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α2-chimaerin controls neuronal migration and functioning of the cerebral cortex through CRMP-2

Jacque P K Ip[1–3,7], Lei Shi[1–3,7], Yu Chen[1–3], Yasuhiro Itoh[4], Wing-Yu Fu[1–3], Andrea Betz[5], Wing-Ho Yung[6], Yukiko Gotoh[4], Amy K Y Fu[1–3] & Nancy Y Ip[1–3]

Disrupted cortical neuronal migration is associated with epileptic seizures and developmental delay. However, the molecular mechanism by which disruptions of early cortical development result in neurological symptoms is poorly understood. Here we report α2-chimaerin as a key regulator of cortical neuronal migration and function. *In utero* suppression of α2-chimaerin arrested neuronal migration at the multipolar stage, leading to accumulation of ectopic neurons in the subcortical region. Mice with such migration defects showed an imbalance between excitation and inhibition in local cortical circuitry and greater susceptibility to convulsant-induced seizures. We further show that α2-chimaerin regulates bipolar transition and neuronal migration through modulating the activity of CRMP-2, a microtubule-associated protein. These findings establish a new α2-chimaerin-dependent mechanism underlying neuronal migration and proper functioning of the cerebral cortex and provide insights into the pathogenesis of seizure-related neurodevelopmental disorders.

Altered cortical cytostructure and circuit property are linked to a number of neurological disorders, such as epileptic seizures, mental retardation and cognitive dysfunction[1,2]. The precise function of the mammalian cerebral cortex is directed by a highly patterned lamination, formed through a series of coordinated developmental processes including neurogenesis, neuronal migration and differentiation[3]. In particular, neuronal migration is the fundamental step directing newborn neurons to their proper destinations, a process that is crucial for the formation of distinct lamina-specific microcircuitry and thus the function of the entire cortex[4]. Disruption of neuronal migration is among the most common causes of cortical malformation disorders and is associated with neuropsychiatric diseases such as schizophrenia and autism[4–8]. Nonetheless, the molecular control of cortical neuronal migration is not fully understood. Furthermore, how disruptions of neuronal migration during embryogenesis lead to the onset of severe neurological symptoms after birth or in adulthood remains largely enigmatic.

Cortical neuronal migration occurs after the generation of neurons from progenitor cells at the ventricular zone. The migrating neurons may initially show various morphologies, but will eventually adopt a bipolar shape that enables them to move radially toward the pial surface through coordinated process extension and nuclear translocation[3,9–11]. In particular, a notable multipolar stage is evident in the subventricular zone and lower intermediate zone, which is characterized by the rapid extension and retraction of several short neurites and a relatively immobile nucleus[10,12]. This highly dynamic stage has been suggested to represent a phase when migrating neurons actively explore the microenvironment for directional cues[3,11]. Several signaling pathways that modulate actin and microtubule cytoskeletal rearrangements are involved in the transition to bipolar shape and radial migration, highlighting cytoskeletal function in this process[3,13]. Nonetheless, it remains unclear how the cytoskeletal network responds to upstream signals to regulate neuronal polarity and direct radial migration.

α2-chimaerin, a Rho GTPase-activating protein (GAP), was previously identified as a critical cytoskeletal regulator in early neuronal development including neuritogenesis and axon pathfinding[14–19]. Mutations of the α2-chimaerin gene *CHN1* cause human Duane's retraction syndrome, an eye movement disorder caused by disrupted innervation of extraocular muscles by ocular motor axons[19]. Moreover, α2-chimaerin is essential for corticospinal axon guidance in rodents[15–17]. Notably, studies from various research groups have reported *CHN1* as a candidate susceptible gene in several neurodevelopmental diseases, such as epilepsy, schizophrenia and autism[20–22]. It is thus likely that α2-chimaerin is involved in neuronal developmental processes whose disruptions are considered risk factors for these diseases. Given its high expression in the embryonic cerebral cortex[14], we were interested in investigating whether α2-chimaerin acts during cortical development.

Here we report that α2-chimaerin is essential for cortical neuronal migration and functional circuit formation. Mice with genetic deletion of α-chimaerin showed impaired neuronal migration. Depletion of α2-chimaerin *in utero* specifically perturbed the transition of neurons from the multipolar to bipolar stage and arrested

[1]Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China. [2]Molecular Neuroscience Center, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China. [3]State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China. [4]Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan. [5]Department of Pathology, University of Otago at Christchurch, Christchurch, New Zealand. [6]School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China. [7]These authors contributed equally to this work. Correspondence should be addressed to N.Y.I. (boip@ust.hk).

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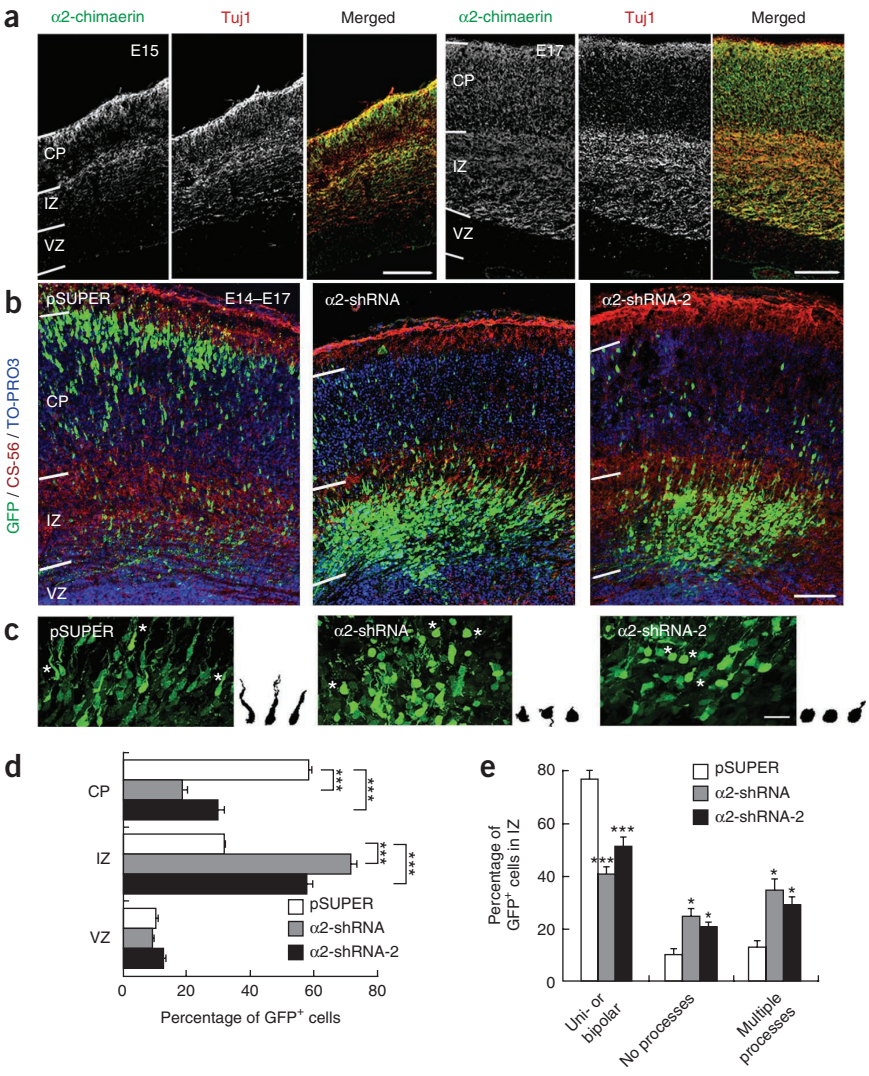

Figure 1 α2-chimaerin is essential for neuronal migration during neocortex development. (**a**) α2-chimaerin was localized to the intermediate zone and the cortical plate in the developing mouse neocortex. E15 and E17 cortical sections were immunostained with antibodies to α2-chimaerin (green) and Tuj1 (red). CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Scale bars, 100 μm. (**b**) Results of *in utero* electroporation of E14 mouse embryos with GFP plasmids together with pSUPER vector (pSUPER) or pSUPER-based shRNAs targeting α2-chimaerin (α2-shRNA or α2-shRNA-2). Representative coronal brain sections at E17 were stained with antibodies to GFP (green) and CS-56 (red), a subplate marker, and counterstained with TO-PRO3, a nuclear marker (blue). Scale bar, 100 μm. (**c**) Morphological defects of migrating neurons in the intermediate zone after depletion of α2-chimaerin. Right subpanels: drawings of representative neurons denoted by asterisks in the left subpanels. Scale bar, 20 μm. (**d**) Quantification of the distribution of GFP$^+$ neurons. $^{***}P < 0.001$; Student's *t*-test. More than 1,000 GFP$^+$ neurons from three to five brains were analyzed in each group. (**e**) Quantification of percentage of neurons with uni- or bipolar morphology, no processes or multiple (≥3) minor processes. $^*P < 0.05$, $^{***}P < 0.001$ versus pSUPER control; Student's *t*-test. More than 100 GFP$^+$ neurons from three brains were examined in each group. Error bars, s.e.m.

neurons in the deep layers of the cortex. Mice with such migration defects showed altered cortical excitability and enhanced seizure susceptibility when treated with a convulsant. Notably, we found that α2-chimaerin regulates neuronal migration through modulating the activity of the microtubule-associated protein CRMP-2. This study establishes a role of α2-chimaerin in neuronal migration and polarity acquisition, thereby advancing the molecular understanding of cortical development during both normal and pathophysiological conditions.

