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Zebrafish Prickle1b mediates facial branchiomotor neuron migration via a farnesylation-dependent nuclear activity

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SUMMARY

The facial branchiomotor neurons (FBMNs) undergo a characteristic tangential migration in the vertebrate hindbrain. We previously used a morpholino knockdown approach to reveal that zebrafish *prickle1b* (*pk1b*) is required for this migration. Here we report that FBMN migration is also blocked in a *pk1b* mutant with a disruption in the consensus farnesylation motif. We confirmed that this lipid modification is required during FBMN migration by disrupting the function of farnesyl biosynthetic enzymes. Furthermore, farnesylation of a tagged Pk1b is required for its nuclear localization. Using a unique rescue approach, we have demonstrated that Pk1b nuclear localization and farnesylation are required during FBMN migration. Our data suggest that Pk1b acts at least partially independently of core planar cell polarity molecules at the plasma membrane, and might instead be acting at the nucleus. We also found that the neuronal transcriptional silencer REST is necessary for FBMN migration, and we provide evidence that interaction between Pk1b and REST is required during this process. Finally, we demonstrate that REST protein, which is normally localized in the nuclei of migrating FBMNs, is depleted from the nuclei of Pk1b-deficient neurons. We conclude that farnesylation-dependent nuclear localization of Pk1b is required to regulate REST localization and thus FBMN migration.

KEY WORDS: Facial branchiomotor neurons, Prickle1b, Neuronal migration, REST, Farnesylation, Zebrafish

INTRODUCTION

The facial branchiomotor neurons (FBMNs) undergo a characteristic tangential migration through the segmented vertebrate hindbrain. In the zebrafish (*Danio rerio*), FBMNs are born beginning at 16 hours post-fertilization (hpf) in rhombomere (r) 4. Shortly after birth, these neurons undergo caudal migration to r6 and r7 (for reviews, see Chandrasekhar, 2004; Song, 2007). Among many proteins known to regulate this migration in zebrafish are several core planar cell polarity (PCP) components (reviewed by Wada and Okamoto, 2009a; Wada and Okamoto, 2009b). These molecules include Vang-like 2 (Vangl2) (Jessen et al., 2002; Bingham et al., 2002); Frizzled 3a (Fzd3a), Cadherin EGF LAG seven-pass G-type receptor 1a (Celsr1a), Celsr1b, Celsr2 (Wada et al., 2006); Scribble 1 (Scrib) (Wada et al., 2005); Prickle1a (Pk1a) (Carreira-Barbosa et al., 2003); and Pk1b (Rohrschneider et al., 2007). Several characteristics of Pk1b make it unique among these PCP components. First, the expression of *pk1b* transcripts is elevated in migrating FBMNs, whereas other components are expressed more broadly throughout the neuroepithelium. Second, overexpression of *pk1b* mRNA neither disrupts FBMN migration nor rescues the Pk1b morphant phenotype (this study). This is in contrast to results with other PCP components and is inconsistent with the prevailing model of PCP interactions (for reviews, see Klein and Mlodzik, 2005; Wang and Nathans, 2007): the expression and localization of core PCP proteins are tightly regulated among cells, such that reductions or elevations of protein levels result in the same mispolarization phenotype. Third, transplantation experiments indicate that Pk1b functions primarily cell-autonomously within FBMNs (Rohrschneider et al., 2007), whereas other PCP components function primarily non-cell-autonomously (Jessen et al., 2002; Wada et al., 2006). Together, these observations suggest that Pk1b might function independently of other PCP components.

The human PRICKLE1 (PK1) homolog has been demonstrated to interact directly with the transcriptional repressor RE1-silencing transcription factor (REST) (Shimojo and Hersh, 2003; Shimojo and Hersh, 2006). REST plays numerous roles in many cell types, including repression of neuronal genes in non-neuronal cells and regulation of the terminal differentiation of neurons (reviewed by Ballas and Mandel, 2005; Qureshi and Mehler, 2009). Interaction with PK1 influences REST nuclear localization and therefore its repressive ability in cell culture (Shimojo and Hersh, 2003; Shimojo and Hersh, 2006; Bassuk et al., 2008). Recently, a mutation in PK1 that reduces binding to REST has been linked to the autosomal recessive syndrome progressive myoclonus epilepsy with ataxia (PME) (Bassuk et al., 2008). Although PK1 expression has been observed in several neuronal subtypes in human cortex and cerebellum, it is currently unclear how PK1 and/or REST function in vivo, and how their dysfunction might contribute to the PME syndrome.

We have undertaken a structure-function analysis of zebrafish Pk1b to better understand the mechanism through which this molecule influences FBMN migration. We find that Pk1b is capable of localizing to the nucleus, and that this localization requires Pk1b farnesylation, a type of post-translational protein prenylation that directs farnesyl lipid attachment and facilitates association with membranes (reviewed by McTaggart, 2006). Further, we find that cell-autonomous nuclear localization of Pk1b

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is required for FBMN migration. Consistent with the apparent necessity of Pk1b farnesylation for FBMN migration, we describe a new zebrafish mutant with a disruption in the *pk1b* farnesylation motif that blocks FBMN migration. Furthermore, we demonstrate that REST function is required during FBMN migration, and that REST is expressed in FBMNs. Finally, we present evidence suggesting that Pk1b interacts with REST to localize this transcriptional silencer to FBMN nuclei. We propose that REST functions in these neurons to suppress their terminal maturation and thus to maintain them in an immature migratory state until they reach their final destination within the hindbrain.

MATERIALS AND METHODS

Fish lines and husbandry

Zebrafish were maintained following standard procedures. Embryos were maintained at 28.5°C and staged as described (Kimmel et al., 1995). Transgenic lines were used as described: Tg(*islet1*:GFP) (Higashijima et al., 2000) and Tg(*zCREST1*:membRFP) (Mapp et al., 2010). *pk1b*^{fh122} fish were generated in a forward screen for FBMN migration defects (see Fig. S1 in the supplementary material). *hmgcrb*^{s617} mutants were isolated from a forward screen of enhancers of the *wnt11* (*slb*) gastrulation phenotype (M.T., unpublished). Mapping confirmed that this mutation was equivalent to that described by D'Amico et al. (D'Amico et al., 2007).

Morpholino design and microinjection

Translation-blocking morpholinos (MOs; Gene Tools) were designed against *fntb* (5′-GCTCTCAAAACTCATGCACTGGGAC-3′) and *pggt1b* (5′-ATCCACCGACTCAAAATCCGCCATC-3′). Other MOs were injected as previously described: splice-blocking Pk1b (Rohrschneider et al., 2007); translation-blocking Hmgcrb (D'Amico et al., 2007); and splice-blocking REST (Gates et al., 2010). MOs were injected at 2 ng/nl.

Microscopy and data analysis

Fixed embryos were imaged on a Zeiss LSM510 confocal microscope. Data were analyzed using ImageJ (NIH) and statistical analysis was performed using Prism (GraphPad).

Generation of Pk1b full-length and mutant constructs

Full-length *pk1b* cDNA (accession NM_001030098) was isolated from a zebrafish shield library as previously described (Carreira-Barbosa et al., 2003).

Venus-Pk1b was generated by inserting the coding sequence for Venus, plus a flexible linker (TSGGSGGGGSGGGSSGEF), upstream of Pk1b in pCS2+. Pk1b mutant constructs were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). For primers used in construction, see Table S1 in the supplementary material. myr/palmVenus-Pk1bΔNLS was generated by inserting the myristoylation/palmitoylation sequence from the murine Lck protein (MGCVCS; accession CAQ51633) immediately upstream of the Venus coding sequence.

mCherry-zhREST4 generation

The mCherry-tagged full-length zebrafish REST (accession DQ377344) construct was kindly provided by Howard Sirotkin (Gates et al., 2010). The mCherry-zhREST4 construct is a hybrid of the zebrafish REST sequence (amino acids 1-315) and the final six amino acids of human REST4 (ECDLVG). For primers used in construction, see Table S1 in the supplementary material.

mRNA generation and microinjection

Capped mRNA was generated using the MEGAscript Sp6 Kit (Ambion). mRNA constructs were injected at 50 ng/μl, with the exception of mCherry-zhREST4 (400 ng/μl).

Subcellular localization measurements

To determine subcellular localization of Venus-Pk1b constructs, injected embryos were incubated until 5 hpf and fixed. Nuclei were counterstained with TO-PRO-3 (Invitrogen) and plasma membrane (cortical actin) was labeled with Rhodamine phalloidin (Invitrogen). Fluorescence intensity was measured in the nuclei, in the cytoplasm and at the plasma membrane of single confocal sections, and average ratios calculated.

