-6 (aa 1-587) and UNC-104 (aa 1-653). NC, CC1 and CC2 domains are indicated. Prediction was performed using the Marcoil algorism.

(B) Schematic drawing of the domain organization of mScarlet-I(mSca), KLP-6CC2-mSca and UNC-104CC2-mSca analyzed in panel C.

(C) Size exclusion chromatography of mSca (blue), KLP-6CC2-mSca (plum) and UNC-104CC2-mSca (orange). The SDS-PAGE of the elution fractions is shown beneath the profiles. The number shown at the left side indicates molecular weight standard.

Figure 7 The CC2 domain is essential for the stable dimer formation in UNC-104

(A) Schematic drawing of the domain organization of UNC-104(1-653) and UNC-104(1-594). Calculated molecular weight is shown at the right side.

(B) Size exclusion chromatography of UNC-104(1-594) (blue) and UNC-104(1-594)(E412K) (orange). The SDS-PAGE image of the elution fractions is shown beneath the profiles. The number shown at the left side indicates molecular weight standard. Both proteins show almost identical profile.

(C) Mass photometry of UNC-104(1-594) (blue) and UNC-104(1-594)(E412K) (orange) at 10 nM. Linges show Gaussian fits. Both UNC-104(1-594) and UNC-104(1-594)(E412K) have a single peak which is Gaussian distributed within 98 ± 14.3 kDa range and within 99 ± 13.6 kDa range, respectively (mean ± S.D.).

(D) Representative kymographs showing the motility of 2 nM UNC-104(1-594) and UNC-104(1-594)(E412K) in the presence of 2 mM ATP. Horizontal and vertical bars show 10 µm and 10 seconds, respectively.

(E) Dot plots showing the landing rate of UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K). For comparison, data for UNC-104(1-653) and UNC-104(1-653)(E412K) are replotted from Figure 5E. Each dot shows a single datum point. Green bars represent median value and interquartile range. N = 31, 32, 31, 30 microtubules for UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K), respectively. Kruskal-Wallis test followed by Dunn's multiple comparison test. ***, $p < 0.001$. ****, $p < 0.0001$. ns, $p > 0.05$ and statistically not significant.

(F) Dot plots showing the velocity of UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K). For comparison, data for UNC-104(1-653) and UNC-104(1-653)(E412K) are replotted from Figure 5F. Green bars represent mean ± S.D.. n = 467, 609, 603, 624 particles for UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K), respectively. One-way ANOVA test followed by Šidák's multiple comparison test. ns, $p > 0.05$ and statistically not significant.

(G) Dot plots showing the run length of UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K). For comparison, data for UNC-104(1-653) and UNC-104(1-653)(E412K) are replotted from Figure 5G. Each dot shows a single datum point. Green bars represent median value and interquartile range. Kruskal-Wallis test followed by Dunn's multiple comparison test. n = 467, 609, 603, 624 particles for UNC-104(1-594), UNC-104(1-594)(E412K), UNC-104(1-653) and UNC-104(1-653)(E412K), respectively. ****, $p < 0.0001$. ns, $p > 0.05$ and statistically not significant.

