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A functional interaction between Na,K-ATPase β_2-subunit/AMOG and NF2/Merlin regulates growth factor signaling in cerebellar granule cells

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Abstract

The Na,K-ATPase, consisting of a catalytic α-subunit and a regulatory β-subunit, is a ubiquitously expressed ion pump that carries out the transport of Na^+ and K^+ across the plasma membranes of most animal cells. In addition to its pump function, Na,K-ATPase serves as a signaling scaffold and a cell adhesion molecule. Of three β-subunit isoforms, β_1 is found almost in all tissues, while β_2 expression is mostly restricted to brain and muscle. In cerebellar granule cells, the β_2-subunit, also known as Adhesion Molecule on Glia (AMOG), has been linked to neuron-astrocyte adhesion and granule cell migration, suggesting its role in cerebellar development. Nevertheless, little is known about molecular pathways that link the β_2-subunit to its cellular functions. Using cerebellar granule precursor cells, we found that the β_2-subunit, but not the β_1-subunit, negatively regulates expression of a key activator of the Hippo/YAP signaling pathway, Merlin/neurofibromin-2 (NF2). The knockdown of the β_2-subunit resulted in increased Merlin/NF2 expression and affected down-stream targets of Hippo signaling, i.e. increased YAP phosphorylation and decreased expression of N-Ras. Further, the β_2-subunit knockdown altered the kinetics of Epidermal Growth Factor Receptor (EGFR) signaling in a Merlin-dependent mode and impaired EGF-induced reorganization of the actin cytoskeleton. Therefore, our studies for the first time provide a functional link between the Na,K-ATPase β_2-subunit and Merlin/NF2 and suggest a role for the β_2-subunit in regulating cytoskeletal dynamics and Hippo/YAP signaling during neuronal differentiation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

Na,K-ATPase beta2-subunit; AMOG; cerebellum; Merlin; epidermal growth factor

INTRODUCTION

The Na,K-ATPase is a ubiquitously expressed integral membrane protein that carries out the extrusion and uptake of Na^+ and K^+ across the plasma membranes of most animal cells. It is well established that the pump function of Na,K-ATPase is essential to maintain intracellular ion homeostasis, to control cell volume, and to restore the resting membrane potential in excitable cells. The Na,K-ATPase is composed of a catalytic α-subunit and a structural β-subunit that is essential for folding, targeting and ion-transporting function of the α-subunit. An optional regulatory γ-subunit can be represented by one of the seven members of the FXYD protein family that are expressed in a tissue-specific manner. There are four isoforms of the α-subunit and three isoforms of the β-subunit. Whereas the α_1 and β_1 isoforms are expressed ubiquitously, suggesting important housekeeping roles in most cell types, the α_2 isoform is found mainly in muscle and nervous tissue, α_3 mostly in neurons, and α_4 in sperm and testes. The β_2 isoform is expressed predominantly in brain and muscle tissue, while β_3 is found in the lung testes, skeletal muscle, and liver [1]. Aside from the ion-transporting function, the Na,K-ATPase serves as a signaling scaffold [2-5]. In addition, a cell adhesion function has been ascribed to both the β_1 and the β_2 isoforms [6-11]. However, the differences between the β_1 and β_2 isoforms in sequence, glycosylation, and tissue-specific expression patterns suggest that the two isoforms may have distinct cellular functions. While the functions of the β_1-subunit have been extensively investigated in epithelial and cardiac cells, little is known about the cellular roles and molecular functions of the β_2 isoform.

The β_2-subunit was initially described as Adhesion Molecule On Glia (AMOG) [6] and later identified as a subunit of Na,K-ATPase [12,13], The β_2 isoform is predominantly expressed in astrocytes and cerebellar granule cells in the brain and also in photoreceptors [14-17]. Mice deficient in the β_2-subunit exhibit motor incoordination at postnatal day 15, followed by tremor and paralysis of extremities, and die at 17-18 days of age [15]. Knock-in of the β_1 isoform in AMOG/β_2 knockout mice under the control of the regulatory elements of the β_2 gene resulted in a normal life-span. The β_1-subunit knock-in also improved but did not completely restore the swelling of astrocytic end feet, and the degeneration of photoreceptor cells observed in AMOG/β_2 knockout mice. Thus, it appears that the β_1 isoform can substitute for some but not all functions of the β_2-subunit, most likely in a cell type-specific manner [18].