To quantify localization of REST protein, individual neurons in each rhombomere were scored. Localization was characterized based on REST localization in the nuclei relative to the cytoplasm. The percentage of neurons displaying each localization pattern was calculated.

Pharmacological treatments

All pharmacological inhibitors were suspended in DMSO and stored at –20°C. Embryos were injected at the 1- to 2-cell stage with 6 mM L-744,832 (L-744; Calbiochem, 422720), 3 mM GGTI-2147 (Calbiochem, 345885), or DMSO (control). For subcellular localization experiments, embryos were soaked in 25 μM atorvastatin (LKT Laboratories, L7658), 100 μM terbinafine (Sigma, T8826), or DMSO diluted in embryo medium, beginning at the 2- to 4-cell stage. For FBMN migration experiments, embryos were soaked in 25 μM atorvastatin, 200 μM terbinafine, or DMSO, beginning at 16 hpf.

Cranial branchiomotor neuron (CBMN)-specific expression rescue experiment

To generate rescue constructs, sequences encoding full-length and mutant Venus-Pk1b were inserted downstream of the *zCREST1* enhancer (Uemura et al., 2005) and a minimal promoter, in a plasmid containing Tol2 transposition sequences (Kawakami and Shima, 1999). DNA encoding each rescue construct (40 ng/μl) and Tol2 *transposase* mRNA (40 ng/μl), with or without Pk1bMO (2 ng/nl), were injected into Tg(*zCREST1*:membRFP) embryos at the 1- to 2-cell stage. Embryos were screened at 48 hpf for Venus expression in the CBMNs.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, and immunohistochemistry was performed as previously described (Prince et al., 1998) using the following primary antibodies: EphA4 [generous gift from David Wilkinson (Irving et al., 1996)] and REST (Abcam, ab21635).

RESULTS

A mutation in the Pk1b farnesylation motif blocks FBMN migration

Pk1b contains one PET domain, three LIM domains, three nuclear localization signals (NLSs) and a farnesylation motif (CaaX), as has been reported previously for other Pk homologs (Jenny et al., 2003; Shimojo and Hersh, 2003) (Fig. 1A). Cell culture studies have demonstrated that the CaaX and NLS motifs are important for nuclear localization (Shimojo and Hersh, 2003; Shimojo and Hersh, 2006); however, the importance of these domains has not been studied extensively in vivo.

In a forward genetic screen using Tg(*islet1*:GFP) transgenic zebrafish, which express GFP in branchiomotor neurons (Higashijima et al., 2000), we identified a mutation in *pk1b*, designated in the screen as *fh122*, that displays a specific defect in the caudal migration of FBMNs. Morphologically, *pk1b*$^{fh122/fh122}$ embryos appear normal at 48 hpf (Fig. 1B,C). However, FBMNs in *pk1b* $^{fh122/fh122}$ mutants do not migrate out of r4 (Fig. 1D,E), consistent with our previously reported Pk1b morpholino (MO) knockdown phenotype (Rohrschneider et al., 2007). The *pk1b*$^{fh122/fh122}$ mutation was mapped to nucleotide 2606 in the *pk1b* coding sequence (see Fig. S1 in the supplementary material), and results in a missense mutation in the farnesylation motif (C869F; Fig. 1A,F). As this cysteine is obligatory for attachment of the farnesyl group (for a review, see McTaggart, 2006), it is expected that the *pk1b*^{fh122} mutants will completely lack farnesylation of the Pk1b protein.

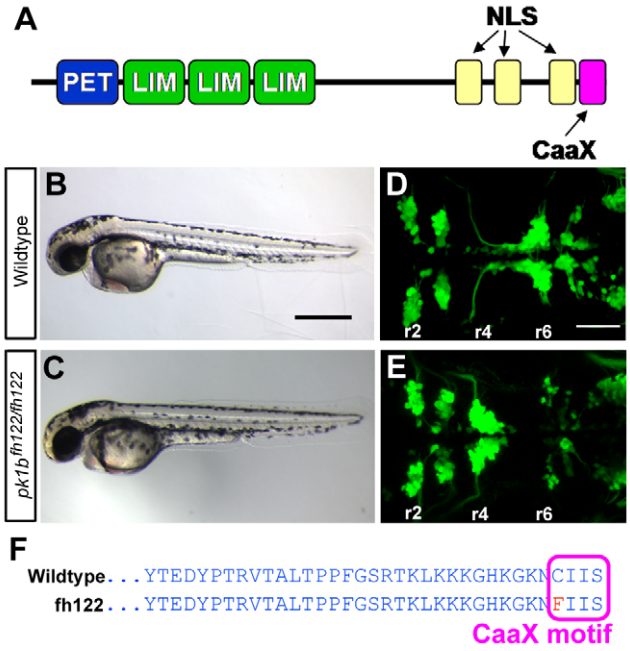

Fig. 1. *prickle1b* is disrupted in zebrafish *fh122* mutants. (**A**) The domain structure of Pk1b. NLS, nuclear localization signal. (**B**,**C**) Lateral brightfield views of 48 hpf wild-type (WT) (A) and *pk1b*$^{fh122/fh122}$ (B) embryos. *pk1b*$^{fh122/fh122}$ embryos are morphologically normal. Anterior is to the left. (**D**,**E**) Maximum projection dorsal views of cranial branchiomotor neurons (CBMNs) in 48 hpf Tg(*islet1*:GFP) embryos. Rhombomeres are indicated (r2-r6). In WT embryos (C), facial branchiomotor neuron (FBMN) cell bodies migrate from r4 to r6. In *fh122* mutants (D), FBMNs fail to undergo caudal tangential migration and cell bodies cluster in r4. (**F**) Amino acid sequence of Pk1b in WT and *pk1b*$^{fh122/fh122}$ fish. The *fh122* mutation results in a C-to-F transition in the Pk1b farnesylation (CaaX) motif. Scale bars: 500 μm in B; 50 μm in D.

HMG-CoA reductase and Farnesyltransferase activities are required for FBMN migration

Disruption of FBMN migration in *pk1b* mutants suggests that farnesylation of Pk1b is important for its function. To investigate further the importance of farnesyl moiety synthesis and attachment during FBMN migration, we used a combination of genetic and pharmacological tools to analyze embryos with disrupted farnesylation (Fig. 2A). We first analyzed embryos mutant in the gene that encodes the upstream, rate-limiting biosynthetic enzyme HMG-CoA reductase b (Hmgcrb). Hmgcr activity is required for primordial germ cell migration and heart morphogenesis in *Drosophila*, mice and zebrafish (Van Doren et al., 1998; Thorpe et al., 2004; Yi et al., 2006; D'Amico et al., 2007; Ding et al., 2008). In comparison to wild-type (WT) and Pk1bMO embryos (Fig. 2B,C), FBMN migration was partially disrupted in *hmgcrb* mutants (Fig. 2D,I). We define partial migration as embryos displaying FBMNs spread between r4 and r6, instead of clustered in r6 (complete migration). We also disrupted Hmgcrb function using a translation-blocking MO (D'Amico et al., 2007) and by treating embryos with the potent Hmgcr inhibitor atorvastatin (Lipitor). Both of these treatments phenocopied the *hmgcrb* mutant: migration was partially disrupted in 84.4% of Hmgcrb morphants (Fig. 2E,I) and in 100% of atorvastatin-treated embryos (Fig. 2F,I). These results demonstrate that Hmgcrb activity is required for normal FBMN migration.