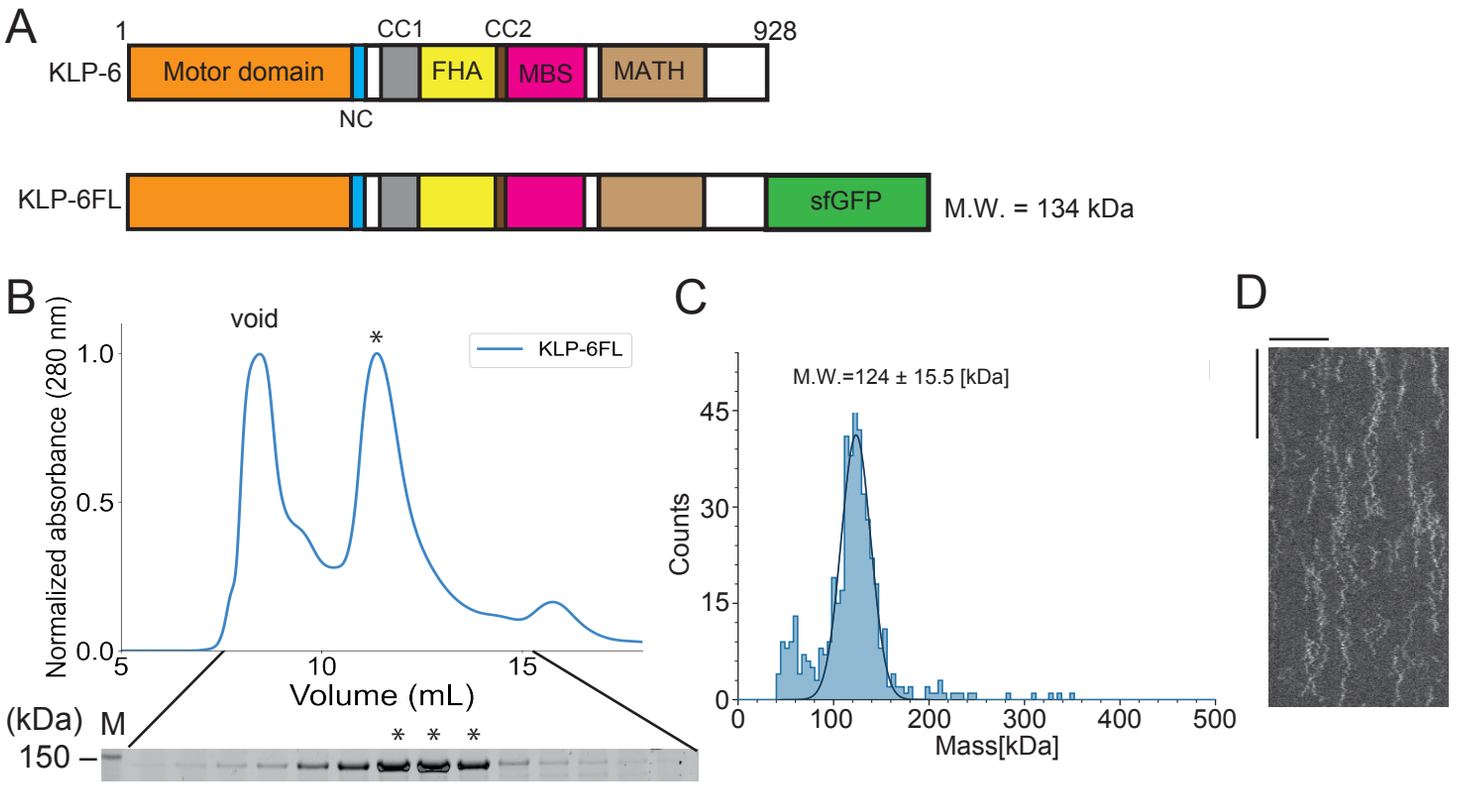
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