In mouse brain, the β_2 isoform is first detectable in late embryogenesis. In the cerebellum, the β_2-subunit levels increase during the first two postnatal weeks and it is expressed at the critical developmental stages of granule neuron maturation [6,19,20]. In *in vitro* assays, AMOG/β_2 antibodies inhibited granule cell migration in cerebellar folium explants of 10-day-old mice and reduced attachment of neurons to astrocytes in adhesion assays [6]. In co-cultures of primary granule cells and L-cells engineered to express the β_1-subunit or β_2-

subunit, the β_2 isoform but not the β_1-subunit increased neurite outgrowth, suggesting isoform specific functions in neuronal growth and differentiation [21]. However, the molecular pathways describing how AMOG/β_2 may be involved in cell adhesion or transmitting extracellular signals remain to be identified.

In this study, we identified Merlin, the gene product of neurofibromatosis type 2 (NF2), as a mediator of isoform-specific functions of the β_2-subunit in the regulation of growth factor signaling in cerebellar granule cells. Merlin is an ERM (ezrin, radixin, and moesin)-like protein that is critical for the regulation of cell proliferation, survival, motility and differentiation [22,23]. Merlin is a key regulator of the development and maintenance of the nervous system. Particularly, it is important for neuronal wiring, process formation, axon guidance and corpus callosum development [24-28]. The involvement of Merlin in the development of the nervous system has been linked to its role in the regulation of the Hippo/YAP signaling cascade [29,30,28]. In addition, Merlin regulates cellular localization and activity of epidermal growth factor receptor (EGFR) [31]. Here we demonstrated an inverse correlation between levels of β_2-subunit and Merlin in primary mouse cerebellar granule cell precursor (CGP) cultures during postnatal cerebellar development. Further, using a pediatric brain tumor cell line derived from CGPs, we established that this inverse relationship is causal and functional. Knockdown of the β_2, but not the β_1 isoform, resulted in increased mRNA and protein levels of Merlin, which, in turn, affected down-stream targets of Merlin-activated Hippo signaling, YAP and N-Ras, and altered the kinetics of EGFR activation. In addition, knockdown of the β_2, but not the β_1 isoform, prevented the formation of EGF-induced actin stress fibers. Therefore, our studies for the first time provide a link between Na,K-ATPase expression and the Hippo/YAP signaling cascade.

MATERIALS AND METHODS

Cell culture and transfection

Human medulloblastoma DAOY cells obtained from American Type Culture Collection (ATCC, Manassas, VA) were cultured in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin-glutamine at 37° C and 5% CO_2. HEK-293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin-glutamine at 37°C and 5% CO_2. All cell lines were authenticated by short tandem repeat (STR) analysis. For treatment with epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), DAOY cells were serum-starved overnight and then treated with 10 ng/mL EGF for indicated times. DAOY cells were treated for 4 hours with 50 μM ouabain (Sigma Aldrich, St. Louis, MO) after overnight serum-starvation.

For knockdown studies, two shRNA sequences that target human Na,K-ATPase β_2-subunit (5 '-CCTTGATGTCATTGTCAAT-3', sh-NaKβ_2-cl1; 5'-CCGGGTCATCAACTTCTAT-3', sh-NaKβ_2-cl2) were cloned into the pSIREN-DNR-DsRed-Express vector (Clontech, Mountain View, CA) using the BamHI and EcoRI restriction sites and the inserts were confirmed by sequencing. The empty pSIREN-DNR-DsRed-Express vector was used as a control vector (ShV). DAOY cells were transfected with the knockdown and control vectors using Lipofectamine 3000 (Thermo-Fisher Scientific, Pittsburgh, PA) and single clones were

selected after treatment with 10 μg/mL puromycin. Knockdown of the β_2-subunit was confirmed by immunoblotting, with each clone representing β_2-subunit knockdown with separate shRNA sequences.

Sh-NaKβ_2-cl1 cells were transfected with YFP (pEYFP-C1; Clontech Laboratories Inc./ TaKaRa, Mountain View, CA) for a Na,K-β_2 rescue control cell line or a YFP-β_2 vector containing silent mutations in the β_2-subunit shRNA targeting sequence for a Na,K-β_2 rescue cell line. The YFP-β_2 vector encoding a fusion protein of YFP linked to the amino terminus of the human Na,K-ATPase β_2-subunit was described previously [32]. Site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA) was performed on the YFP-β_2 vector to introduce three silent point mutations in the Sh-NaKβ_2-cl1 targeting sequence to prevent shRNA targeting to the rescue vector using the following mutagenesis primers: forward 5'-TCACTGACATTGACAATGACGTCTAAGTTCTCAGTCTTGGGGCGAA-3', reverse 5'-TTCGCCCCAAGACTGAGAACTTAGACGTCATTGTCAATGTCAGTGA-3'. Successful mutagenesis was confirmed by sequencing. Transfection was performed as described above using Lipofectamine 3000, single clones were selected after treatment with 200 μg/mL of G418, and rescue of Na,K-ATPase β_2-subunit expression was verified by immunoblot analysis and immunofluorescence microscopy.