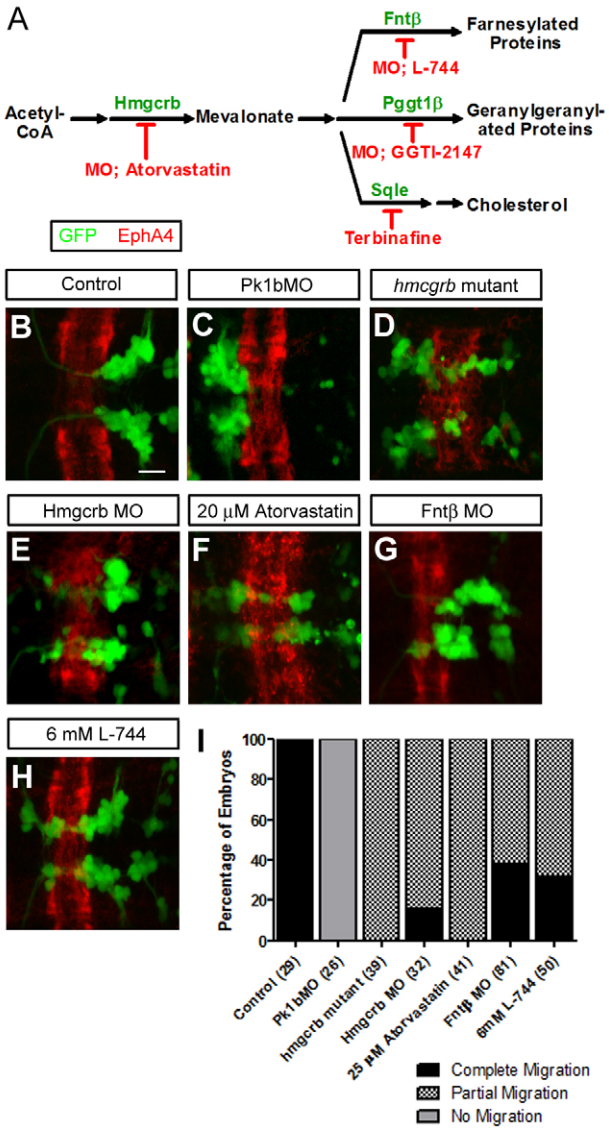

Fig. 2. Hmgcrb and Fntb are required for FBMN migration. (**A**) Summary of the Hmgcrb biosynthetic pathway, including enzymes (green) and disrupting agents used in this study (red). Hmgcrb catalyzes the first rate-limiting step in the generation of farnesylated and geranylgeranylated proteins, as well as in the synthesis of cholesterol. (**B-H**) Maximum projection dorsal views of FBMNs (green) in 42 hpf Tg(*islet1*:GFP) zebrafish embryos, immunostained for EphA4 to mark r5 (red). FBMNs migrate to r6 in control embryos (B), whereas migration is completely blocked in Pk1b morphants (C). Disruption of Hmgcrb function in *hmgcrb* mutants (D), by injection of Hmgcrb MO (E) or by treatment with atorvastatin (F), partially blocks migration. Migration is also partially blocked in embryos injected with Fntβ MO (G) or with the Fnt inhibitor L-744 (H). Scale bar: 20 μm. (**I**) Extent of FBMN migration following the treatments indicated. The number of embryos scored is indicated in parentheses.

As Hmgcrb is required for the synthesis of not only farnesyl moieties but also the related geranylgeranyl molecule and cholesterol, we specifically blocked farnesyl protein modification. We designed a translation-blocking MO targeted to the β-subunit of Farnesyltransferase (Fntβ) and observed partial disruption of migration in 61.7% of morphants (Fig. 2G,I). We also treated

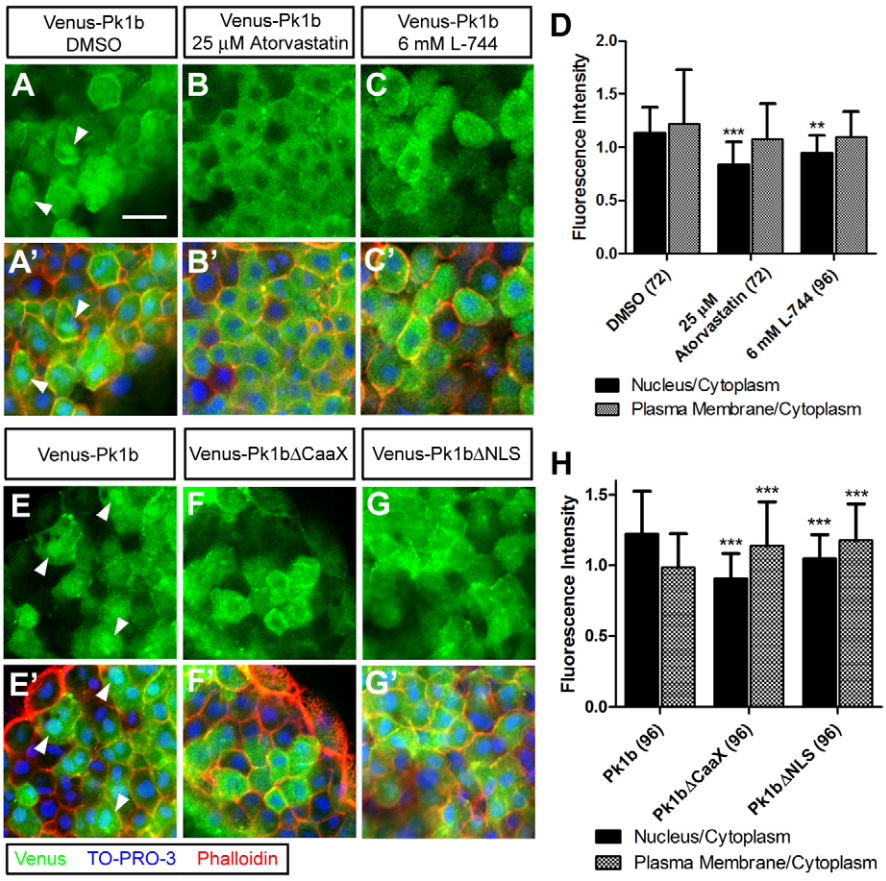

Fig. 3. Nuclear localization of Venus-Pk1b is mediated by farnesylation and NLS motifs. (**A-C′**) Single confocal sections of zebrafish blastulae expressing Venus-Pk1b (green) treated with DMSO (A,A′, control), atorvastatin (B,B′) or L-744 (C,C′), with nuclei labeled by TO-PRO-3 (A′-C′, blue) and plasma membrane labeled by Rhodamine phalloidin (A′-C′, red). Arrowheads highlight cells with elevated nuclear localization. (**D**) Ratio of fluorescence intensity in nuclei versus cytoplasm (black bars) and plasma membrane versus cytoplasm (gray bars) for the treatments indicated. The number of cells scored is indicated in parentheses. (**E-G′**) Blastulae expressing Venus-Pk1b (E), Venus-Pk1bΔCaaX (F) or Venus-Pk1bΔNLS (G) (green), with nuclei (E′-G′, blue) and plasma membrane (E′-G′, red) labeled. Arrowheads highlight cells with elevated nuclear localization. (**H**) Ratio of fluorescence intensity in nuclei versus cytoplasm and plasma membrane versus cytoplasm for the constructs indicated. **, $P<0.01$; ***, $P<0.001$; one-way ANOVA. Error bars indicate s.d. Scale bar: 20 μm.

embryos with the potent Fnt inhibitor L-744 (Kohl et al., 1995) and 68.0% of embryos displayed partially disrupted migration (Fig. 2H,I).

Our results demonstrate that Hmgcrb and Fnt activities play an important role in FBMN migration. Migration was likely to be only partially disrupted in these ‘lipid-deficient’ embryos because we were unable to further reduce the function of these enzymes without interfering with the many diverse developmental processes and basic cellular functions that require the products of Hmgcrb and Fntβ activities. Interestingly, we also found that the inhibition of Protein geranylgeranyltransferase 1β (Pggt1β) and Squalene epoxidase (Sqle) activities (Petranyi et al., 1984; Vasudevan et al., 1999) partially blocked FBMN migration (see Fig. S2 in the supplementary material), suggesting that both geranylgeranyl protein modification and cholesterol synthesis are required during this process. However, as Pk1b function is not expected to be altered by the activity of Pggt1β or Sqle, we focused our efforts on understanding the role of farnesylation.

Farnesylation of Pk1b mediates its nuclear localization

As Fnt activity is required for complete FBMN migration and mutation of the Pk1b farnesylation consensus motif disrupts FBMN migration, we hypothesized that farnesylation of Pk1b is important for its function. Consistent with this idea, we found that *hmgcrb* and *pk1b* genetically interact during migration (see Fig. S3 in the supplementary material). To test this model further, we first analyzed how the subcellular localization of Pk1b is altered by disrupted processing. We generated a Venus-tagged Pk1b construct and observed its localization in late blastula stage embryos. Injection of mRNA encoding Venus-Pk1b resulted in Venus localization throughout the cells, including in the nuclei, in the cytoplasm and at the plasma membrane (Fig. 3A,A′,E,E′). To test whether Hmgcr or Fnt activities influence Pk1b localization, we treated Venus-Pk1b-injected embryos with atorvastatin or L-744. Nuclear localization of Venus-Pk1b, relative to the cytoplasm, was decreased in both atorvastatin-treated and L-744-treated embryos (Fig. 3B-D). Neither Pggt1 nor Sqle inhibition significantly disrupted Venus-Pk1b nuclear or plasma membrane localization (see Fig. S4A-B′,D in the supplementary material). These data indicate that inhibition of Hmgcr or Fnt activities alters Venus-Pk1b localization, suggesting that farnesylation of Pk1b stabilizes its nuclear localization.