RESULTS

α2-chimaerin is essential for radial migration

As a first step in studying whether α2-chimaerin functions in cortical development, we examined the expression profile of α2-chimaerin in the developing mouse brain. α2-chimaerin protein was prominently expressed in different developmental stages of the mouse brain (embryonic day (E) 12 to postnatal day (P) 15; **Supplementary Fig. 1a**). Immunohistochemical study showed that α2-chimaerin was highly expressed in the intermediate zone and cortical plate of the developing cerebral cortex at E15 and E17 but was barely detected in the ventricular zone (**Fig. 1a**). We then investigated whether neuronal migration is affected in a mutant mouse line (*Chn1*$^{-/-}$) that lacks the expression of both α2-chimaerin and its isoform α1-chimaerin[17]. We used bromodeoxyuridine (BrdU) to label ventricular proliferating cells at E14 and traced the migration of these cells at E17. Whereas most of the BrdU-labeled cells migrated into the cortical plate in wild-type cortex, significant numbers of labeled cells accumulated in the intermediate zone in *Chn1*$^{-/-}$ cortex ($P < 0.01$; Student's *t*-test; **Supplementary Fig. 1b**,**c**). Furthermore, fewer labeled cells reached the upper regions of cortical plate in mutant cortex (**Supplementary Fig. 1b**,**c**). The effect of *Chn1* on neuronal migration was specific, as knockout of *Chn1* did not affect the differentiation of intermediate progenitor cells (IPCs; positive for the marker Tbr2 (T-box transcription factor Eomes)) or caspase-3–induced apoptosis (**Supplementary Fig. 1d–f**). As α1-chimaerin is weakly expressed in the embryonic central nervous system[14], the impaired neuronal migration found in *Chn1*$^{-/-}$ mice is probably due to the absence of α2-chimaerin.

We then studied the regulatory role of α2-chimaerin in neuronal migration by suppressing its expression in ventricular cells at E14 using *in utero* electroporation. Two short hairpin RNAs (α2-shRNA or α2-shRNA-2), which target different sequences of α2-chimaerin, were used to study the effect of α2-chimaerin knockdown on migration (**Supplementary Fig. 2a–c**). Whereas most of the neurons expressing pSUPER vector or scrambled shRNA migrated into the upper regions of the cortical plate at E17, knockdown of α2-chimaerin by either α2-shRNA or α2-shRNA-2 led to accumulation of migrating neurons in the intermediate zone (**Fig. 1b–d** and **Supplementary Fig. 2d**,**f**). Morphological analysis revealed that control neurons showed a typical unipolar or bipolar shape with one prominent leading process orienting toward the marginal zone. By contrast, most of the intermediate zone–trapped

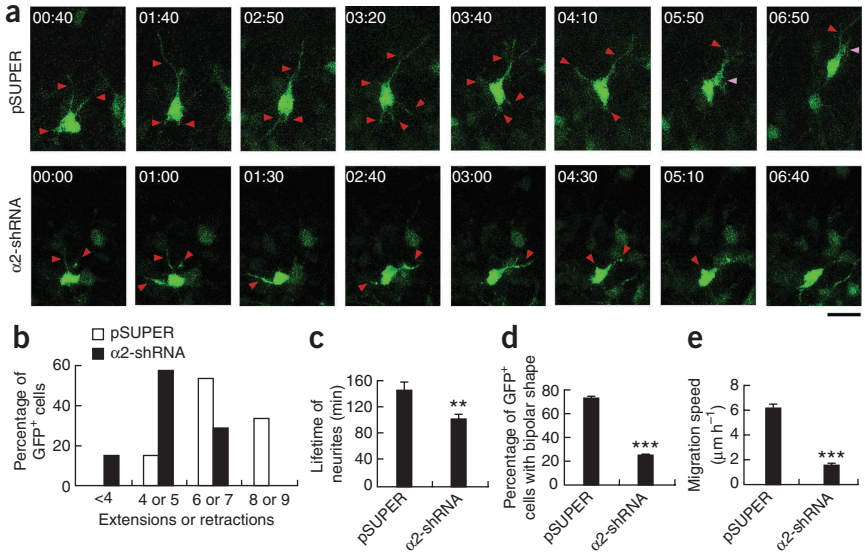

Figure 2 α2-chimaerin regulates multipolar-bipolar transition. (**a**) α2-chimaerin knockdown impairs the transition to a bipolar morphology. E14 mouse embryos were electroporated *in utero* with GFP plasmids together with pSUPER vector or α2-chimaerin shRNA. Cortical slice cultures were prepared at E16 and the migration of GFP$^+$ cells were monitored for 7 h. Red arrowheads, neurites extending from cell bodies. Pink arrowheads, swelling of leading process of the pSUPER electroporated neuron (see also **Supplementary Movie 3**). Scale bar, 20 μm. (**b**) Total number of process extension and retraction events was decreased in α2-chimaerin knockdown neurons ($n = 20$ neurons in each group). (**c**) The average neurite lifetime was shortened in α2-chimaerin knockdown neurons. $^{**}P < 0.01$; Student's *t*-test ($n = 79$ and 72 neurites in pSUPER and α2-shRNA group, respectively). (**d**) Percentage of multipolar cells changing to bipolar shape in the imaging period. $^{***}P < 0.001$; Student's *t*-test ($n = 82$ and 79 neurons in pSUPER and α2-shRNA groups, respectively). (**e**) The migration rate of α2-chimaerin knockdown neurons was significantly reduced. Data are presented as migration distance divided by time. $^{***}P < 0.001$; Student's *t*-test. Error bars, s.e.m.

α2-chimaerin-knockdown neurons extended multiple minor processes or even showed a rounded morphology with no processes (**Fig. 1c**,**e** and **Supplementary Fig. 2e**,**g**). These morphologically abnormal neurons did not show caspase-3–induced apoptosis (**Supplementary Fig. 3a**). We also confirmed that both the morphology of brain lipid binding protein (BLBP)-labeled radial glia (that is, the basal processes and endfeet) and the number of Tbr2$^+$ IPCs were unchanged upon α2-chimaerin knockdown (**Supplementary Fig. 3b–e**).

To address whether α2-chimaerin regulates migration of neurons from different layers, we electroporated α2-chimaerin shRNA *in utero* at earlier stages (E12 or E13). These knockdown neurons were similarly arrested at the intermediate zone when examined 3 d later (**Supplementary Fig. 4a–d**), suggesting that α2-chimaerin-mediated function is required for migration of neurons from different cortical layers.

α2-chimaerin regulates multipolar–bipolar transition

Before the unidirectional radial migration toward the pial surface, migrating neurons undergo a multipolar stage in the intermediate zone, where they rapidly extend and retract processes until they transform into the bipolar shape[10]. To directly study whether α2-chimaerin is important for the multipolar–bipolar transition, we performed time lapse analysis on cortical slices to track the migratory behavior of pSUPER or α2-chimaerin shRNA–electroporated cells. Most of the control neurons attained bipolar shape, underwent nuclear translocation and migrated to the cortical plate (**Supplementary Movie 1**). In contrast, α2-chimaerin knockdown neurons were unable to enter into the cortical plate and instead moved locally within the intermediate zone (**Supplementary Movie 2**).