Merlin knockdown in β_2-subunit knockdown cells was established by transiently transfecting Sh-NaKβ_2-cl1 cells with NF2 siRNA. Cells were seeded in 60 mm dishes and transfected the next day with either SMARTpool NF2 siRNA or control non-targeting SMARTpool siRNA using DharmaFECT 1 transfection reagent all purchased from GE Dharmacon (Lafayette, CO). Merlin knockdown was confirmed by immunoblotting. Cells were incubated for 48 hours with the transfection reagents, serum starved overnight, then treated with EGF as indicated the next day.

Production of soluble forms of the β_1-subunit and β_2-subunit

The vector encoding for a soluble secreted protein comprised of the extracellular domain of the β_1-subunit (M. Hamrick, unpublished data) and constructed similar to the procedure described previously [33] was a generous gift from Dr. Liora Shoshani. This construct encodes for the chimera containing a cytoplasmic and a transmembrane domain of dipeptidyl peptidase 4 (DPPIV, amino-acid residues 1-29) and an extracellular domain of the dog Na,K-ATPase β_1 subunit (amino-acid residues 63 to 303). When expressed in eukaryotic cells, this chimeric protein is inserted in the endoplasmic reticulum (ER) membrane as a type II transmembrane protein (N-terminus in cytoplasm and C-terminus in the ER lumen), allowing co-translational addition of N-glycans to the extracellular domain of the β_1-subunit followed by cleavage of this domain by signal peptidase(s) in the ER lumen and secretion of the β_1 extracellular domain into media [34,32]. The replacement of the region coding for the β_1-subunit with the region coding for an extracellular domain of the human Na,K-ATPase β_2-subunit (amino-acid residues 68 to 290) resulted in a vector for expressing a soluble secreted protein comprised of the extracellular domain of the β_2-subunit. HEK-293 cells were transfected with vectors coding for secreted forms of the extracellular domain of the dog β_1-subunit or human β_2-subunit. 24 hours after transfection, media was changed to

DMEM media containing 1% FBS and, 8 hours later, the culture medium containing a respective secreted protein was collected. The medium of mock-transfected cells was used as a control. The immunoblot analysis of cell culture media, using β_1- and β_2-specific antibodies detected proteins of ~ 45 and 55 kDa, respectively (Supplemental Figure 1).

Primary culture of CGP cells and β_2-subunit knockout mice

Cerebella dissected from C57BL/6J mice at postnatal day 6 were dissociated into single cells using the Papain Dissociation System kit (Worthington Biochemical Corp, Freehold, NJ) as described previously [35] according to the manufacturer's protocol. After filtering through a 70 μm nylon mesh, the cell suspension was plated twice to remove astroglia cells and fibroblasts. CGP cells were cultured in poly-D-lysine coated 6-well plates in Neurobasal media containing 0.25 mM KCl and B-27 supplement. Cells were treated with Smoothened Agonist (SAG) (MilliporeSigma, Burlington, MA) at 1 μM for indicated time points, and media was completely changed every 2-3 days, β_2-subunit knockout ($AMOG^{0/0}$) mice on a C57BL/6J background were described previously [15].

RNA extraction and qPCR

Total RNA was extracted from cerebellum of wildtype C57BL/6J or $AMOG^{0/0}$ β_2-subunit knockout mice or from DAOY cells using TRIzol reagent (Life Technologies, Carlsbad, CA) as per manufacturer's instructions. Total RNA was treated with the TURBO DNA free kit (Ambion, Life Technologies) to remove genomic DNA. 1 μg of RNA was used to produce cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR analysis was performed with a SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The following primer sets were used: 5'-ACCAATCTTACCATGGACACTGAA-3' (human Na,K-β_1 forward); 5'-CGGTCTTTCTCACTGTACCCAAT-3' (human Na,K-β_1, reverse); 5'-CAAACGTGCCTGCCAATTCA-3' (human Na,K-β_2 forward); 5'-CTTCATCTCGCTTCCCAGCA-3' (human Na,K-β_2 reverse); 5'-AATGTGGCCGAGGACTTTGATTGC-3' (β-actin forward); 5'-AGGATGGCAAGGGACTTCCTGTAA-3' (β-actin reverse); 5′-TTCTGGTGCTTGTCTCACTGA-3′ (beta2 microglobulin forward); 5′-CAGTATGTTCGGCTTCCCATTC-3′ (beta2 microglobulin reverse); 5′-TGATCAGCATGGCCTATGGACAGA-3′ (mouse Na,K-α_1 forward); 5′-TGAAAGGGCAGGAAACCGTTCTCA-3' (mouse Na,K-α_1 reverse); 5′-GGTGGTAGTTGGTTTAAGATCC-3' (mouse Na,k-β_1 forward); 5′-GGAATCTGTGTCAATCCTGG-3′ (mouse Na,k-β_1 reverse); 5'-ATTGAGCTGTCCTGTGGCTC-3' (mouse Na,K-α_2 forward); 5'-TCTCGTGGATGGAGAGCTGA-3 ' (mouse Na,K-α_2 reverse); 5'GCCTCCAAGATGGTCATCCA-3' (mouse Na,K-β_2 forward); 5'-AGAGGAGGATGAAGGCCCAG-3' (mouse Na,K-β, reverse); 5'-GCTGAGGAGGAGCTAGTTCAA-3' (mouse Merlin forward); 5'-TAGTCGCCATACTTGGCCTG-3' (mouse Merlin reverse).