To test this idea further, we generated a variant of Venus-Pk1b with the farnesylation motif deleted (Venus-Pk1bΔCaaX). As Pk homologs contain NLSs, we also generated a Venus-Pk1bΔNLS construct with all three NLSs deleted. We compared nuclear and plasma membrane localization in blastulae after injection of each construct, and observed that deletion of the CaaX motif (and to a lesser extent the NLS motifs) resulted in significantly decreased nuclear localization (Fig. 3E-H). Simultaneous deletion of the CaaX and NLS motifs further decreased Venus localization (see Fig. S4C,E in the supplementary material). These results further demonstrate that farnesylation of Pk1b mediates its nuclear localization. We also found increased plasma membrane localization in Venus-Pk1bΔCaaX-injected and Venus-Pk1bΔNLS-injected embryos. Although it is unclear whether this increase in plasma membrane localization is simply due to more protein being available to shuttle between the cytoplasm and plasma membrane, we can nevertheless conclude that the farnesylation and NLS motifs are not required for Pk1b plasma membrane localization.

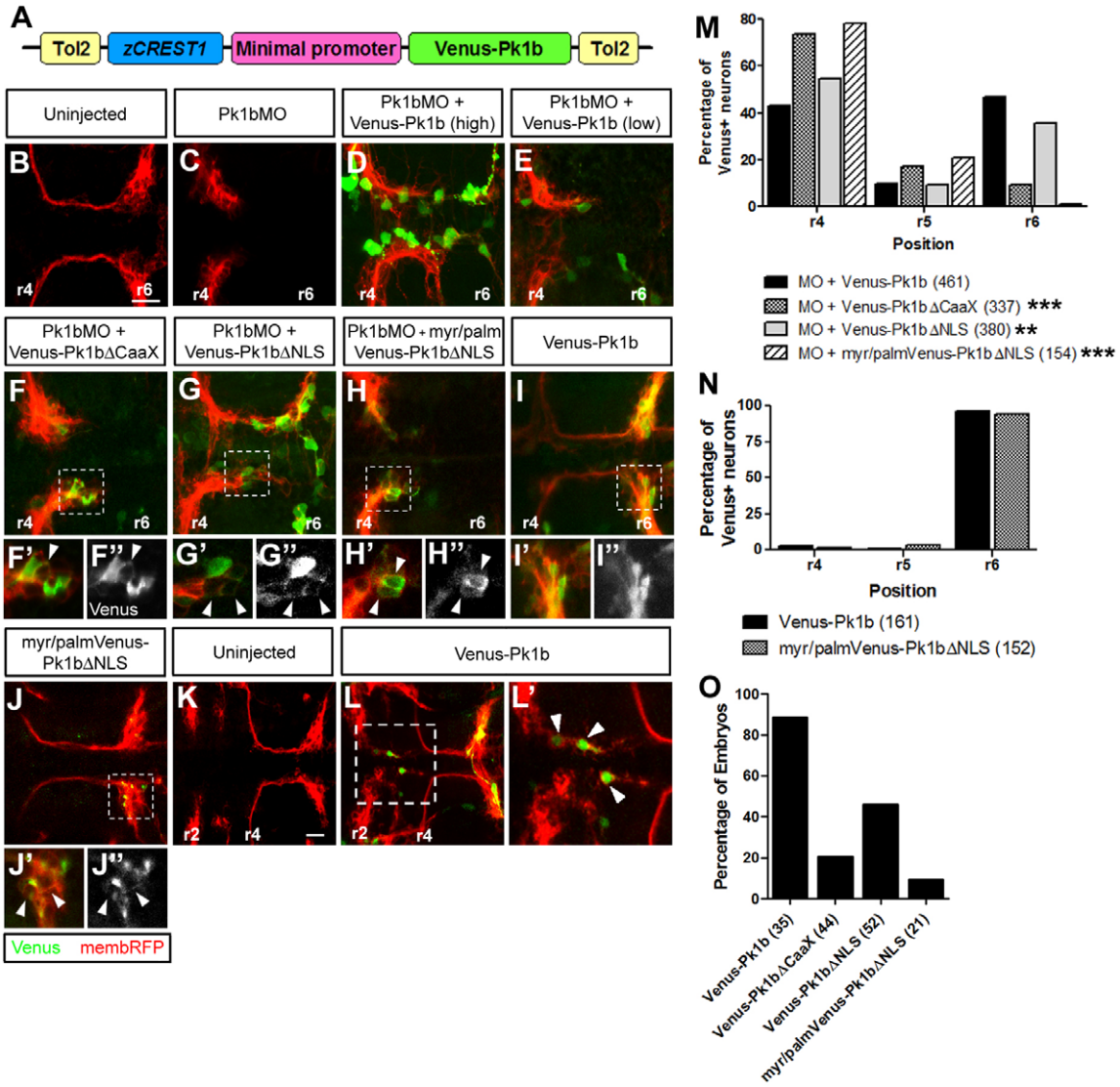

Fig. 4. Nuclear localization of Pk1b is required during FBMN migration. (**A**) The rescue construct. (**B-J″**) Maximum projection dorsal views of FBMNs (red) in 48 hpf Tg(*zCREST1*:membRFP) zebrafish embryos. (B,C) FBMNs migrate to r6 in uninjected embryos (B), whereas migration is completely blocked in Pk1b morphants (C). (D-J) Representative embryos injected with Pk1bMO and/or Venus-tagged rescue constructs (green). CBMN-specific expression of full-length Venus-Pk1b can partially rescue FBMN migration to varying extents; embryos with higher transgenesis efficiency (D) generally display greater rescue capacity than those with lower efficiency (E). Expression of Venus-Pk1bΔCaaX (F), Venus-Pk1bΔNLS (G) or myr/palmVenus-Pk1bΔNLS (H) significantly reduces rescue capacity. Neither Venus-Pk1b (I) nor myr/palmVenus-Pk1bΔNLS (J) expression significantly disrupts migration in the absence of Pk1bMO. (F′-J″) Magnified, single-slice views of boxed regions of F-J, showing nuclear exclusion of Venus in a subset of neurons (arrowheads). (**K-L′**) Dorsal views of CBMNs (red) in representative 48 hpf Tg(*zCREST1*:membRFP) embryos uninjected (B) and injected with Venus-Pk1b (green). Rhombomeres are indicated. (L′) Magnified view of boxed region in L. Arrowheads highlight TgBMNs migrating out of r2 into r3 and r4. (**M**,**N**) Venus-positive neurons were scored for their anteroposterior position. The percentage of neurons in r4-6 is shown. The number of neurons scored is indicated in parentheses. ***, $P<0.001$; **, $P<0.01$; χ^2 test (relative to MO + Venus-Pk1b). (**O**) Embryos that displayed at least one Venus-positive TgBMN were scored. The percentage of embryos with at least one Venus-positive migratory TgBMN is displayed. The number of embryos scored is indicated in parentheses. Scale bars: 20 μm.

Our results are substantially different from those reported for Pk1a by Veeman et al. (Veeman et al., 2003), who found that deletion of the CaaX motif caused increased localization of GFP-Pk1a in the nucleus relative to the cytoplasm. Although the farnesylation and nuclear localization motifs are conserved between Pk1a and Pk1b, divergence in other domains might contribute to differences in protein function and localization. Taken together, our analysis of Venus-tagged Pk1b variants in blastulae suggests that farnesylation mediates Pk1b nuclear localization, but is not required for plasma membrane localization.