Further analysis showed that control neurons actively extended and retracted neurites and eventually formed one main leading process guiding the migration of cell body toward the pial surface (**Fig. 2a** and **Supplementary Movie 3**). Process extension and retraction events in α2-chimaerin knockdown neurons were markedly reduced (**Fig. 2b** and **Supplementary Movie 4**). Some of the knockdown neurons even stopped forming neurites after a few rounds of extension and retraction and remained in a relatively rounded shape in the subsequent imaging period (**Fig. 2a** and **Supplementary Movie 4**). These findings suggest that the process dynamics is impaired in α2-chimaerin knockdown neurons. Furthermore, the average lifetime of the processes extending from the knockdown neurons was notably shorter than that of control neurons, indicative of reduced process stability after α2-chimaerin knockdown (**Fig. 2c** and **Supplementary Movie 4**). Consequently, bipolar transition of the knockdown neurons was disrupted (**Fig. 2d**), and they showed substantially reduced migration speed at the radial direction (**Fig. 2e**). Together, these data suggest that α2-chimaerin regulates the formation of a stabilized leading process in migrating neurons which is required for multipolar-bipolar transition.

α2-chimaerin knockdown arrests neurons in the white matter

To determine whether the intermediate zone–accumulated cells could eventually migrate to the cortical plate, we examined the positions of α2-chimaerin–silenced neurons at postnatal stages. Control neurons had settled in layers II–IV of the cortex at P2 and P19 (**Fig. 3a–e**). In contrast, most of the α2-chimaerin–depleted neurons remained stalled in the subcortical region, forming an ectopic neuronal layer in the white matter (**Fig. 3a–e**). This supports the notion that α2-chimaerin knockdown truly arrests neuronal migration, rather than delaying the process. These ectopic neurons were positive for the layer II–IV marker Cutl1 and negative for the layer V–VI marker Ctip2 (**Fig. 3c** and **Supplementary Fig. 5**). Moreover, the ectopic neurons were positive for the neuronal marker NeuN at P19, suggesting that these neurons maintained their identity at later developmental stage (**Fig. 3f**,**g**).

These results indicate that *in utero* suppression of α2-chimaerin may lead to a displacement of layer II–IV neurons into the white matter in the postnatal cortex. When compared to normal layer II–IV neurons, which had extensive dendritic arborization, the white matter-trapped α2-chimaerin knockdown neurons showed severely perturbed morphology, including distorted cell shape, abnormal neurite extension and absence of a directional leading process (**Supplementary Fig. 6**).

α2-chimaerin knockdown alters cortical excitability

Altered cortical cytostructure can disrupt both local and long-range neuronal connections[23,24]. It is therefore likely that the ectopic neurons resulting from knockdown of α2-chimaerin would lead to impaired microcircuitry in the cortex. To test this hypothesis, we performed whole-cell recordings of spontaneous synaptic activity in cortical

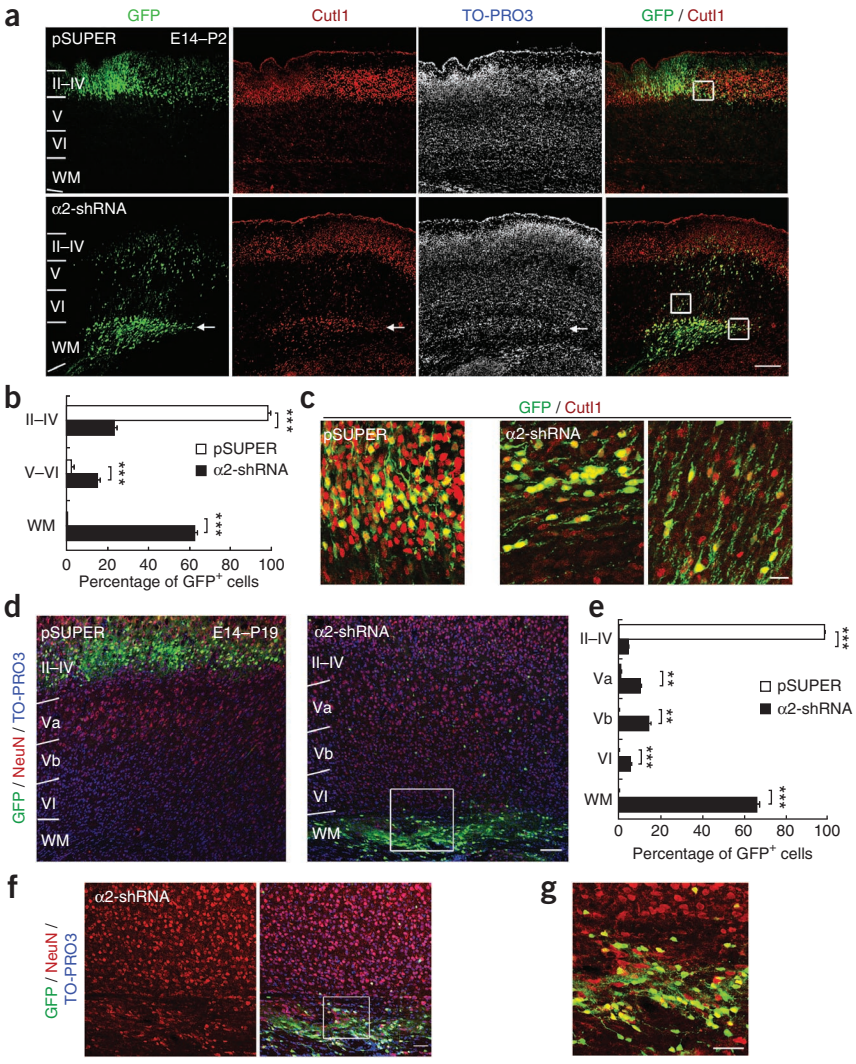

Figure 3 α2-chimaerin knockdown results in accumulations of ectopic neurons in postnatal brain. (**a**) α2-chimaerin knockdown neurons stalled at the deep layers at P2. E14 mouse embryos were electroporated *in utero* with GFP plasmids together with pSUPER vector (pSUPER) or α2-chimaerin shRNA (α2-shRNA). Representative coronal brain sections at P2 were stained with antibodies to GFP (green) and Cutl1 (red), a layer II–IV marker, and counterstained with TO-PRO3 (blue). Arrows indicate the ectopic band of neurons. WM, white matter. Scale bar, 100 μm. (**b**) Layer distribution of pSUPER or α2-shRNA electroporated neurons. $^{***}P < 0.001$; Student's *t*-test. (**c**) Boxed regions from **a** at higher magnification. Scale bar, 20 μm. Despite the abnormal location, most of the α2-chimaerin knockdown neurons expressed Cutl1. (**d**) Silencing of α2-chimaerin caused an ectopic accumulation of neurons in the white matter at P19. Representative brain coronal sections were stained with antibodies to GFP (green) and NeuN (red), a neuronal marker, and counterstained with TO-PRO3 (blue). Scale bar, 100 μm. (**e**) Quantification of the layer distribution of GFP$^+$ neurons in P19 cortex. $^{**}P < 0.01$; $^{***}P < 0.001$; Student's *t*-test. (**f**,**g**) Boxed region from **d** at higher magnification for visualization of the white matter ectopic neurons, which were NeuN-positive. Scale bars, 20 μm. For **b**,**e**, more than 1,000 GFP$^+$ neurons from three to five brains were analyzed in each group. Error bars, s.e.m.

slices prepared at P15–20. The ectopic neurons at the white matter showed notably low frequency of both glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) and GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs; **Fig. 4a–c** and **Supplementary Fig. 7a–c**). However, the layer II–III neurons overlying the ectopic neurons showed markedly enhanced sEPSC frequency and amplitude when compared to the contralateral intact layer II–III neurons or those electroporated with pSUPER vector (**Fig. 4a–c**). Furthermore, synchronous bursts of sEPSCs were occasionally observed in layer II–III neurons after α2-chimaerin knockdown but were not found in control neurons (**Fig. 4a**). In contrast to the hyperactive sEPSCs, the frequency of sIPSCs in the overlying layer II–III neurons was significantly lower ($P < 0.01$, Student's *t*-test; **Supplementary Fig. 7a–c**). These findings suggest that the balance of circuit excitation and inhibition abnormally shifted toward excitation in the upper cortical layers when α2-chimaerin expression was knocked down.