Immunoblotting

Cerebellar tissue from C57BL/6J or AMOG$^{0/0}$ mice, or cell extracts were prepared either with a Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 0.1% Triton X-100, 1 mM sodium orthovanadate supplemented with protease and phosphatase inhibitor cocktails) or SDS lysis buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 95 mM NaCl and 2% SDS supplemented with protease and phosphatase inhibitor cocktails). Equal amounts of protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked in 5% bovine serum albumin (BSA) or 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for one hour at room temperature, and incubated overnight at 4° C with the indicated primary antibody diluted in 5% BSA/TBS-T or 5% milk/TBS-T. HRP-conjugated secondary antibodies were diluted in 5% BSA or 5% milk for one hour at room temperature, and Amersham ECL or ECL Prime Western Blotting Detection Reagents (GE Healthcare, Marlborough, MA) were used to visualize protein bands. Image J software was used for densitometric analysis of the immunoblots. The following antibodies were used: anti-α-tubulin (#3873), anti-GAPDH (#2118), anti-phospho-EGFR Tyr1173 (#4407), anti-EGFR (#4267), anti-phospho-Akt S473 (#9271), anti-Akt (#9272), anti-phospho Erk1/2 Thr202/Tyr204 (#4377), anti-Erk1/2 (#9102), anti-Merlin (#6995), anti-GFP (#2555), anti-YAP (#14074), anti-phospho-YAP Ser127 (#13008), anti-rabbit IgG HRP-linked (#7074), and anti-mouse IgG HRP-linked (#7076) from Cell Signaling Technology (Danvers, MA), anti-Na,K-ATPase α_1-subunit (sc-71638) from Santa Cruz Biotechnology, anti-Na,K-ATPase β_1-subunit (HPA012911) from Sigma-Aldrich, anti-Na,K-ATPase β_2-subunit (ab76509), anti-NRAS (ab154291) and anti-vinculin (ab18058) from Abcam (Cambridge, MA), anti-pan Ras from Cytoskeleton Inc. (Denver, CO), and anti-Na,K-ATPase α_2-subunit (PA525725) from Thermo-Fisher Scientific (Pittsburgh, PA).

Immunofluorescence

Cells seeded in 12-well plates inlaid with glass coverslips were treated as indicated. The coverslips were fixed in 4% paraformaldehyde supplemented with 100 μM $CaCl_2$ and 1 mM of $MgCl_2$ for 15 minutes. Coverslips were blocked in 5% normal goat serum/0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 minutes, and incubated in primary antibody diluted in 1% BSA/0.3% Triton X-100 in PBS for one hour. After washing with PBS, cells were incubated for 30 minutes with the appropriate secondary antibody and/or Alexa Fluor-594 labeled phalloidin (Life Technologies) diluted in 1% BSA/0.3% Triton X-100 in PBS. Secondary antibody used was anti-mouse IgG Alex Fluor-488 from Life Technologies. After washing with PBS, coverslips were mounted on glass slides with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). All incubations occurred at room temperature. Confocal microscopy was performed using a Leica TCS SP5 confocal microscope.

Ouabain-sensitive rubidium uptake assay

Measurement of the ouabain-sensitive $^{86}Rb^+$transport was performed as described previously [8]. Briefly, one set of cells seeded in 12-well plates was pretreated with 50 μM ouabain for 30 minutes at 37° C while a second set of samples was left untreated. Cells were washed with ice cold wash buffer (144 mM NaCl, 10 mM HEPES pH 7.4, 0.5 mM $CaCl_2$), then uptake buffer (144 mM NaCl, 10 mM HEPES pH 7.4, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$,

1 mM $RbCl_2$, 1 mg/mL glucose) containing 1 μCi/mL of $^{86}Rb^+$(PerkinElmer, Waltham, MA) was added to the plates and incubated for 10 minutes at 37° C. After three washes, cells were lysed with 0.5 N NaOH and $^{86}Rb^+$ levels were determined using a Beckman LS 6500 Scintillation Counter (Beckman Coulter, Fullerton, CA). The ouabain-sensitive $^{86}Rb^+$flux was calculated as the difference between $^{86}Rb^+$ counts for ouabain-insensitive uptake and total cellular uptake (untreated cells). This difference was normalized to the total protein concentration as determined by the DC protein assay (Bio-Rad, Hercules, CA). The experiment was done in triplicate.