Nuclear localization of Pk1b is required for its function in FBMN migration

Our results thus far demonstrate that Fnt activity is required for FBMN migration and that farnesylation of Pk1b mediates its nuclear localization. By extension, we hypothesized that farnesylation-mediated nuclear localization of Pk1b is required during FBMN migration. We therefore sought to test the functional requirements for Pk1b structural motifs during FBMN migration. We first attempted a simple rescue approach by co-injecting Pk1bMO with

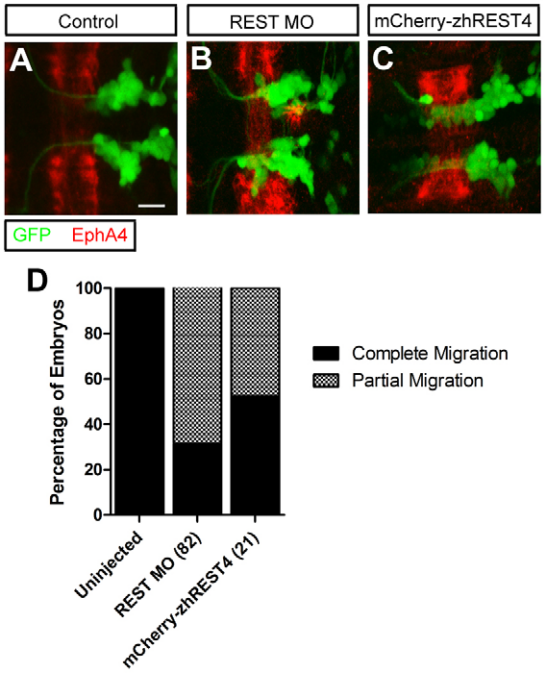

Fig. 5. REST function is required for FBMN migration.
(**A-C**) Maximum projection dorsal views of FBMNs (green) in 42 hpf Tg(*islet1*:GFP) zebrafish embryos, immunostained for EphA4 to mark r5 (red). FBMN migration is partially disrupted in REST morphants (B) and in mCherry-zhREST4-injected embryos (C), relative to controls (A). Scale bar: 20 μm. (**D**) The extent of FBMN migration was scored in embryos with disrupted REST function. The number of embryos scored is indicated in parentheses.

mRNA encoding either Venus-Pk1b or untagged Pk1b; however, we found that neither construct was able to rescue the morphant FBMN phenotype (data not shown).

As we noted previously (Rohrschneider et al., 2007), expression of *pk1b* transcripts is elevated in FBMNs relative to the surrounding neuroepithelium. As this elevated Pk1b expression in the neurons might be crucial for proper function, we next designed a rescue approach that would recapitulate this expression pattern. Using the *zCREST1* enhancer of the *islet1* regulatory elements (Uemura et al., 2005), we drove expression of Venus-tagged Pk1b variants specifically in the cranial branchiomotor neurons (CBMNs; Fig. 4A). Using the Tol2 transposition system (for a review, see Kawakami, 2007), we were able to efficiently generate transient transgenic embryos to analyze the capacity of Pk1b variants to rescue FBMN migration in Pk1b morphants.

CBMN-specific expression of full-length Venus-Pk1b in Pk1b morphant embryos resulted in a partial rescue of FBMN migration (Fig. 4D,E,M; as compared with uninjected controls and Pk1b morphants in Fig. 4B and 4C, respectively). Specifically, 56.8% of Venus-positive FBMNs were able to migrate into r5 and r6, whereas 43.2% remained in r4. Overall, we found that neurons were able to migrate more efficiently in embryos with higher numbers of Venus-positive neurons (compare Fig. 4D with 4E). We attribute this variation to the community effect that we have previously hypothesized (Cooper et al., 2003; Rohrschneider et al., 2007) (G.S.W. and C.B.M., unpublished): FBMNs interact with each other during migration and thus Pk1b-positive neurons migrate more efficiently in cooperation with each other than as individuals. Intriguingly, we found that the presence of Venus-positive neurons was even sufficient to induce the migration of Venus-negative neurons that would otherwise not be expected to migrate.

In contrast to embryos expressing full-length Venus-Pk1b, only 26.4% of neurons expressing Venus-Pk1bΔCaaX were able to migrate into r5 and r6 (Fig. 4F,M). In a similar manner, expression of Venus-Pk1bΔNLS only rescued migration out of r4 in 45.6% of Venus-positive FBMNs (Fig. 4G,M), representing a significant decrease relative to full-length Venus-Pk1b. Deletion of both the CaaX and NLSs had a synergistic effect: only 18.9% of neurons expressing Venus-Pk1bΔNLSΔCaaX migrated out of r4 (see Fig. S5A-B in the supplementary material). In addition, we generated a variant of Venus-Pk1b that is more robustly targeted to the plasma membrane by addition of myristoylation and palmitoylation signals (myr/palmVenus-Pk1bΔNLS). Injection of this construct did not efficiently rescue FBMN migration, as only 22.1% of neurons expressing this construct migrated out of r4 (Fig. 4H,M). Although we did observe neurons with primarily non-nuclear Venus localization, many neurons expressing high levels of Venus-Pk1bΔCaaX, Venus-Pk1bΔNLS or Venus-Pk1bΔNLSΔCaaX were able to override the absence of sequence-encoded localization instructions and subsequently showed enrichment of Venus-tagged protein in the nucleus (Fig. 4F′,F″,G′,G″). We therefore rescored migration in a subset of Venus-Pk1bΔCaaX-expressing or Venus-Pk1bΔNLS-expressing embryos and found that only 13.8% of FBMNs with non-nuclear Venus-Pk1bΔCaaX and 33.0% of those with non-nuclear Venus-Pk1bΔNLS expression were able to migrate out of r4 (see Fig. S5C,D in the supplementary material). Strikingly, all FBMNs with non-nuclear Venus-Pk1bΔNLSΔCaaX remained in r4 (see Fig. S5E in the supplementary material). Taken together, these results demonstrate that nuclear localization, which is dependent on CaaX and NLS motifs, is important for Pk1b function during FBMN migration.

Combined with our analysis of blastulae, these rescue results suggested that nuclear localization of Pk1b is required during migration of FBMNs. However, it remained possible that neurons expressing Pk1bΔCaaX or Pk1bΔNLS migrated less efficiently because of increased Pk1b plasma membrane localization, which we also observed in blastulae. To eliminate this possibility, we tested whether elevated Pk1b expression could disrupt normal FBMN migration. Expression of either full-length Venus-Pk1b or myr/palmVenus-Pk1bΔNLS in FBMNs did not significantly disrupt migration: 96.2% and 94.1% of Venus-positive neurons migrated correctly to r6, respectively (Fig. 4I-J,N). These results demonstrate that elevated plasma membrane localization of Pk1b does not disrupt FBMN migration. Moreover, these data strongly suggest that the ΔCaaX and ΔNLS variants of Venus-Pk1b have reduced capacities to rescue migration because of their reduced nuclear localization.

As a second gauge of Pk1b function, we measured the ability of Venus-Pk1b variants to induce migration of the trigeminal branchiomotor neurons (TgBMNs) from r2 to r3. We have previously shown that overexpression of Hoxb1b causes a transformation of TgBMNs to an FBMN-like identity, including caudal migration capacity (McClintock et al., 2002) and expression of Pk1b (Bingham et al., 2010). This induced migration is at least partially dependent on the expression of Pk1b, as co-injection of Pk1bMO reduces the capacity of these FBMN-like neurons to migrate (Bingham et al., 2010). Remarkably, we found that expression of Venus-Pk1b induced migration of TgBMNs in 92.9% of embryos. This induction of TgBMN migration was reduced in embryos expressing Venus-