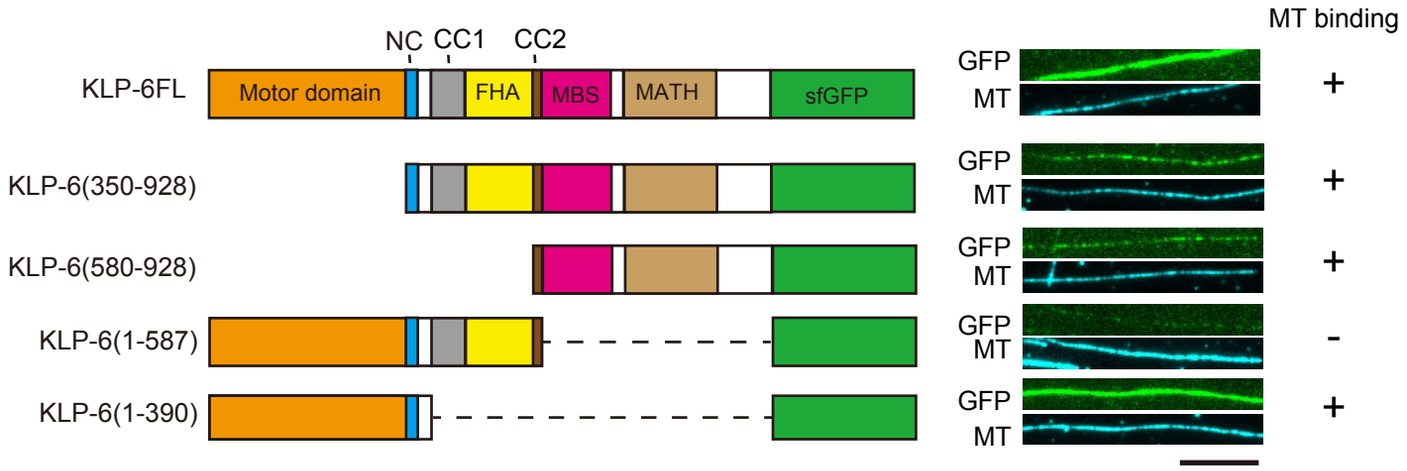
Figure 1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+