Statistical analysis

Data are presented as mean ± standard error (SE) unless otherwise indicated. Differences between means of the two groups were analyzed with the use of a two-tailed unpaired student' t-test. When applicable, p values are stated in the figure legends.

RESULTS

Expression of Na,K-ATPase β-subunit isoforms increases during cerebellar granule cell differentiation

Cerebellar granule cells, which are the most common neurons in the cerebellum, are one of the few cell-types simultaneously expressing the β_1 and β_2 isoforms [16] and are uniquely suited to study isoform-specific functions of Na,K-ATPase β-subunits. Consistent with previous studies [6,19,35,20], we found that both β_1 and β_2-subunits are expressed in mouse cerebella with levels increasing during postnatal maturation (Fig. 1). Levels of Na,K-ATPase α-and β-subunit protein (Fig. 1a) and mRNA (Fig. 1b) in cerebellar tissue from postnatal pups gradually increased during cerebellar development from day 4 to day 21 when the mouse cerebellum is fully formed [36]. Nevertheless, it appeared that a notable increase in the β_2 levels may occur at a later stage than the β_1 isoform. In primary cultures of cerebellar granule cells, β_1 and β_2 mRNA (Fig. 1c) and protein (Fig. 1d, DMSO) levels increased during differentiation as well. We previously showed that the β_1 subunit is a target of the sonic hedgehog (Shh) pathway and that activation of Shh signaling represses β_1-subunit levels [35], As expected, treatment of cerebellar granule cell primary cultures with SAG, an activator of Shh signaling [37,35], prevented the increase in β_1-subunit expression that was observed in DMSO-treated control cells (Fig. 1d-e). However, this treatment did not significantly affect β_2-subunit protein (Fig. 1d) or mRNA (Fig. 1e) expression, indicating that the β_2-subunit is not a target of the Shh signaling pathway. Together, these data suggest that the β_1 and β_2 isoforms are regulated by independent mechanisms during cerebellar granule neuron maturation and might have isoform-specific functions in these cells.

Knockdown of Na,K-ATPase β_2-subunit in a cerebellar granule cell precursor-derived cell line alters levels of other Na,K-ATPase isoforms and ion-transporting activity

We previously generated β_1-subunit knockdown cell lines from DAOY cells, a pediatric medulloblastoma cell line originating from cerebellar granule cell precursors. Like cerebellar granule cells, DAOY cells express not only the β_1-subunit but also the β_2 isoform (Fig. 2a, b). Therefore, to better understand isoform specific functions of the β_2-subunit on the molecular level, we generated β_2-subunit knockdown cell lines from DAOY cells using

two different shRNA targeting sequences resulting in more than 90% decrease in β_2-subunit protein levels (Fig. 2a, Sh-Na,Kβ_2-cl1 and Sh-Na,Kβ2-cl2). A comparison of levels of β_1 and β_2 isoforms in two independent sets of β_2-subunit knockdown cell lines and the two previously described β_1-subunit knockdown clones (Sh-Na,Kβ_1-cl1 and Sh-Na,Kβ_1-cl2) [35] revealed that loss of either β-subunit led to a reciprocal increase in protein (Fig. 2a) and mRNA (Fig. 2b) levels of the other isoform. However, β_1 and β_2 knockdown differentially affected expression of α-subunit isoforms. Knockdown of the β_2-subunit significantly increased α_1-subunit protein (Fig. 2a) and mRNA (Fig. 2b), and decreased α_2 protein and mRNA (Fig. 2a, b, respectively). In contrast, β_1-subunit knockdown did not change α_1- and α_2-subunit protein and mRNA levels (Fig. 2a, b). β_1-subunit knockdown, but not β_2-subunit knockdown, resulted in increased α_3-subunit expression (Fig. 2a). Ouabain-sensitive rubidium uptake experiments revealed that both β_1-subunit knockdown and β_2-subunit knockdown decreased ion-pumping activity of the Na,K-ATPase. However, β_2-subunit knockdown had a greater effect on Na,K-ATPase activity than β_1-subunit knockdown when compared to their control cells with a decrease by 52 ± 0.18% and 50 ± 0.14% in Sh-Na,Kβ_2-cl1 and Sh-Na,Kβ_2-cl2 cells, respectively, and a decrease by 23 ± 0.16% and 21 ± 0.13% in Sh-Na,Kβ_1-cl1 and Sh-Na,Kβ_1-cl2 cells, respectively (Fig. 2c). It remains to be determined whether isoform selective changes in α-β complexes may account for the observed differences in Na,K-ATPase pump function in β_1 and β_2 knockdown cells.