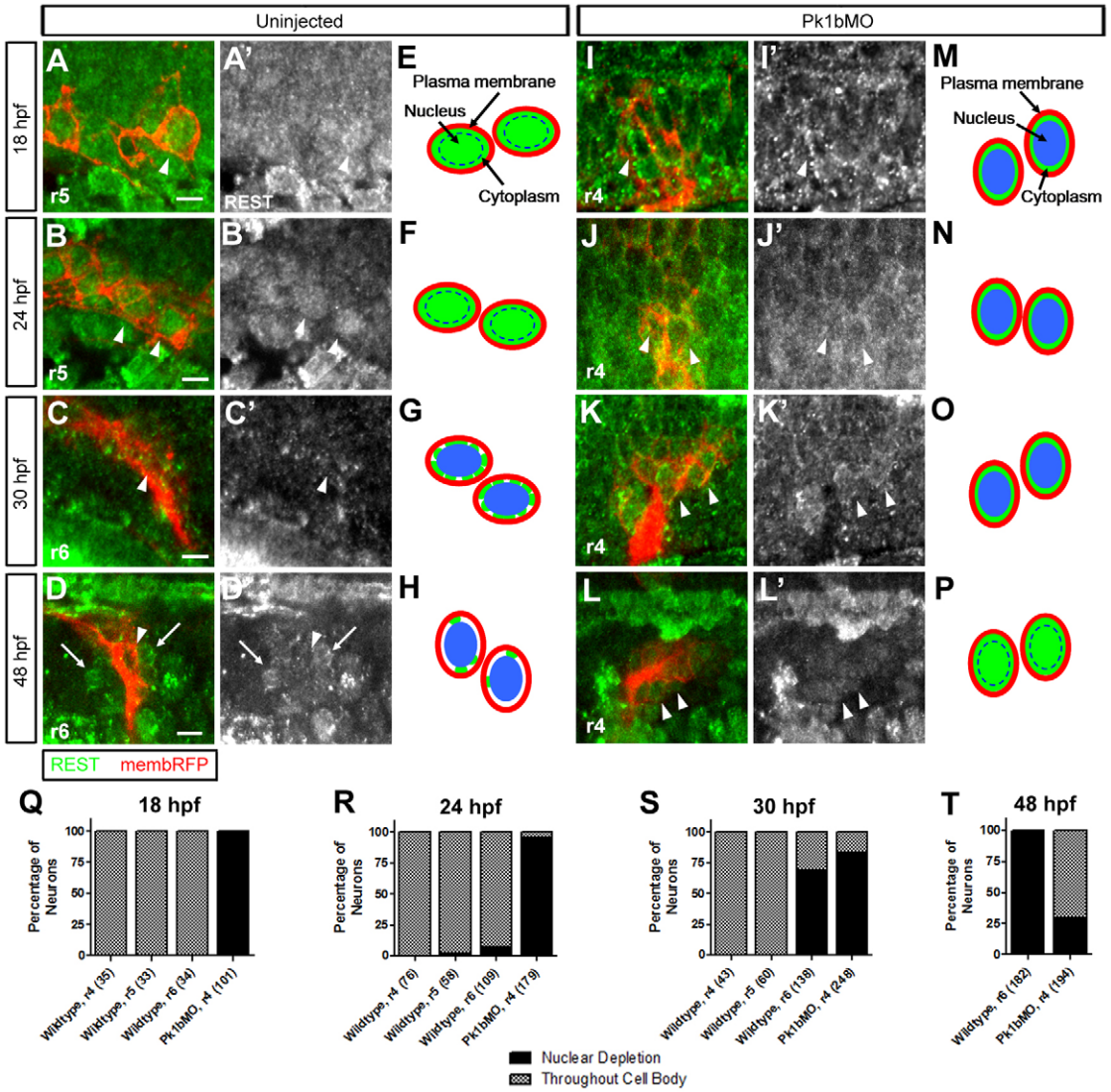

Fig. 6. REST is localized to the nuclei of migrating FBMNs. (**A-D′**,**I-L′**) Single-slice dorsal views of FBMNs (red) in Tg(*zCREST1*:membRFP) zebrafish embryos immunostained for REST (green) at the indicated stages. (A-D) In WT neurons, REST localized throughout the cell body during migration (A,B). As neurons settle in r6, REST becomes depleted from the nuclei (C). By 48 hpf, REST protein is further reduced or absent in FBMN cell bodies (D). (I-L) In Pk1b morphants, REST is depleted from the nuclei of FBMNs and expression is maintained at later embryonic stages. Arrowheads highlight individual FBMNs and arrows indicate non-FBMNs. A′-D′ and I′-L′ show REST immunostaining alone Scale bars: 10 μm. (**E-H**,**M-P**) Schematics illustrating REST localization (green) in FBMNs (red); nuclei are indicated (blue). See also Fig. S6 in the supplementary material. (**Q-T**) Localization of REST was scored in WT and Pk1bMO FBMNs. Percentage of FBMNs with depleted nuclear REST or localization throughout the cell body is displayed. The number of neurons scored is indicated in parentheses.

Pk1bΔCaaX, Venus-Pk1bΔNLS, Venus-Pk1bΔNLSΔCaaX or myr/palmVenus-Pk1bΔNLS (21.1%, 50.0%, 14.3% or 9.5%, respectively; Fig. 4K-L′,O and see Fig. S5F in the supplementary material). These data suggest that: (1) expression of Venus-Pk1b is sufficient to induce the migration of TgBMNs in a WT environment; and (2) the CaaX and NLS motifs are important for Pk1b function during the induced migration of TgBMNs.

Our rescue experiments allow us to draw the following important conclusions. First, expression of Venus-Pk1b specifically in the CBMNs is sufficient to rescue FBMN migration in Pk1b morphants. This result is consistent with our previous transplantation experiments which demonstrated that Pk1b functions primarily cell-autonomously in the FBMNs (Rohrschneider et al., 2007). Second, the farnesylation and NLS motifs contribute to the rescue capacity of Venus-Pk1b. In summary, we conclude that CaaX- and NLS-mediated nuclear localization is required for proper Pk1b function during FBMN migration.

REST function is required for FBMN migration

Although Pk1 homologs are best known as components of PCP signaling that act at the plasma membrane, our results thus far suggest that Pk1b might act at least partially independently of other core PCP components in the nuclei of FBMNs. Previous research has shown that human PK1 functions at the nuclear membrane to regulate nuclear translocation of the transcriptional silencer REST (Shimojo and Hersh, 2003; Shimojo and Hersh, 2006). We