As altered cortical excitability is closely associated with epileptic seizures, we then examined whether young adult mice (P30) showed abnormal seizure activity after *in utero* suppression of α2-chimaerin. We used pentylenetetrazol (PTZ), a convulsant agent, to induce seizures in unelectroporated, pSUPER-electroporated or α2-chimaerin shRNA–electroporated mice (25 mg per kilogram body weight intraperitoneal administration every 10 min)[25]. Minimal motor seizures including abnormal neck flexion and rearing were induced after 23 injections in all groups. However, the cumulative doses of PTZ and the time to induce the onset of generalized tonic-clonic seizures were significantly lower after α2-chimaerin was depleted (**Fig. 4d**,**e**). This suggests that α2-chimaerin knockdown mice are more susceptible to convulsant-induced seizures. Notably, whereas generalized tonic-clonic seizures were induced ~20 min after the first minimal seizure in control or pSUPER-electroporated mice, the generalized seizures occurred much faster (at ~5 min) when α2-chimaerin was knocked down (**Fig. 4f**). The significantly shortened interval between minimal and generalized seizures suggests an elevated seizure severity in α2-chimaerin knockdown mice.

Neuronal migration requires the SH2 domain of α2-chimaerin

α2-chimaerin possesses a GAP domain that inhibits Rac1 and an SH2 domain that mediates phosphotyrosine-dependent protein interactions (**Supplementary Fig. 8a**)[26]. To dissect how α2-chimaerin regulates neuronal migration and the formation of functional cortex, we re-expressed different α2-chimaerin mutants in the α2-chimaerin knockdown cortex and examined neuronal migration (**Supplementary Fig. 8b**,**c**). A GAP-inactive mutant (R304G)[14] of α2-chimaerin restored migration to a degree similar to that achieved by re-expression of wild-type α2-chimaerin (**Fig. 5a–c**). Furthermore, downregulation of Rac1 activity through overexpression of a dominant-negative (DN) mutant of Rac1 could not rescue the migration defects in α2-chimaerin knockdown neurons (**Supplementary Fig. 8d**,**e**). Thus, the GAP activity of α2-chimaerin is dispensable for neuronal migration. By contrast, re-expression of an SH2 mutant of α2-chimaerin (R73L), which shows reduced binding affinity for phosphotyrosine residues[14], did not rescue

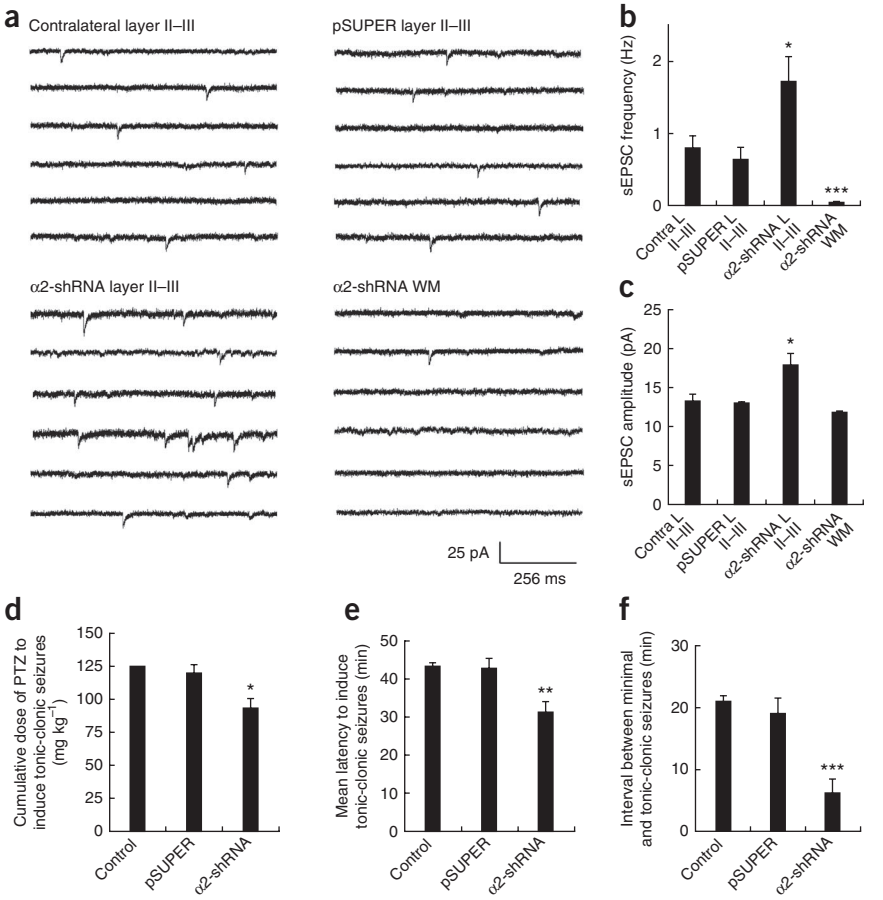

Figure 4 Mice with *in utero* suppression of α2-chimaerin show altered cortical excitability and show enhanced susceptibility to seizures. (**a**–**c**) Increased spontaneous glutamatergic activity (sEPSCs) in the layer II–III cortex after *in utero* suppression of α2-chimaerin. (**a**) Representative traces of sEPSCs recorded at −70 mV. We recorded layer II–III pyramidal neurons of contralateral unelectroporated cortex (contra L II–III), pSUPER vector–electroporated cortex (pSUPER L II–III) or α2-chimaerin shRNA-electroporated cortex (α2-shRNA L II–III) at P15–20. We also recorded ectopic cells accumulated in the white matter after *in utero* electroporation of α2-shRNA (α2-shRNA WM). (**b**) Mean sEPSC frequencies in layer II–III pyramidal neurons of contra, pSUPER, α2-shRNA slices and white matter–trapped ectopic cells of α2-shRNA slices. (**c**) Mean sEPSC amplitudes in layer II–III pyramidal neurons of contra, pSUPER, α2-shRNA slices and white matter-trapped ectopic cells of α2-shRNA slices. The sEPSCs were sensitive to the AMPA-receptor antagonist CNQX, indicating that they were glutamatergic in nature (eight to ten neurons from three or four mice were analyzed in each group). (**d**–**f**) Mice with migration defects after α2-chimaerin knockdown were more susceptible to PTZ-induced seizures. We studied P30 mice with *in utero* electroporation of pSUPER or α2-shRNA, together with age-matched controls (six to eight mice from each group). Data are presented as cumulative PTZ doses (**d**), latency to induce generalized tonic-clonic seizures (**e**) and interval between minimal and generalized seizures (**f**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus pSUPER L II–III for **b**,**c** or versus pSUPER for **d**–**f**; Student's *t*-test. Error bars, s.e.m.

the migration defects in α2-chimaerin knockdown cortex (**Fig. 5a**–**c**). Similarly to what was observed in α2-chimaerin silenced neurons, most of R73L-expressing neurons accumulated in the intermediate zone and lacked a directional leading process (**Fig. 5c**).

To further confirm the critical role of SH2-mediated interaction in neuronal migration, we engineered a short protein fragment containing the N-terminal SH2 domain of α2-chimaerin (α2-SH2-c), which served as a competitive binding partner for phosphotyrosine residues. Ectopic expression of α2-SH2-c in migrating neurons resulted in migration defects similar to those observed when α2-chimaerin was depleted or its SH2 domain was mutated (**Fig. 5d**–**f**). Thus, the SH2 domain–mediated interaction between α2-chimaerin and tyrosine-phosphorylated protein(s) seems to be a key determinant of neuronal migration.

α2-chimaerin modulates Trk-regulated CRMP-2 activity

The SH2 domain of α2-chimaerin is known to couple with receptor tyrosine kinases (RTKs), such as EphA4, or other tyrosine-phosphorylated molecules[15–18,26]. However, we found that knockdown of EphA4 did not disrupt radial migration (data not shown), which is consistent with reports demonstrating that EphA4 does not regulate radial migration during cortical development[27,28]. Another family of RTKs, the Trk receptors, which are activated by the neurotrophins, have been implicated in neuronal migration[29–32]. We found that inhibition of the tyrosine kinase activity of Trk receptors in cortical slices by bath application of a Trk inhibitor, K252a, resulted in significantly retarded migration of electroporated cells (**Fig. 6a**,**b**), confirming the involvement of Trk signaling in radial migration. Consistent with previous findings[30,32], we also found that *in utero* knockdown of TrkB impaired neuronal migration, albeit to a milder extent (**Supplementary Fig. 9a**–**c**). Notably, α2-chimaerin interacted with various neurotrophin receptors and showed relatively stronger binding to TrkB (**Fig. 6c**). This α2-chimaerin-TrkB interaction was mediated by SH2–phosphotyrosine binding and depended on the kinase activity of TrkB (**Fig. 6d** and **Supplementary Fig. 9d**). Furthermore, α2-chimaerin was recruited to the activated TrkB receptors upon treatment with its cognate ligand brain-derived neurotrophic factor (BDNF) in cortical neurons (**Fig. 6e**).