Na,K-ATPase β_2-subunit knockdown alters EGF-induced reorganization of the actin cytoskeleton

Both, the β_1 and the β_2 isoforms have been ascribed cell-cell adhesion functions. When compared to control cells, β_2-subunit knockdown cells (Sh-Na,Kβ_2-cl1 appeared rounded and displayed a pronounced circumferential actin ring (Fig. 3a, top panels). Further, β_2-subunit knockdown prevented the EGF-induced actin stress fiber formation typically observed in vector-transfected control cells (ShV) (Fig. 3a, bottom panels). The cells remained rounded with a pronounced cortical actin ring and discontinuous actin fibers. Immunofluorescence of EGF -treated cells using vinculin as a marker for focal adhesion complexes showed that vinculin was localized to the leading edges of the elongated control cells (ShV) while the focal adhesions in Sh-Na,Kβ_2-cl1 cells did not display specific directionality (Fig. 3b). Interestingly, we found a similar effect when the adhesion function of the β_2-subunit was blocked in untransfected DAOY cells (control) which express the β_2- and β_1-isoforms using a secreted, soluble form of the extracellular domain of human β_2-subunit (Fig. 3c). While soluble β_2 did not affect changes in cell shape in control cells, when DAOY cells were treated with EGF in the presence of soluble β_2 in the culture medium, EGF failed to induce actin stress fibers and prevented the EGF-induced changes in cell shape that were observed in cells treated with the control medium. Nevertheless, incubation of DAOY cells with EGF in the presence of soluble β_1 resulted in an elongated cell shape and the cells displayed actin stress fibers similar to control cells (Fig. 3c). Thus, it is possible that the adhesion function of the β_2-subunit is required for EGF to induce the changes in cell shape and actin organization that are often observed in migrating cells.

To confirm that it is the knockdown of the β_2-subunit and not the off-target effects associated with the shRNA plasmid and selection of stable knockdown cell lines caused the

observed changes in cell phenotype and actin organization, we generated β_2-subunit rescue cells. To establish these cell lines, we expressed a YFP-fusion protein of the β_2-subunit that contained silent mutations in the shRNA targeting sequence in β_2-subunit knockdown cells (Sh-Na,Kβ_2-cl1 pEYFP-Na,Kβ_2R). β_2-subunit knockdown cells transfected with pEYFP plasmid (Sh-Na,Kβ_2-cl1 pEYFP) served as a control for these experiments (Fig. 4a). Confocal microscopy confirmed that the YFP-tagged mutant β_2-subunit localized to the plasma membrane (Fig. 4b). When treated with EGF, YFP-vector transfected control cells (Sh-Na,Kβ_2-cl1 pEYFP) displayed a prominent circumferential actin ring (Fig. 4c) similar to β_2-subunit knockdown cells (Sh-Na,Kβ_2-cl1) (Fig. 3a). Nevertheless, the β_2-subunit rescue cells (Sh-Na,Kβ_2-cl1 pEYFP-Na,Kβ_2R) responded to EGF-treatment with the formation of actin stress fibers (Fig. 4c) as previously observed in ShV control cells (Fig. 3a) and in parental DAOY cells (Fig. 3c, control). These results clearly indicate that the lack of β_2-subunit in DAOY cells is responsible for the loss of EGF-induced reorganization of the cytoskeleton.

We next determined whether reduced β_2-subunit expression affects the activation of the EGFR. Immunoblotting with anti-phospho-EGFR (Y1173) antibody that recognizes the activated form of the EGFR, revealed an increased and prolonged EGFR activation in β_2-subunit knockdown cells (Sh-Na,K$\beta_2$7-cl1) when compared to vector-transfected control cells (ShV) with peak EGFR phosphorylation occurring at 10 minutes in control cells and around 15-30 minutes in β_2-subunit knockdown cells (Fig. 4d). Looking at downstream EGF signaling, we found that Erk1/2 and Akt phosphorylation were unaffected by β_2-subunit knockdown, suggesting that canonical EGFR signaling is independent of β_2-subunit expression, β_2-subunit rescue cells had similar EGFR phosphorylation kinetics as control ShV cells, and the increase in EGFR phosphorylation also more closely resembled that of control ShV cells (compare Figs. 4d and e), indicating that the lack of β_2-subunit rather than off-target effects of shRNA is responsible for the change in EGFR activation.