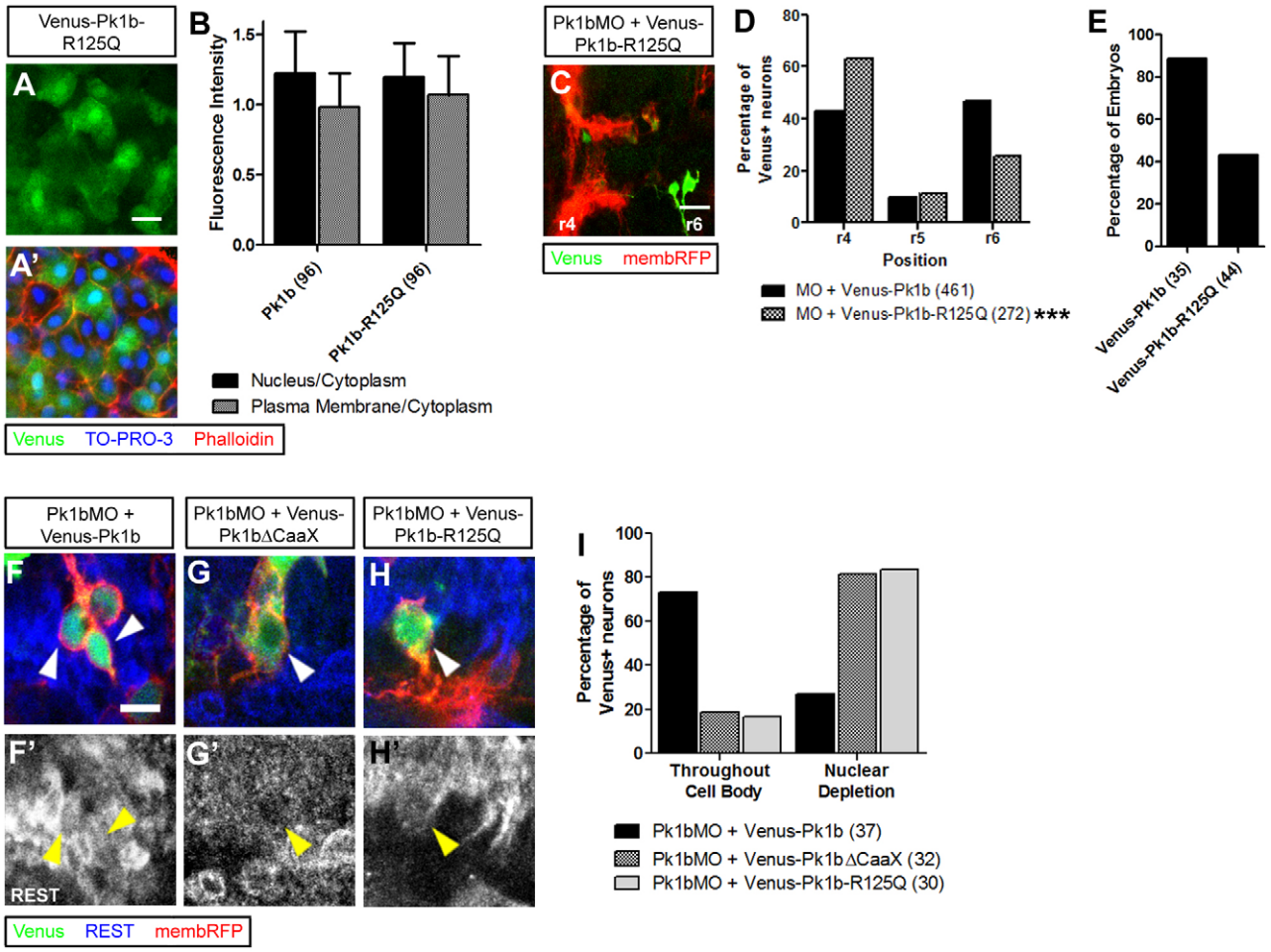

Fig. 7. Interaction with Pk1b is required for proper REST localization during FBMN migration. (**A**,**A′**) Zebrafish blastulae expressing Venus-Pk1b-R125Q (green), with nuclei (A′, blue) and plasma membrane (A′, red) labeled. (**B**) Ratio of fluorescence intensity in nuclei versus cytoplasm (black bars) and plasma membrane versus cytoplasm (gray bars). Number of cells scored is indicated in parentheses. Error bars indicate s.d. (**C**) Dorsal view of FBMNs (red) in representative 30 hpf Tg(*zCREST1*:membRFP) embryo injected with Pk1bMO and Venus-Pk1b-R125Q (green). (**D**) Venus-positive neurons were scored for their anteroposterior position and totaled. The percentage of neurons in r4-6 is shown. Numbers in parentheses indicate number of neurons scored. ***, $P<0.001$; χ^2 test. (**E**) Percentage of embryos with migratory TgBMNs was scored. The number of embryos scored is indicated in parentheses. (**F-H′**) Dorsal views of FBMNs (red) in r4 of representative 30 hpf Tg(*zCREST1*:membRFP) embryos injected with Pk1bMO and Venus-Pk1b (E), Venus-Pk1bΔCaaX (F) or Venus-Pk1b-R125Q (G) (green). Embryos were immunostained for REST (blue). (E) REST is correctly localized in nuclei of Venus-Pk1b-positive neurons (arrowheads). (F,G) REST is depleted from nuclei of FBMNs expressing Venus-Pk1bΔCaaX or Venus-Pk1b-R125Q. F′-H′ show REST immunostaining alone (**I**) Venus-positive neurons in r4 were scored for localization of REST and totaled. The percentage of neurons is shown. The number of neurons scored is indicated in parentheses. Scale bars: 20 μm in A,C; 10 μm in F.

hypothesized that this role is conserved in zebrafish Pk1b, and therefore first sought to determine whether REST functions during FBMN migration.

Using a previously described splice-blocking MO (Gates et al., 2010), we knocked down REST function. At a low dose, REST MO injection partially disrupted FBMN migration without significantly disrupting overall patterning of the embryo (compared with stage-matched embryos; Fig. 5A,B,D). In most embryos, neurons were distributed between r4 and r6. We were unable to inject REST MO at higher doses without causing early-stage lethality; this is likely to reflect the role of REST in regulating the specification of non-neuronal cells (Chen et al., 1998). We also generated a truncated version of mCherry-tagged REST (mCherry-zhREST4), which is similar to the neuron-specific REST4 splice variant. In mammals, REST4 is thought to act as a natural dominant-negative by preventing full-length REST from binding to DNA (Palm et al., 1998; Shimojo et al., 1999). Injection of mRNA encoding mCherry-zhREST4 resulted in a partial disruption of FBMN migration in 47.6% of embryos (Fig. 5C,D). Together, our results indicate that REST function is required for normal FBMN migration.

Nuclear localization of REST protein in FBMNs is dependent on Pk1b

As we hypothesized that Pk1b and REST function together and we have demonstrated that Pk1b function is required primarily in the migrating neurons, we next examined whether REST is expressed in the FBMNs. To visualize REST protein, we immunostained embryos with an antibody that specifically recognizes full-length REST; as expected, we saw a dramatic reduction in labeling in REST morphants (see Fig. S6A-D in the supplementary material). As predicted based on in situ hybridization data (Gates et al.,

2010), levels of REST protein were highest in non-neural tissues (data not shown) and in the floor plate (see Fig. S6E in the supplementary material). Within the neural tube, expression of REST was elevated in several subsets of neurons, including the FBMNs (Fig. 6A-B′ and see Fig. S6C in the supplementary material). Elevated REST expression in FBMNs was observed as early as 18 hpf, shortly after the neurons are born. At 18 and 24 hpf, REST protein was distributed throughout the cell bodies, including in the nuclei, of neurons actively undergoing tangential migration (Fig. 6A-B′,E,F,Q,R and see Fig. S6F-G in the supplementary material). As neurons reached r6 and switch from tangential to dorsolateral migration, REST was depleted from nuclei and localized only in the cytoplasm (Fig. 6C,G,S). By 48 hpf, expression of REST was greatly reduced or absent in FBMNs; neurons still expressing REST completely lacked detectable nuclear REST protein (Fig. 6D,H,T). This expression pattern is consistent with REST functioning in the nucleus to regulate gene expression in tangentially migrating neurons and then being exported out of the nucleus once FBMNs reach their final destination along the anteroposterior axis.

In Pk1b morphants, the pattern of REST localization was significantly different. As early as 18 hpf, there was an increase in the number of FBMNs exhibiting depleted nuclear REST localization (Fig. 6I-K,M-O,Q-S and see Fig. S6H-I in the supplementary material). Depletion of REST was also observed in the surrounding neuroepithelium, suggesting that low levels of Pk1b in the neuroepithelium (Rohrschneider et al., 2007) might also regulate REST localization. Intriguingly, REST localized to nuclei of morphant FBMNs at 48 hpf, suggesting that the mislocalization of the neurons might ultimately lead to changes in neuronal character (Fig. 6L,P,T).

In summary, our analysis demonstrates that loss of Pk1b function is associated with depletion of REST from the nucleus. This result is consistent with our hypothesis that nuclear Pk1b function is required in FBMNs to mediate REST translocation.

Interaction between Pk1b and REST is required for FBMN migration

Previous work in cell culture has established that PK1 can regulate the nuclear translocation of REST (Shimojo and Hersh, 2003; Bassuk et al., 2008). Bassuk et al. identified a *PK1* mutation in human patients with PME (Bassuk et al., 2008). This mutation, R104Q, lies in the PET domain of the protein, severely disrupts interaction with REST and compromises the ability of PK1 to mediate REST nuclear translocation. We examined whether interaction with REST is important for zebrafish Pk1b subcellular localization and function in vivo.

We generated a Venus-Pk1b-R125Q construct, which contains a mutation analogous to PK1-R104Q. The subcellular localization of protein encoded by this construct was indistinguishable from that of full-length Venus-Pk1b, both in the nucleus and at the plasma membrane (Fig. 7A-B), suggesting that this mutation does not disrupt trafficking of Pk1b. This result precisely parallels the behavior of full-length human PK1 and PK1-R104Q in HeLa cells (Bassuk et al., 2008). However, we found that Venus-Pk1b-R125Q had a reduced capacity to rescue FBMN migration in Pk1b morphants, such that only 33.7% of Venus-positive neurons migrated to r5 and r6, whereas 66.3% of neurons remained in r4 (Fig. 7C,D). In addition, migration of TgBMNs was induced in only 43.2% of embryos (Fig. 7E). These results are consistent with the hypothesis that interaction with the transcriptional silencer REST is necessary for Pk1b to function during FBMN migration.

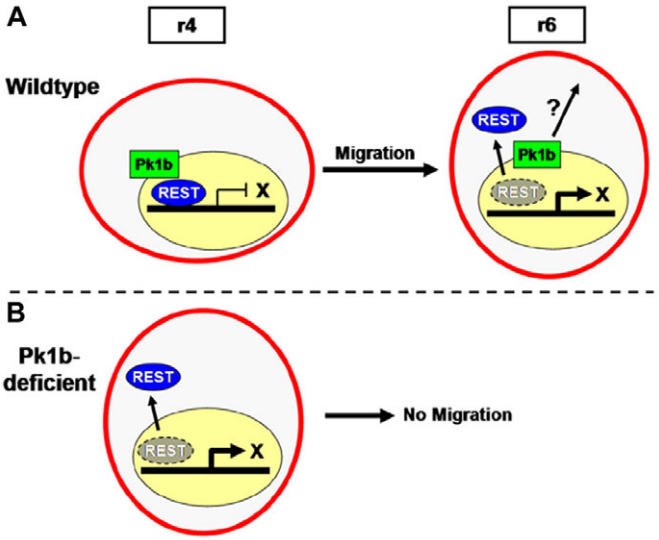

Fig. 8. Model of Pk1b and REST interaction during FBMN migration. (**A**) Pk1b nuclear and/or nuclear membrane localization is required to keep REST in the nuclei during tangential migration of FBMNs. Once neurons reach r6, REST is depleted from the nuclei and terminal differentiation genes (X) are expressed. Pk1b might be removed from the nucleus during this process (question mark). (**B**) In the absence of Pk1b at the nucleus, REST prematurely exits the nucleus, resulting in the expression of terminal differentiation genes in r4 and preventing a response to migratory cues.