We then asked whether α2-chimaerin participates in neurotrophin-mediated pathways during neuronal migration. Neurotrophin-dependent Trk activation was previously shown to increase the inhibitory phosphorylation of the kinase GSK3β at Ser9, which in turn leads to increased activity of its downstream effector CRMP-2, a microtubule-associated protein, through a decrease in inhibitory phosphorylation at Thr514 (refs. 33,34). We found that both Trk receptor autophosphorylation and GSK3β inhibitory phosphorylation were attenuated in α2-chimaerin knockdown neurons upon BDNF stimulation, suggesting impaired signaling downstream of neurotrophin receptors (**Fig. 6f**–**h** and **Supplementary Fig. 9e**). Unexpectedly, although GSK3β was less inhibited in α2-chimaerin knockdown neurons, CRMP-2 showed no increased phosphorylation. Instead, α2-chimaerin depletion resulted in a further reduction in phosphorylated CRMP-2 (pCRMP-2) upon BDNF stimulation, leading to an abnormally high proportion of activated CRMP-2 (**Fig. 6f**,**i** and **Supplementary Fig. 9e**). A similar effect was observed in α2-chimaerin knockdown neurons when treated with two other neurotrophins, NT3 and NT4, which

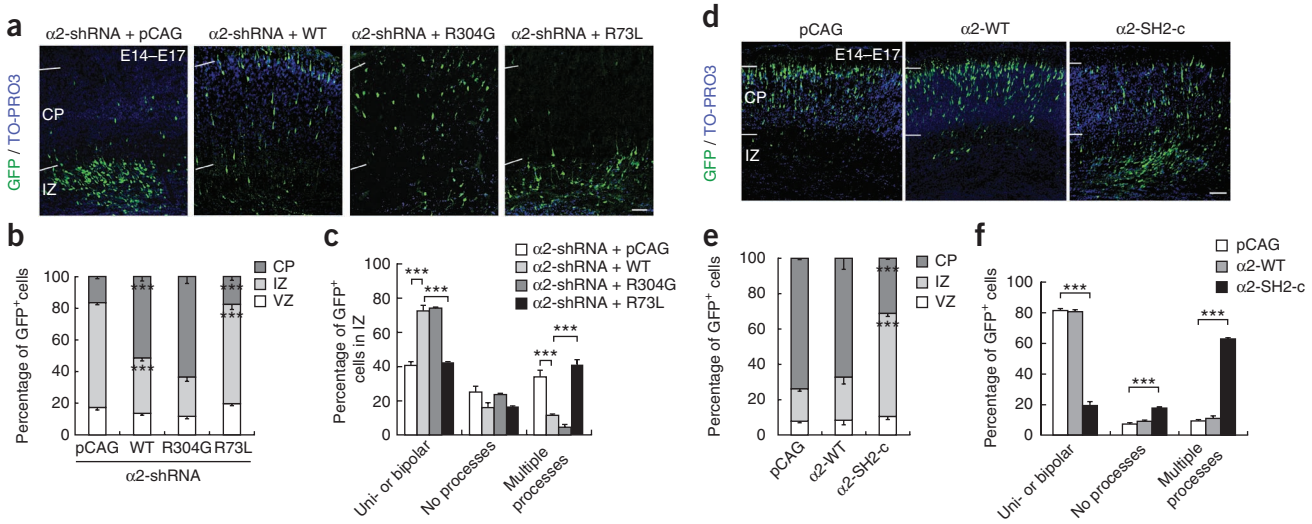

Figure 5 The SH2 domain of α2-chimaerin is required for neuronal migration. (**a**) Expression of R73L α2-chimaerin did not rescue the migration defects after α2-chimaerin knockdown. E14 mouse embryos were electroporated *in utero* with α2-shRNA together with pCAG GFP vector only or GFP plasmids expressing α2-chimaerin wild-type (WT), R304G or R73L. Representative coronal sections at E17 were stained with antibodies to GFP (green) and counterstained with TO-PRO3 (blue). Scale bar, 50 μm. CP, cortical plate; IZ, intermediate zone. (**b**) Quantification of the distribution of GFP$^+$ neurons expressing different α2-chimaerin mutants at E17. VZ, ventricular zone. ***$P < 0.001$ versus α2RNAi + pCAG vector group; Student's *t*-test. (**c**) Percentage of neurons in the IZ with uni- or bipolar morphology, no processes or multiple minor processes. ***$P < 0.001$; Student's *t*-test. (**d**–**f**) Overexpression of α2-SH2-c disrupted radial migration in the cerebral cortex. E14 mouse embryos were electroporated *in utero* with GFP vector or GFP plasmid expressing α2-SH2-c. (**d**) Representative coronal sections at E17 stained with antibodies to GFP (green) and counterstained with TO-PRO3 (blue). Scale bar, 50 μm. (**e**) Quantification of the distribution of GFP$^+$ neurons at E17. ***$P < 0.001$ versus pCAG vector group; Student's *t*-test. (**f**) Quantification of cell morphology at intermediate zone after overexpression of α2-SH2-c. ***$P < 0.001$; Student's *t*-test. More than 1,000 cells for analyzing distribution of GFP$^+$ cells, and more than 150 cells for morphological analysis from three to five brains in each group. Error bars, s.e.m.

are cognate ligands of the TrkC and TrkB receptors, respectively (**Fig. 6f–i**, **Supplementary Fig. 9e** and data not shown).

We further studied whether α2-chimaerin knockdown alters the localization of pCRMP-2 in neurons. pCRMP-2 was normally localized to perinuclear regions and co-localized with stabilized microtubules (marked by acetylated tubulin) on the longest neurite of cultured neurons (**Fig. 6j**). However, in α2-chimaerin knockdown neurons, pCRMP-2 failed to localize to distal ends of any neurite (**Fig. 6j**). α2-chimaerin knockdown neurons were characterized by the absence of a prominent neurite with high level of acetylated tubulin, suggesting impaired microtubule organization (**Fig. 6k**).

Thus, we found that both CRMP-2 phosphorylation and microtubule stabilization were dysregulated in α2-chimaerin silenced neurons. Notably, we showed that precise regulation of the phosphorylation of CRMP-2, and thus its activity, is important for neuronal migration. CRMP-2 was abundantly expressed and highly phosphorylated throughout the developing cortex (**Supplementary Fig. 10a**). Furthermore, overexpression of either a phosphodeficient mutant (T514A) of CRMP-2 or a phosphomimetic mutant (T514E) *in utero* caused substantial inhibition of neuronal migration (**Supplementary Fig. 9b**,**c**), suggesting that either hyperactivation or inactivation of CRMP-2 in neurons disrupts migration.

CRMP-2 acts downstream of α2-chimaerin in radial migration

To further demonstrate the function of CRMP-2 in neuronal migration, we studied whether migration is affected when CRMP-2 is suppressed. *In utero* knockdown of CRMP-2 resulted in accumulation of migrating neurons in the intermediate zone, which distinctly resembled the migration defects generated by α2-chimaerin knockdown (**Fig. 7a**–**b**). Morphological analysis showed that these intermediate zone–trapped CRMP-2–silenced neurons extended multiple minor processes or showed a rounded morphology with no processes (**Fig. 7c**). Consistent with the α2-chimaerin knockdown results, we confirmed that CRMP-2 knockdown did not affect the differentiation of Tbr2$^+$ IPCs (**Supplementary Fig. 11a–c**). Thus, these findings support the notion that CRMP-2 is a downstream target of α2-chimaerin that mediates its function in neuronal migration.

Next we asked whether α2-chimaerin regulates neuronal migration through modulating the activity of CRMP2. As α2-chimaerin knockdown led to abnormally low pCRMP-2 upon BDNF stimulation, we examined whether restoration of pCRMP-2 levels by ectopically expressing CRMP-2 wild-type or T514E could rescue the migration defects generated by α2-chimaerin knockdown. We found that overexpression of CRMP-2 wild-type was sufficient to restore normal migration in α2-chimaerin-knockdown cortex (**Fig. 7d–f**). Of note, whereas expression of CRMP-2 T514E in intact cortex impaired neuronal migration, overexpressing it in α2-chimaerin knockdown cortex restored normal migration (**Fig. 7d–f**). In contrast, CRMP-2 T514A did not rescue the migration defects (**Fig. 7d–f**). Thus, replenishment of phosphorylated CRMP-2 protein indeed reversed the migration defects in α2-chimaerin knockdown cortex. Together, these findings show that the α2-chimaerin-regulated CRMP-2 phosphorylation and activation is a crucial molecular event that controls neuronal migration.