β_2-subunit knockdown increases expression of Merlin

We next sought to identify molecular mechanisms that could possibly integrate the cell adhesion function of the β_2-subunit with the observed changes in EGFR activation and associated actin reorganization. The ERM-like protein NF2/Merlin has previously been found to link the actin cytoskeleton to membrane proteins, to mediate contact inhibition, and to regulate EGFR signaling [31,38]. Consistent with a previous study [24], we found that Merlin protein (Fig. 5a) and mRNA (Fig. 5b) decreased throughout postnatal cerebellar development and inversely correlated with β-subunit protein expression (Fig. 1a) and mRNA levels (Fig. 1b). Further, β_2-subunit knockdown resulted in increased protein expression of Merlin, while in β_1-subunit knockdown cells (Sh-Na,Kβ_1-cl1, Sh-Na,Kβ_1-cl2)the Merlin levels were comparable to vector-transfected control cells (ShV) (Fig. 5c). Increased Merlin expression in β_2 knockdown cells was at least in part regulated transcriptionally as Merlin mRNA levels were 1.7 (Sh-Na,Kβ_2-cl1) and 1.8 (Sh-Na,Kβ_2-cl2) times higher in β_2 knockdown cells as compared to control cells (ShV) (Fig. 5d). In addition, the Merlin protein levels in β_2-subunit rescue cells (Sh-Na,Kβ_2-cl1 pEYFP-Na,Kβ_2R) returned to levels similar to ShV control cell levels (Fig. 5e), suggesting that the β_2-subunit indeed regulates Merlin expression in cells originating from cerebellar granule neuron precursors. We next

tested if upregulation of Merlin is responsible for the shift in EGFR phosphorylation kinetics that we observed in β_2-subunit knockdown cells (Fig. 4d). To test this, we knocked down Merlin in β_2-subunit knockdown cells (Sh-Na,Kβ_2-cl1 NF2/Merlin siRNA) and performed a time course experiment with EGF. We found that Merlin knockdown in β_2-subunit knockdown cells prevented both the shift in kinetics and the increase in the degree of EGFR phosphorylation observed in the β_2 alone knockdown cells, while phosphorylated Erk1/2 and Akt levels were unaffected (Fig. 5f). These data demonstrate that the increase in Merlin in β_2-subunit knockdown cells is responsible for the increase and a temporal shift in EGFR phosphorylation observed in these cells. It has been shown previously that Merlin can associate with, and sequester EGFR into a separate membrane compartment [38]. Whether this is the case in β_2-subunit knockdown cells as well as the molecular links between β_2-subunit and Merlin function remain to be determined.

Knockdown of the β_2-subunit results in YAP inactivation and reduced N-Ras expression

Merlin is an important regulator of the transcription coactivator YAP (yes-associated protein) within the Hippo pathway [30]. Indeed, upregulation of Merlin in β_2-subunit knockout cells was accompanied by increased phosphorylation of YAP at serine 127, a marker of YAP inactivation. Increased YAP phosphorylation was not detected in cells treated with the cardiac glycoside ouabain, a pharmacological inhibitor of the Na,K-ATPase pump function (Fig. 6a), suggesting that the reduced pump activity observed in β_2-subunit knockdown cells (Fig. 2c) does not mediate the changes in YAP activation. It remains to be determined whether the cell adhesion function of the β_2-subunit regulates YAP activity in cerebellar granule cells. It has been shown previously that Merlin decreases RAS gene expression through YAP-dependent transcriptional inactivation of the promoters of RAS genes, including H-Ras, K-Ras and N-Ras [39]. In our study, knockdown of Na,K-ATPase β_2-subunit, but not the β_1-subunit, resulted in a decrease in pan-Ras protein expression (Fig. 6b), and this decrease in overall Ras levels was seen in both β_2-subunit knockdown clones (Fig. 6c). The changes in pan-Ras expression may be due to the loss of N-Ras as decreased N-Ras protein levels were observed in both clones of β_2-subunit knockdown cells with no change in β_1-subunit knockdown cells (Fig. 6d). N-Ras protein expression in β_2-subunit rescue cells were similar to N-Ras levels in control cells (Fig. 6e), further supporting that loss of β_2-subunit expression results in decreased N-Ras expression. In addition, we found an inverse relationship between β_2-subunit expression and Merlin levels accompanied by decreased N-Ras levels in cerebellar tissue from β_2-subunit knockout mice compared to wild-type C57BL/6J mice (Fig. 6f) confirming the results from our *in vitro* studies. Together, these data suggest that loss of β_2-subunit expression does not only affect the cellular organization of the actin cytoskeleton (Fig. 3) and kinetics of EGFR activation (Figs. 3, 4), but also induces changes in cell signaling cascades often associated with Merlin function.