To analyze further the relationship between Pk1b and REST, we characterized REST localization in Pk1b morphants expressing Pk1b variants in the rescue assay described above. Embryos were analyzed at 30 hpf; at this stage, later-born FBMNs in r4 and r5 of WT embryos still display REST localization throughout their cell bodies (Fig. 6S). We found that in FBMNs expressing full-length Pk1b, REST was properly localized to nuclei in 73.0% of neurons in r4 (Fig. 7F,I). This was equally the case for Venus-positive neurons surrounded by Venus-negative neurons (data not shown), suggesting that the interactions among FBMNs that underlie the community effect do not perturb REST localization. However, in neurons expressing Venus-Pk1bΔCaaX or Venus-Pk1b-R125Q, we observed nuclear depletion of REST in 81.2% and 83.3% of Venus-positive neurons in r4, respectively (Fig. 7G-I). Our results demonstrate that proper REST localization is lost in FBMNs expressing Pk1b-R125Q, and suggest that interaction between Pk1b and REST is required to properly regulate REST localization.

DISCUSSION

In this study, we have demonstrated that farnesylation mediates nuclear localization of the Pk1b protein, and that this localization is required for FBMN migration. Furthermore, we have identified a role for an interaction between Pk1b and REST in this process, and demonstrated that REST is mislocalized in Pk1b-deficient embryos. Our data lead us to a model in which Pk1b functions at least partially independently of core PCP components at the plasma membrane, and instead functions at the nucleus to mediate REST nuclear translocation (Fig. 8; see below).

Our investigations of the role of lipid biosynthesis in FBMN migration have revealed that, in addition to a crucial role for farnesylation, there is also an important contribution of

geranylgeranylation and cholesterol synthesis to FBMN migration. However, consistent with Pk1b being a substrate of Fntβ, we found that neither Pggt1β nor Sqle inhibition disrupted Venus-Pk1b localization. This suggests that geranylgeranylation and cholesterol synthesis contribute to FBMN migration independently of regulation of Pk1b localization. We hypothesize that Hedgehog signaling is affected by the loss of these lipid products. Several Hedgehog pathway components are required for the specification and migration of FBMNs (Chandrasekhar et al., 1998; Chandrasekhar et al., 1999; Vanderlaan et al., 2005). Furthermore, Hedgehog proteins are modified by cholesterol (for a review, see Bürglin, 2008; Porter et al., 1996). It is also likely that the disruption of other heterotrimeric G-protein-dependent signaling pathways, including Sdf1 (Cxcl12)/Cxcr4 (Sapède et al., 2005; Cubedo et al., 2009), contributes to FBMN migration defects in Pggt1β-deficient embryos. Finally, the disrupted geranylgeranylation of small GTPases might cause cytoskeletal disorganization and further impair migration.

We have demonstrated that FBMN migration can be rescued in Pk1b morphants by driving expression of Pk1b specifically in the facial neurons. Our results confirm that Pk1b functions predominantly cell-autonomously, consistent with our previous transplantation results (Rohrschneider et al., 2007). Our rescue experiments also lend support to the community effect model that we have previously described (Cooper et al., 2003; Rohrschneider et al., 2007). Intriguingly, misexpression of Venus-Pk1b in the TgBMNs is sufficient to drive their tangential migration. This suggests that TgBMNs possess both the cues and machinery for migration, but that the absence of Pk1b prevents migration from occurring.

Although we have shown that Pk1b functions with REST during FBMN migration, we cannot exclude a second function of Pk1b that is dependent on interactions with core PCP components at the plasma membrane. Our subcellular localization measurements demonstrate that Venus-Pk1b can be enriched at the plasma membrane, and it is possible that Pk1b shuttles between cellular compartments. Since we have shown that this localization is not dependent on farnesylation, it is possible that Venus-Pk1b is recruited to the plasma membrane by the transmembrane protein Vangl2 (Jenny et al., 2003). *pk1b* and *vangl2* genetically interact during FBMN migration, and we have found that exogenously supplied Pk1b and Vangl2 can mutually influence their subcellular localization in vivo (O.M.M. and V.E.P., unpublished). An intriguing possibility is that Pk1b is recruited away from the nucleus in r6 by Vangl2 and/or other core PCP components to help terminate tangential migration.

Based on the expression pattern of REST in WT and Pk1b morphants, we hypothesize that Pk1b-mediated nuclear REST localization is required during the early stages of FBMN migration to repress terminal differentiation of the neurons (Fig. 8A). Once the neurons reach r6, REST relocates to the cytoplasm and repression of terminal differentiation genes is relieved. Consistent with our hypothesis and our data, previous work has demonstrated a role for Hdac1 during FBMN migration (Nambiar et al., 2007), which can be recruited by REST to targets of repression (Belyaev et al., 2004). In future work, it will be essential to identify the transcriptional targets of REST and characterize their role in FBMN migration. Interestingly, a survey of the human genome (Johnson et al., 2007) has identified REST binding sites near several genes implicated in FBMN migration, including *CELSR3* (Qu et al., 2010), contactin 2 (also known as *TAG1*) (Sittaramane et al., 2009) and those encoding components of the DAB1/reelin/CDK5 signaling pathway (Ohshima et al., 2002). Molecules upregulated in the final stages of FBMN migration, when radial migration commences, such as cRet, Tag1 and cadherin 8 in mouse (Garel et al., 2000), are strong candidates to be repressed by REST during tangential migration. Our preliminary analysis of the zebrafish genome has identified REST binding sites in the promoter regions of *cdk5r1b* and *cdk5r2a*, orthologs of which have been implicated in regulating the radial migration of FBMNs in mouse (Ohshima et al., 2002).

In the absence of Pk1b function, REST is depleted from the nucleus, which might lead to the premature expression of terminal differentiation genes in r4 (Fig. 8B). We suggest that premature maturation prevents FBMNs from interpreting and/or responding to tangential migration cues, such that Pk1b-deficent FBMNs in r4 inappropriately display cell behaviors that are characteristic of neurons in r6, as we have previously described (Mapp et al., 2010). Although our data do not allow us to discriminate between Pk1b function in the nucleus versus at the nuclear membrane, we hypothesize that Pk1b nuclear membrane localization is necessary to regulate REST entry and exit. It will be important in future studies to determine the precise localization of endogenous Pk1b during and after FBMN migration.

Interestingly, REST itself has at least one nuclear localization sequence (Grimes et al., 2000; Shimojo et al., 2001; Shimojo, 2006); however, interactions with other REST splice isoforms as well as with other non-REST proteins might influence its localization. It is thus unclear whether Pk1b functions to actively import REST into the nucleus or to prevent REST from exiting the nucleus. Notably, huntingtin has been reported to sequester REST in the cytoplasm (Zuccato et al., 2003), and PK1, REST, huntingtin and the dynactin subunit $p150^{Glued}$ can complex together in cell culture (Shimojo, 2008). In addition, the mechanism by which REST is depleted from the nucleus once neurons reach r6 remains unclear. It might be that Pk1b is removed from the nuclear membrane, which would subsequently reverse the nuclear accumulation of REST. This could occur either by a change in the phosphorylation status of Pk1b (Shimojo and Hersh, 2006) or by recruitment to the cytoplasm and/or plasma membrane. Alternatively, a Pk1b-independent mechanism might be responsible for exporting REST from the nucleus.

Given the apparent complexities of REST functions and interactions (reviewed by Ballas and Mandel, 2005; Qureshi and Mehler, 2009), several questions remain. However, our work provides the first in vivo evidence that Pk1b and REST function in concert to regulate neuronal migration. We hypothesize that this mechanism might play a broader role in regulating nervous system development, and might be at the root of nervous system dysfunction in PME patients.

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Competing interests statement
The authors declare no competing financial interests.

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