Figure 2

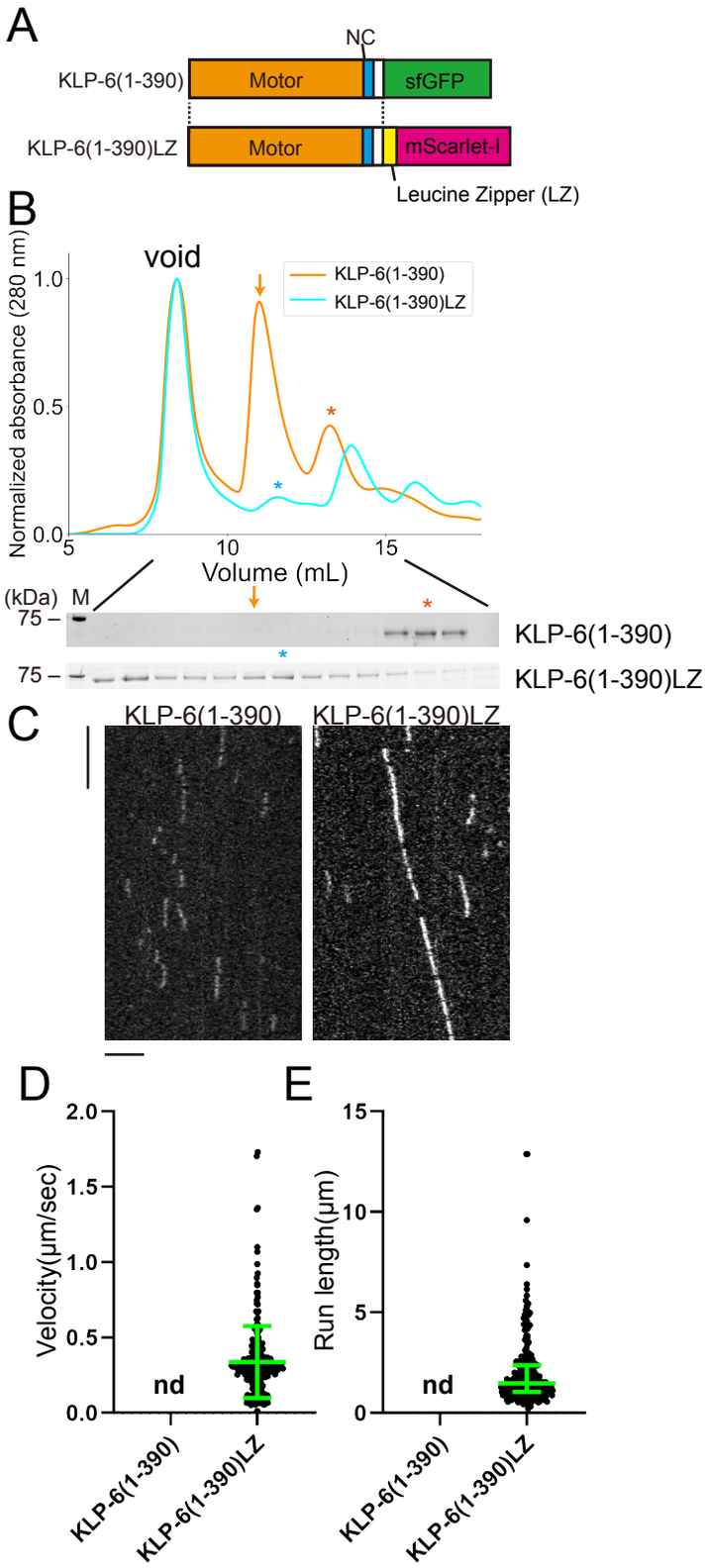
A
NC
KLP-6(1-390)
Motor
sfGFP
KLP-6(1-390)LZ
Motor
mScarlet-I
Leucine Zipper (LZ)
B
void
KLP-6(1-390)
KLP-6(1-390)LZ
Normalized absorbance (280 nm)
1.0
0.5
0.0
5
10
15
Volume (mL)
(kDa)
M
75 –
KLP-6(1-390)
75 –
KLP-6(1-390)LZ
C
KLP-6(1-390)
KLP-6(1-390)LZ
D
Velocity(µm/sec)
2.0
1.5
1.0
0.5
0.0
nd
KLP-6(1-390)
KLP-6(1-390)LZ
E
Run length(µm)
15
10
5
0
nd
KLP-6(1-390)
KLP-6(1-390)LZ