DISCUSSION

The brain-specific protein α2-chimaerin was previously identified as an essential RacGAP for axon guidance in both motor and ocular system[15–17,19]. Our findings here identify α2-chimaerin as a key regulator of radial migration. We show that the function of α2-chimaerin in neuronal migration is not mediated by its GAP activity but rather through its regulation of CRMP-2. Notably, disruption of α2-chimaerin–mediated neuronal migration leads to aberrant communication in the

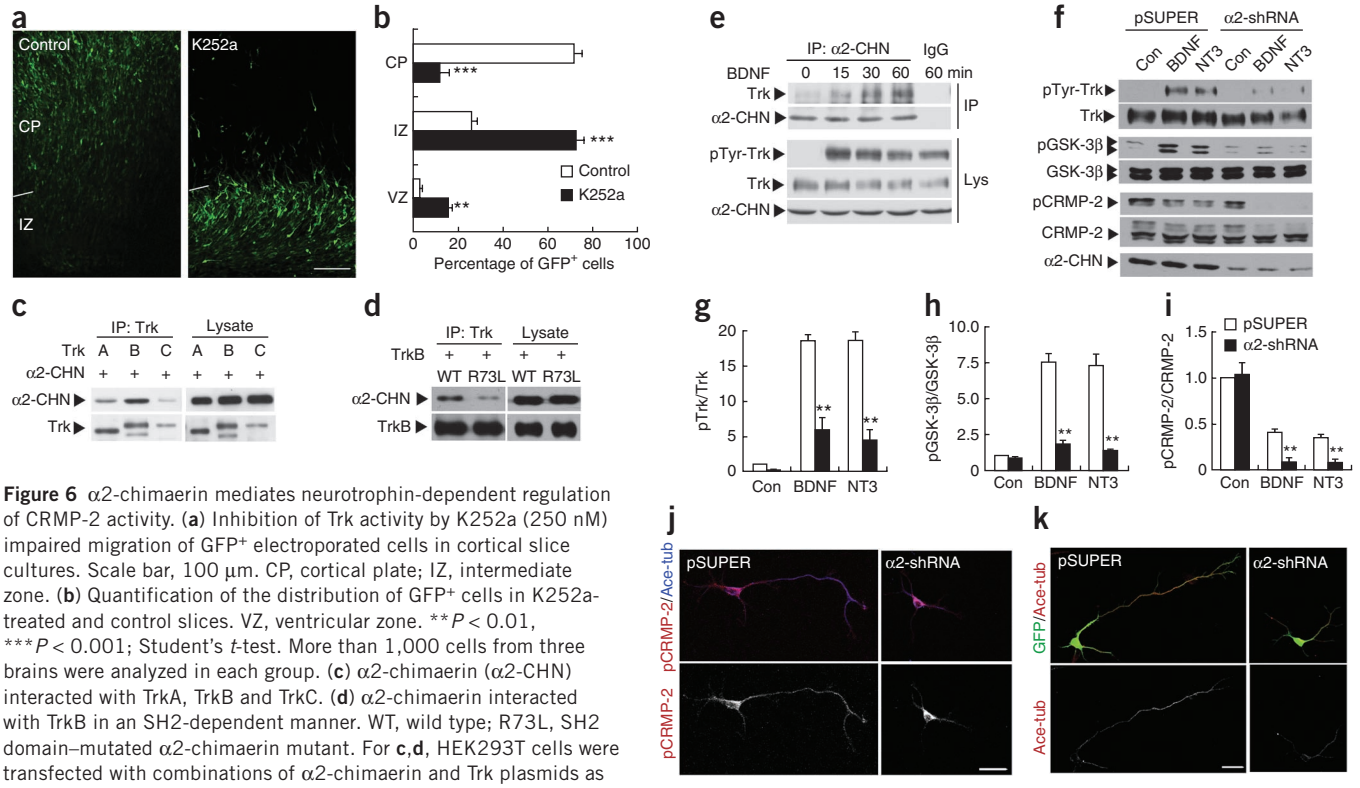

Figure 6 α2-chimaerin mediates neurotrophin-dependent regulation of CRMP-2 activity. (**a**) Inhibition of Trk activity by K252a (250 nM) impaired migration of GFP$^+$ electroporated cells in cortical slice cultures. Scale bar, 100 μm. CP, cortical plate; IZ, intermediate zone. (**b**) Quantification of the distribution of GFP$^+$ cells in K252a-treated and control slices. VZ, ventricular zone. $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test. More than 1,000 cells from three brains were analyzed in each group. (**c**) α2-chimaerin (α2-CHN) interacted with TrkA, TrkB and TrkC. (**d**) α2-chimaerin interacted with TrkB in an SH2-dependent manner. WT, wild type; R73L, SH2 domain–mutated α2-chimaerin mutant. For **c**,**d**, HEK293T cells were transfected with combinations of α2-chimaerin and Trk plasmids as indicated, followed by co-immunoprecipitation (IP). (**e**) α2-chimaerin was recruited to TrkB in cortical neurons upon BDNF treatment. Cortical neurons (4 d *in vitro*) were treated with BDNF (100 ng ml^{-1}) at the indicated time points, followed by co-IP. Lys, lysate; IgG, immunoglobulin G. (**f**) α2-chimaerin regulated neurotrophin-mediated signaling. pSUPER- or α2-shRNA–transfected cortical neurons (4 d *in vitro*) were treated with BDNF or NT3 or were untreated (Con) (100 ng ml^{-1}) for 30 min. (**g**–**i**) Quantification of tyrosine-phosphorylated (pTyr)-Trk (**g**), phosphorylated GSK-3β (pGSK-3β) (**h**) and pCRMP-2 (**i**) after α2-chimaerin knockdown and BDNF or NT3 treatment (Con, untreated) (fold change). $^{**}P < 0.01$; Student's *t*-test. (**j**) Cortical neuron cultures (2 d *in vitro*) from cortex electroporated with GFP and pSUPER or α2-shRNA, stained for pCRMP-2 (red) and acetylated tubulin (ace-tub; blue). Scale bar, 20 μm. (**k**) α2-chimaerin knockdown neurons were characterized by the absence of one acetylated tubulin–positive neurite. Scale bar, 20 μm. Error bars, s.e.m.

cortical network and increased seizure susceptibility in mice. Together, these data establish a new role of α2-chimaerin in neuronal migration, a process that is critical for normal cortical development and balanced excitation and inhibition in cortical circuits. We show that α2-chimaerin targets the multipolar-to-bipolar transition during neuronal migration. The multipolar stage is highly dynamic and has been suggested as a vulnerable point for disruptions that are associated with neocortical developmental disorders[11]. The transition from multipolar to bipolar cells requires active rearrangement of microtubules. In particular, stabilization of microtubules by local acetylation of tubulins is important for the establishment and maintenance of leading processes. Formation of such stabilized leading processes is required for controlling centrosome positioning and forward movement, as well as providing structural supports for intracellular vesicle transport[4,35]. Although several microtubule regulators, such as Cdk5, Dcx and Lis1, are essential for the exit of neurons from the multipolar stage[36–38], the detailed mechanism underlying the regulation of process stability is not fully understood. Here we identify α2-chimaerin as a critical regulator of bipolar transition through the control of neurite stability. This notion is supported by our time-lapse experiments demonstrating that α2-chimaerin–suppressed neurons show impaired process dynamics, reduced process lifetime and a lack of stabilized microtubule (marked by the absence of acetylated tubulin).

CRMP-2 is a microtubule-associated protein previously reported as a critical regulator of neuronal polarization *in vitro*[39]. Nonetheless, the *in vivo* role of CRMP-2 in cortex development has remained unclear. Here we demonstrate that CRMP-2 is essential for neuronal migration. Gain-of-function studies further show that CRMP-2 phosphorylation, and thus its activity, is tightly regulated to mediate proper neuronal migration (**Supplementary Fig. 10b**,**c**). Replenishment of phosphorylated CRMP-2 restored normal migration in α2-chimaerin knockdown cortex, strongly suggesting that precise regulation of CRMP-2 activity in migrating neurons at the multipolar stage is sufficient for breaking the cell symmetry and stabilizing one process as the leading process for radial migration. We thus propose that α2-chimaerin directs the formation of a stabilized leading process by regulating the precise activity and localization of CRMP-2. We speculate that α2-chimaerin may function to modulate the interaction of CRMP-2 with its upstream kinases. α2-chimaerin has been shown to exist as a signaling complex with Cdk5 and CRMP-2 (ref. 40). We also found that α2-chimaerin interacts with GSK-3β in cortical neurons in response to BDNF stimulation (data not shown). Whether these interactions are involved in the molecular mechanism underlying α2-chimaerin-mediated CRMP-2 regulation awaits further investigation.