Figure 3

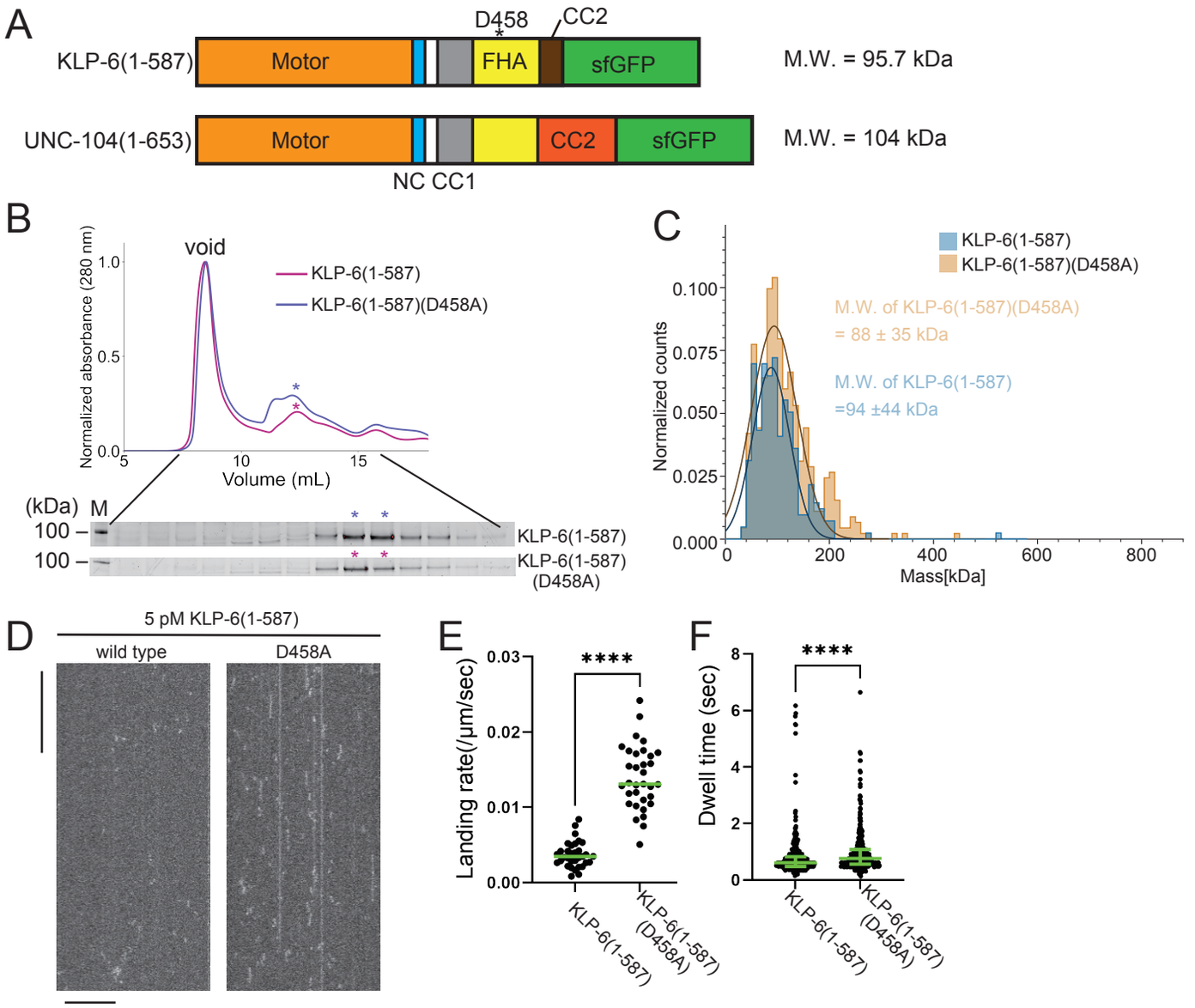
A
D458
CC2
KLP-6(1-587)
Motor
FHA
sfGFP
M.W. = 95.7 kDa
UNC-104(1-653)
Motor
CC2
sfGFP
M.W. = 104 kDa
NC CC1
B
void
KLP-6(1-587)
KLP-6(1-587)(D458A)
Normalized absorbance (280 nm)
Volume (mL)
(kDa) M
100
100
KLP-6(1-587)
KLP-6(1-587)
(D458A)
C
KLP-6(1-587)
KLP-6(1-587)(D458A)
M.W. of KLP-6(1-587)(D458A)
= 88 ± 35 kDa
M.W. of KLP-6(1-587)
=94 ±44 kDa
Normalized counts
Mass[kDa]
D
5 pM KLP-6(1-587)
wild type
D458A
E
Landing rate(/μm/sec)
****
KLP-6(1-587)
KLP-6(1-587)
(D458A)
F
Dwell time (sec)
****
KLP-6(1-587)
KLP-6(1-587)
(D458A)

Figure 4

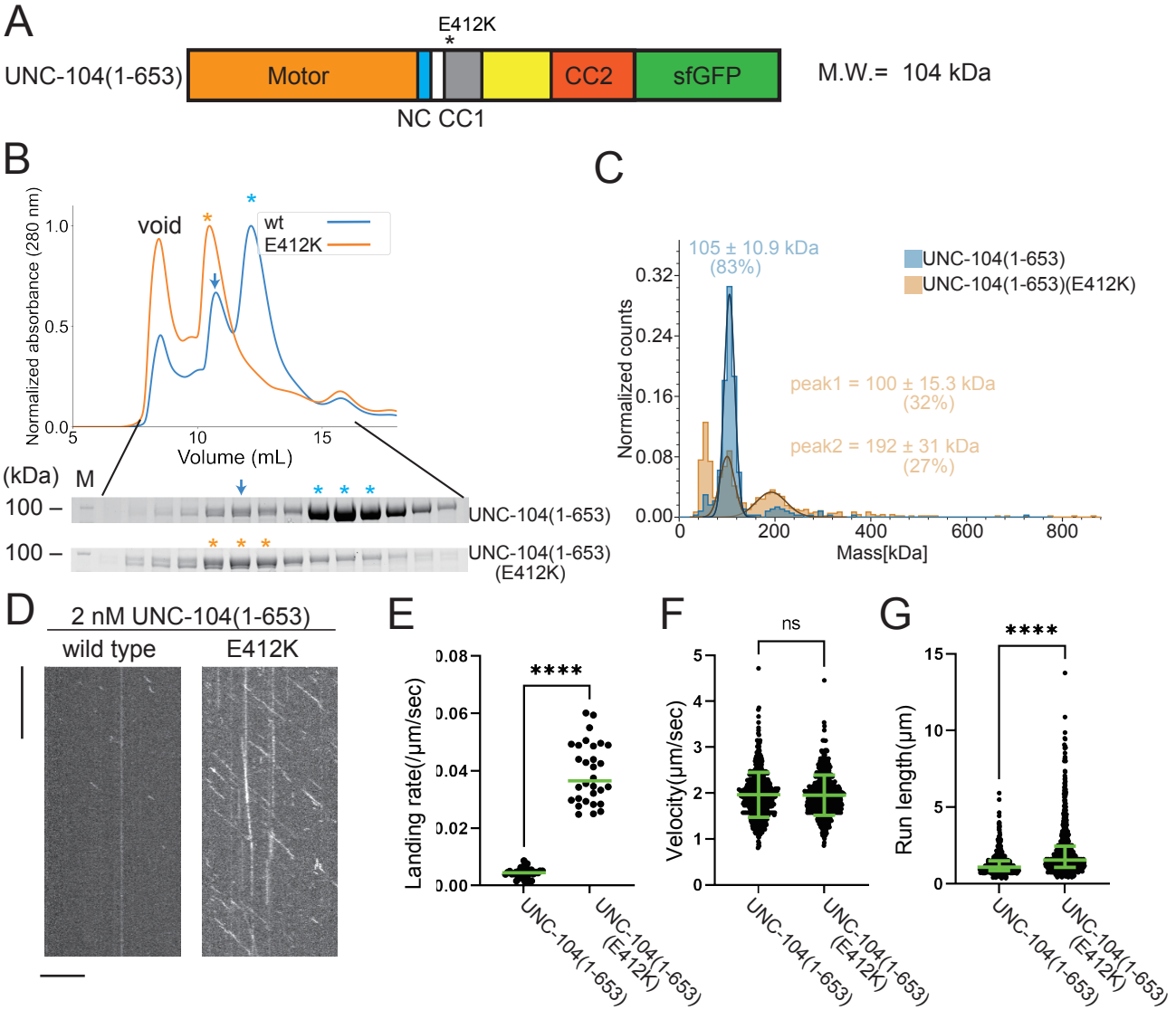
A
E412K
UNC-104(1-653)
Motor
CC2
sfGFP
M.W.= 104 kDa
NC CC1
B
Normalized absorbance (280 nm)
void
wt
E412K
Volume (mL)
(kDa)
M
100 –
UNC-104(1-653)
UNC-104(1-653)
(E412K)
C
105 ± 10.9 kDa
(83%)
UNC-104(1-653)
UNC-104(1-653)(E412K)
Normalized counts
peak1 = 100 ± 15.3 kDa
(32%)
peak2 = 192 ± 31 kDa
(27%)
Mass[kDa]
D
2 nM UNC-104(1-653)
wild type
E412K
E
Landing rate(/µm/sec)
****
UNC-104(1-653)
UNC-104(1-653)
(E412K)
F
ns
Velocity(µm/sec)
G
****
Run length(µm)