Polarity establishment and directional movement of neurons during migration are triggered by extracellular signals in the microenvironment[3]. We have shown that α2-chimaerin requires binding of its SH2 domain to phosphotyrosine to trigger exit from the multipolar stage and initiate migration, suggesting that tyrosine phosphorylation is an essential molecular event for neuronal migration. Indeed, the SH2 domain is a common scaffolding module linking RTKs and tyrosine-phosphorylated cytoplasmic signaling molecules to a variety

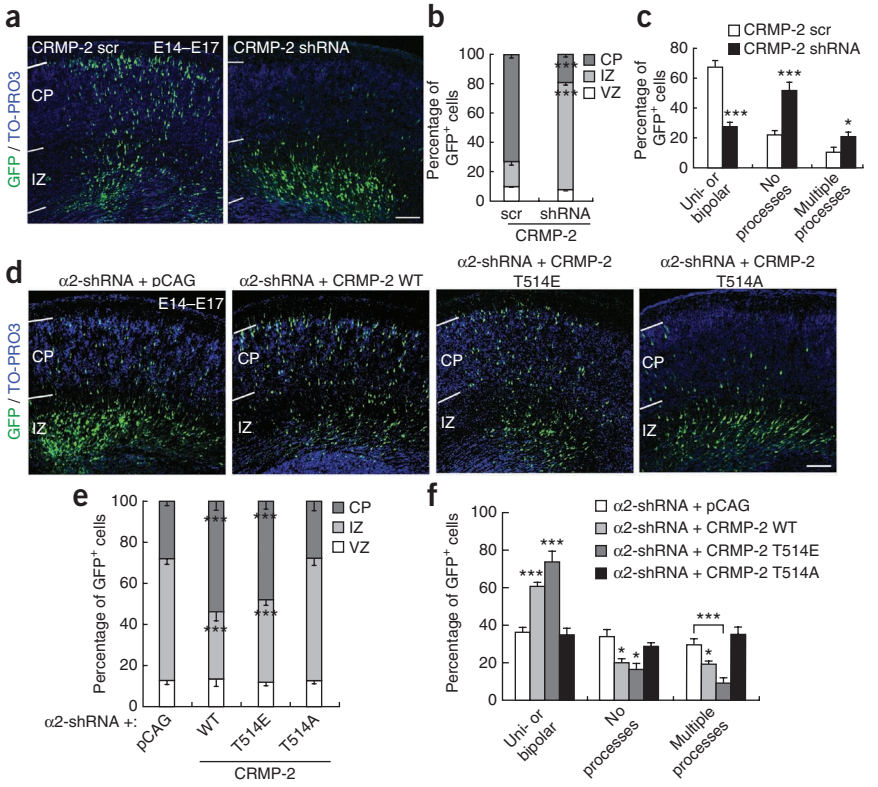

Figure 7 α2-chimaerin mediates neuronal migration through precise regulation of CRMP-2 activity. (**a**) *In utero* knockdown of CRMP-2 disrupted radial migration in the cerebral cortex. E14 mouse embryos were electroporated *in utero* with GFP plasmid together with CRMP-2 scrambled shRNA (scr) or CRMP-2 shRNA. Representative coronal sections at E17 were stained with GFP antibody (green) and counterstained with TO-PRO3 (blue). CP, cortical plate; IZ, intermediate zone. Scale bar, 100 μm. (**b**) Quantification of the distribution of GFP$^+$ neurons at E17. VZ, ventricular zone. $^{**}P < 0.01$; $^{***}P < 0.001$; Student's *t*-test. (**c**) Quantification of cell morphology in the IZ after CRMP-2 knockdown. $^{***}P < 0.001$; Student's *t*-test. (**d**) GFP plasmids expressing CRMP-2 wild-type (WT), T514A or T514E mutants or GFP vector only were electroporated *in utero* together with α2-chimaerin shRNA (α2-shRNA) at E14. Representative coronal sections at E17 were stained with antibodies to GFP (green) and counterstained with TO-PRO3 (blue). Scale bar, 100 μm. (**e**) Quantification of the distribution of GFP$^+$ neurons at E17. $^{***}P < 0.001$ versus α2-shRNA + pCAG vector group; Student's *t*-test. (**f**) Quantification of cell morphology in the IZ. $^{*}P < 0.05$, $^{***}P < 0.001$, versus α2-shRNA + pCAG vector group; Student's *t*-test. More than 1,000 cells for analysis of distribution of GFP$^+$ cells, and more than 150 cells for morphological analysis from three to five brains in each group. Error bars, s.e.m.

of downstream effectors[41]. We demonstrate that α2-chimaerin is recruited to activated neurotrophin receptors in an SH2-dependent manner, thus participating in neurotrophin-dependent regulation of CRMP-2 activity (**Fig. 6c–i**). It is noteworthy that knockdown of TrkB leads to a mild delay in neuronal migration, whereas blockade of Trk activation by the general inhibitor K252a disrupts migration. This could be due to the incomplete knockdown of endogenous TrkB or the presence of redundant pathways, such as those mediated by other neurotrophin receptors, in neuronal migration. Furthermore, other tyrosine kinase–dependent signaling pathways, including those induced by Reelin, semaphorin and β-amyloid precursor protein, are essential for cortex development[42–44]. Thus, it remains to be addressed whether α2-chimaerin transduces signals from a divergent signaling network by means of its SH2-mediated binding to regulate migratory behavior of neurons.

Cortical dysplasia and epileptic seizures are observed in humans with psychiatric disorders such as schizophrenia and autism. Emerging evidence suggests that imbalance between excitatory and inhibitory synaptic activity in cortical microcircuits is one of the underlying causes contributing to the clinical symptoms of these diseases[4]. In support of this idea, the present study reveals that impaired neuronal migration by *in utero* suppression of α2-chimaerin results in imbalance of excitation and inhibition in cortical microcircuits and increased seizure susceptibility. Notably, we find that the presence of ectopic neurons in the deep layers leads to reduced inhibitory drive in both the ectopic layers and the overlying cortex, likely contributing to the increased cortical excitability and the epileptic activity. This finding is consistent with the understanding that locally misplaced neurons, even though present only in small numbers, may result in errors in cortical excitation and lead to epileptic seizures[45]. The current demonstration of the role of α2-chimaerin in neuronal migration thus opens a new avenue for research on epileptic seizures. It is of interest that migration defects in *Chn1*$^{-/-}$ mice were not comparable to those induced by α2-chimaerin knockdown, which is reminiscent of the discrepant phenotypes when other important regulators, such as Dcx and β-amyloid precursor protein, are genetically knocked out versus *in utero* knocked down[36,43]. Thus, the potential compensatory mechanisms by which normal migration is maintained in *Chn1*$^{-/-}$ mice will be worth investigating further. *CHN1* has been reported as a susceptibility gene in both schizophrenia and autism[20,21]. Moreover, mRNA expression of *Chn1* in rodent frontal cortex is regulated in response to antipsychotic drugs[46]. In this context, further studies on the genetic involvement of α2-chimaerin in psychotic conditions and understanding of the molecular and cellular events mediated by α2-chimaerin may provide insights to the pathogenesis of psychiatric disorders.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

N.Y.I. supervised the project. J.P.K.I., L.S., Y.C., W.-Y.F., A.K.Y.F. and N.Y.I. designed the experiments. J.P.K.I. and L.S. conducted the majority of experiments.

J.P.K.I., L.S., Y.C., A.K.Y.F. and N.Y.I. performed the data analyses. Y.I. and Y.G. provided technical support on *in utero* electroporation and live-imaging experiments. J.P.K.I. and Y.I. performed live-imaging experiments. W.-H.Y. performed the electrophysiology experiments and subsequent data analyses. A.B. provided *Chn1*$^{-/-}$ mice. J.P.K.I., L.S., A.K.Y.F. and N.Y.I. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Constructs, antibodies and chemicals. pSUPER shRNA targeting α2-chimaerin (α2-shRNA) and expression constructs including α2-chimaerin wild-type, R73L and R304G have been described[18]. α2-chimaerin-SH2-c (amino acids 1–262) was generated by PCR amplification. The corresponding scrambled sequence of α2-shRNA was 5′-GTTCATATGTTCACCTATT-3′. The shRNA target sequence of α2-shRNA-2 was 5′-ACATATGCCAGTCCTGAAA-3′. The shRNA target sequence for CRMP-2 was 5′-CAGTGCTCTTGAGTACAAC-3′, and its scrambled sequence was 5′-GCACTTGAATCGGTACCAT-3′. Wild-type CRMP-2 was a generous gift from C. Hall. shRNA-resistant constructs of wild-type and mutants of α2-chimaerin and CRMP-2 were generated with the QuikChange mutagenesis kit (Stratagene). The shRNA target sequence for TrkB was 5′-CAAGCTGACGAGTTTGTCC-3′, and its corresponding scrambled sequence was 5′-CAGTCGCGTTTGCGACTGG-3′ (ref. 32). TrkA, TrkB wild-type and kinase-dead, and TrkC constructs have been described[47]. For *in utero* electroporation, α2-chimaerin and CRMP-2 expression constructs were subcloned into pCAG2IG vector, which contains an insert site followed by an IRES and the GFP coding sequence under the control of the CAG promoter[48].