Figure 5

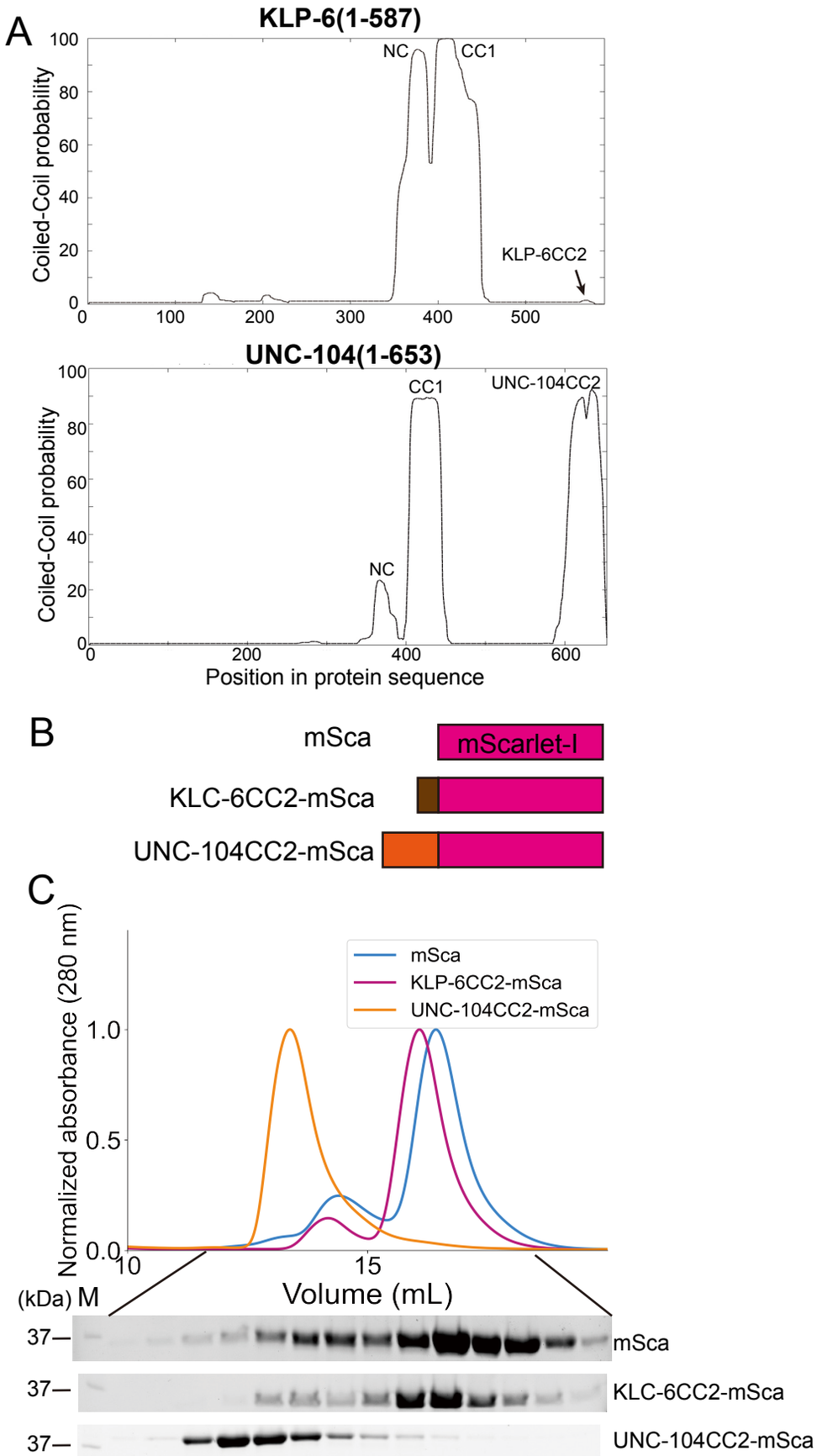
A
KLP-6(1-587)
Coiled-Coil probability
NC
CC1
KLP-6CC2
UNC-104(1-653)
CC1
UNC-104CC2
NC
Position in protein sequence
B
mSca
mScarlet-I
KLC-6CC2-mSca
UNC-104CC2-mSca
C
Normalized absorbance (280 nm)
mSca
KLP-6CC2-mSca
UNC-104CC2-mSca
Volume (mL)
(kDa) M
37—
mSca
KLC-6CC2-mSca
UNC-104CC2-mSca