Tuj1, CS-56, α-tubulin and acetylated tubulin antibodies were purchased from Sigma; MAP2, NeuN and CRMP-2 antibodies were from Chemicon; Cutl1 and pan-Trk antibodies were from Santa Cruz; tyrosine-phosphorylated Trk and activated-caspase-3 antibodies were from Cell Signaling; GFP antibody was from MBL; BLBP, Ctip2 and Tbr2 antibodies were from Abcam; BrdU antibody was from Abdserotec. Phospho-Thr514 CRMP-2 antibody was a generous gift from K. Kaibuchi, and α2-chimaerin antibody was previously described[18]. PTZ and BrdU were purchased from Sigma, TO-PRO3 was from Invitrogen, 4′,6-diamidino-2-phenylindole (DAPI) was from Zymed and K252a was from Calbiochem.

***In utero* electroporation and immunohistochemistry.** E14 ICR-strain mice were used for *in utero* electroporation as described previously[48]. Briefly, the mixed plasmid DNA solution (1 μl) was injected into the lateral ventricle of each embryo in a pregnant ICR mouse at E14. pSUPER shRNA was mixed with pCAG2IG vector expressing GFP at 1:1 ratio (1 μg: 1 μg) with a final concentration of 2 μg μl^{-1}. In rescue experiments, pSUPER shRNA was mixed with indicated pCAG2IG expressing constructs at a 1:3 ratio (1 μg: 3 μg) with a final concentration of 4 μg μl^{-1}. An electrode (CUY21E, Nepagene) was positioned flanking the ventricular zones of each embryo and pulsed four times at 35 V for 50 ms, with intervals of 1 s, using an electroporator (EM830, BTX). The uterine horn was then returned to the abdominal cavity to allow the embryos to continue to develop. The embryos were removed at the indicated day after electroporation. The brains were perfused, dissected out and fixed with 4% (wt/vol) paraformaldehyde for 2 h. After fixation, the brains were moved to 20% (wt/vol) sucrose for 4 h, followed by 30% sucrose overnight. Coronal sections were prepared by cryostat and subjected to immunohistochemistry. Brain sections were permeabilized and blocked in Tris-buffered saline (TBS) with 0.1% Triton X-100 and 3% (wt/vol) BSA for 30 min at room temperature 25 °C, followed by incubation with specific primary antibodies at 4 °C overnight. Sections were washed with TBS with 0.1% Triton X-100 for 20 min and incubated with appropriate fluorescence-conjugated secondary antibodies for 1 h at room temperature 25 °C. *z*-series confocal images were collected at 1-μm steps with ten optical sections in Fluoview (Olympus). Subregions of the cortex were identified on the basis of cell density (nuclear staining by DAPI or TO-PRO3). All animal studies were approved by the Animal Care Committee at the Hong Kong University of Science and Technology in accordance with institutional guidelines.

Statistical analyses. Data are presented as mean ± s.e.m. Statistical significance was determined using unpaired Student's *t*-test.

BrdU injection. E14 pregnant female *Chn1*$^{+/-}$ mice mated with *Chn1*$^{+/-}$ male mice[17] received a single injection of BrdU at 50 mg kg^{-1}. Brains were perfused, fixed and sectioned at E17. Sections were treated with 1 M HCl at 45 °C for 30 min, then incubated at 4 °C overnight with mouse antibody to BrdU and rabbit antibody to MAP2. The genotypes of the embryos were determined.

Cortical slice cultures and live-imaging analysis. *In utero* electroporation was performed at E14. Two days after electroporation, electroporated brains were rapidly removed and coronal slices (300 μm) were prepared using a vibratome. Cortical slices were then grown on Millicell inserts (Millipore) in Neurobasal medium (Invitrogen) supplemented with 2% (vol/vol) B27 (Invitrogen). Time-lapse images were captured every 15 min for 13 h for low magnification images (10× objective) or every 10 min for 7 h for high magnification images (40× objective) with a confocal microscope (Leica SP5). *z*-series confocal images were collected at 3-μm steps with 10–15 optical sections. For drug treatment, cortical slices were settled for 2 h after preparation and treated with K252a or DMSO by bath application for 18 h. To visualize distribution of GFP^+ cells, *z*-series confocal images were collected at 3-μm steps with 10 optical sections with a confocal microscope (Nikon A1).

Electrophysiology. *In utero* electroporation was performed at E14. Electrophysiological recordings were performed on cortical slices prepared at P15–20. Briefly, mice were anesthetized and brains were rapidly dissected in ice-cold artificial cerebrospinal fluid (ACSF) with the composition 126 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $NaHPO_4$ and 10 mM glucose (95% O_2, 5% CO_2, pH 7.4). Coronal brain slices (250 μm) prepared using a vibratome were kept in oxygenated ACSF at 25 °C for 1 h before recordings. The slices were transferred to a chamber mounted onto a fixed-stage upright microscope (Olympus BX51WI) equipped with an epifluorescence attachment and were superfused with ACSF at physiological temperature. Conventional whole-cell recordings were made from target cortical neurons, including GFP^+ neurons, aided by differential interference contrast optics. Microelectrodes had a resistance 6–8 MΩ. All electrophysiological data were digitized by the Digidata-pClamp system and analyzed with MiniAnalysis (Synaptosoft).

Seizure induction with PTZ. For PTZ-induced seizures, *in utero* electroporation of pSUPER vector or α2-chimaerin shRNA was performed in E14 ICR mice. Examination of PTZ-induced seizures was performed at P30[25]. Mice without electroporation or electroporated with pSUPER vector or α2-chimaerin shRNA were injected intraperitoneally with PTZ (25 mg kg^{-1}) every 10 min until generalized seizures occurred (normally within 1 h). After injection, mice were immediately returned to the test cage for seizure activity recording. Seizure behavior was scored based on Racine's scale[49]. After recordings, mice were killed to confirm the success of electroporation of pSUPER or α2-chimaerin shRNA by visualizing the distribution of GFP^+ cells.

Cell cultures and transfection. Primary cortical neurons were prepared and cultured from E18 rat embryos as previously described[50]. Transfection of dissociated neurons was performed by the Amaxa Nucleofector system (Lonza AG). Cortical neurons (1×10^7 per plate) were plated on 100-mm culture dishes coated with poly-D-lysine (0.1 mg ml^{-1}; Sigma) and fed with Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen). Coimmunoprecipitation and western blot analysis were performed 3–5 d after plating. For studying the distribution of pCRMP-2 and stabilized microtubules, primary cortical neuron cultures were prepared from E16 ICR mice 2 d after *in utero* electroporation of pSUPER or α2-chimaerin shRNA. Cells were fixed and immunostained for GFP, α2-chimaerin, pCRMP-2 and acetylated tubulin after 2 d *in vitro*.

HEK-293T cells were cultured in DMEM (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS plus antibiotics. Transfections of HEK-293T cells were performed by mixing Lipofectamine Plus (Invitrogen) with DNA in serum-free medium. After 5 h, serum-free medium was replaced by DMEM plus 10% heat-inactivated FBS. After 24 h, cells were collected and subjected to co-immunoprecipitation and western blot analysis.

Protein extraction and immunoprecipitation. For coimmunoprecipitation studies, HEK-293T cells, cultured neurons, or brain tissues were lysed in lysis buffer (100 mM Tris, pH 8.5, 100 mM NaCl, 1 mM EDTA) with 0.5% Nonidet P-40 and a cocktail of protease inhibitors. The lysates were then incubated with the appropriate antibody (1–2 mg) at 4 °C for 3 h and incubated with 20 ml of protein G Sepharose (GE) at 4 °C for 1 h. The samples were washed with lysis buffer and resuspended in SDS sample buffer.

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