Figure 6

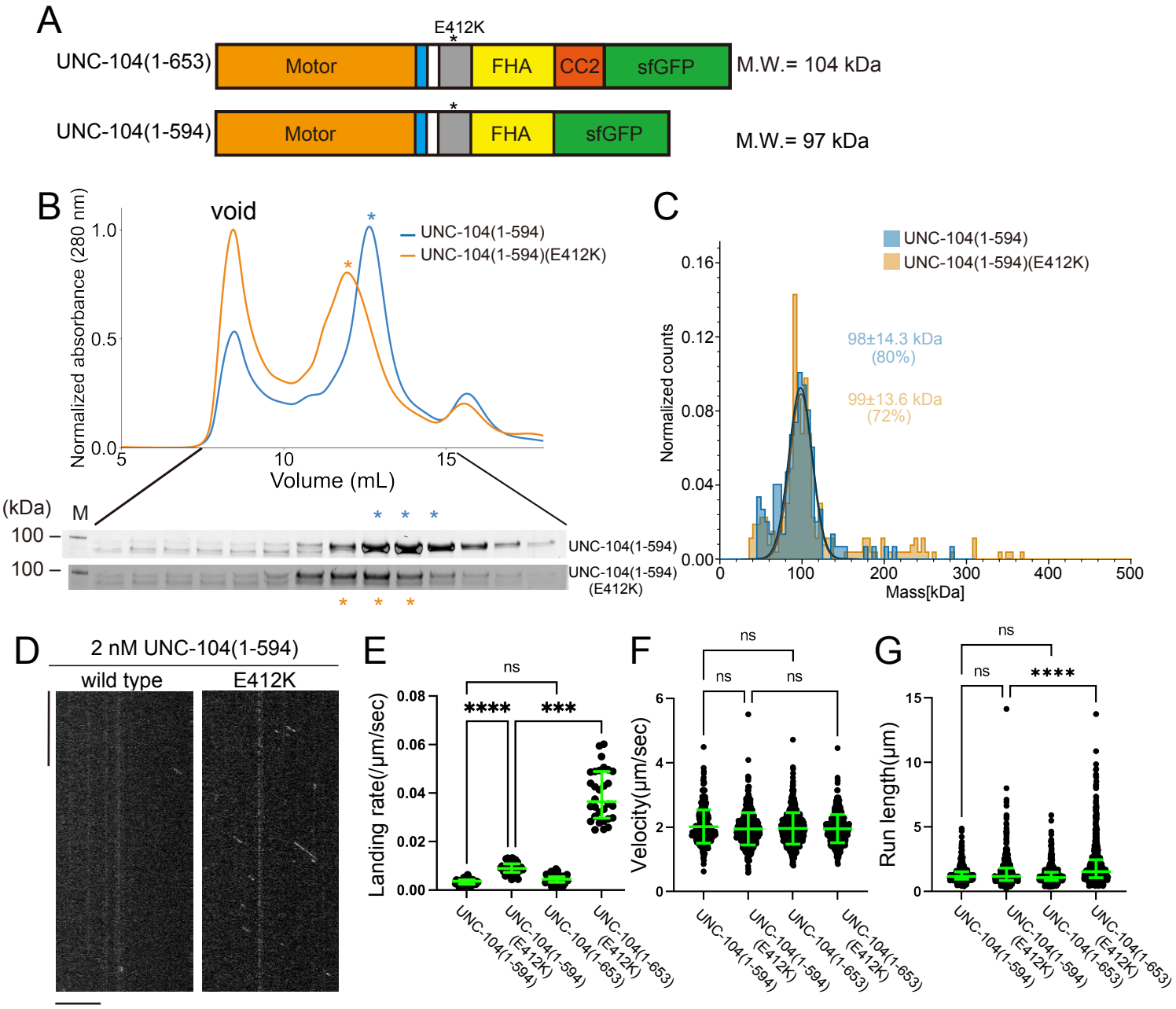
A
E412K
UNC-104(1-653)
Motor
FHA
CC2
sfGFP
M.W.= 104 kDa
UNC-104(1-594)
Motor
FHA
sfGFP
M.W.= 97 kDa
B
void
UNC-104(1-594)
UNC-104(1-594)(E412K)
Normalized absorbance (280 nm)
Volume (mL)
(kDa)
M
100
UNC-104(1-594)
UNC-104(1-594) (E412K)
C
UNC-104(1-594)
UNC-104(1-594)(E412K)
98±14.3 kDa (80%)
99±13.6 kDa (72%)
Normalized counts
Mass[kDa]
D
2 nM UNC-104(1-594)
wild type
E412K
E
Landing rate(/μm/sec)
ns
****
***
F
Velocity(μm/sec)
ns
ns
ns
G
Run length(μm)
ns
ns
****
UNC-104(1-594)
UNC-104(1-594) (E412K)
UNC-104(1-653)
UNC-104(1-653) (E412K)

Figure 7