DISCUSSION

Despite structural differences and varied tissue expression patterns, little is known about isoform-specific roles of the β_1 and β_2 subunit of Na,K-ATPase or the molecular pathways that may relate these functions to intracellular signals. Cerebellar granule cells present an ideal system to study isoform-specific roles of the β_1 and the β_2 subunits, as they are one of

the few cell types that express both proteins, thus allowing for defining isoform-specific functions within the same cell type. Work published previously from our laboratory showed that activation of Shh signaling, a major developmental pathway regulating CGP proliferation, decreased β_1-subunit expression and reduced β_1-subunit levels were associated with increased cell proliferation and *in vivo* tumorigenesis of DAOY cells [35]. However, the β_2 subunit was not suppressed by activation of Shh signaling (Fig. 1d, e), and knockdown of the β_2 isoform did not alter DAOY cell proliferation (Supplemental Fig. 2). Beyond this, only the knockdown of the β_2 isoform, but not the β_1-subunit, led to changes in actin stress fiber formation. Further, only in β_2 knockdown cells, we observed a shift in EGFR phosphorylation kinetics that could be attributed to the upregulation of NF2/Merlin, a ERM-like protein known to relate cell adhesion to the actin cytoskeleton. Together, our studies for the first time demonstrate independent cellular functions for the β_1 and β_2 subunits of Na,K-ATPase and link the β_2-subunit to Merlin expression and function in cerebellar granule cells.

The β-subunits of Na,K-ATPase are multifunctional proteins with both pump-dependent and pump-independent roles. They are involved in the translation of and trafficking of the catalytic α-subunit to the plasma membrane, regulate the kinetic properties of the pump, and function as cell adhesion molecules and cell signaling scaffolds [40,1,4]. Individual isoforms of the β-subunit preferentially associate with certain α-subunits and different α-/β-subunit combinations have different K^+affinities and turn-over-rates with $\alpha 2/\beta_2$ having a remarkably reduced affinity for K^+. Thus, it has been suggested that the α_2/β_2 complex in cerebellar granule cells may have unique functional characteristics to respond to $Na^+$and K^+gradients in the cerebellum which has a high neuronal density together with a relatively low abundance of astrocytes [41,40,32]. Initial characterization of our knockdown clones indicated that loss of the β_1- or β_2-subunit was accompanied by a reciprocal increase in the β_2 or β_1 isoform, respectively. The increase of the β_2-isoform in β_1-subunit knockdown cells was accompanied by an increase in α_3, and not its preferred binding partner α_2, with the overall Na,K-ATP activity as measured by $^{86}Rb^+$ uptake being around 80% of the activity of vector control cells. On the other hand, while the increase in the β_1 isoform in β_2-subunit knockdown cells was accompanied by an increase in α_1,the increase in the α_1/β_1 complex did not completely restore the pump activity (Fig. 2 and 3). It is possible that the compensatory increase of the α_1/β_1 complex was simply not sufficient to substitute for the loss of the activity of the $\alpha 2/\beta_2$ heterodimer or that the β_2-subunit has additional functions in cerebellar granule cells beyond its role in regulating the catalytic activity of the pump that cannot be replaced by the β_1 isoform.

We reported previously that inhibition of Na,K-ATPase activity by the cardiac glycoside ouabain attenuated the formation of EGF-induced actin stress fibers in DAOY cells [42], similar to what we observed in the studies presented here. Nevertheless, in cells with pharmacological inhibition of the Na,K-ATPase activity, EGFR activation patterns determined 15 minutes after EGF treatment were similar to vehicle control cells, a time point when we observed clear differences in the levels of phosphorylated EGFR between β_2-subunit knockdown and empty vector control cells (Fig. 4d). Further, ouabain treatment inhibited not only EGF-induced stress fiber formation but also the activation of Erk1/2 and Akt, an effect we did not observe in β_2-subunit knockdown cells. Thus, acute changes in Na,K-ATPase activity achieved by pharmacological inhibition of the pump activity may

directly affect EGFR downstream signaling pathways and actin stress fiber formation without changes in the activation kinetics of the receptor itself However, the high concentrations of ouabain used in our previous studies are not sustainable in cell culture over an extended period of time or in an *in vivo* environment. It is likely that in a physiological environment, chronic inhibition of Na,K-ATPase pump activity and/or the loss of additional functions of the β_2-subunit, such as its cell adhesion function, lead to additional, sustained cellular changes that allow for a more subtle control of the organization of the actin cytoskeleton. Towards this, knockdown of the β_2-subunit resulted in an increase in Merlin/NF2, a protein that links adhesion molecules to the actin cytoskeleton and is known to regulate EGFR activation through spatial regulation of the receptor [31,38]. Indeed, knockdown of Merlin rescued EGFR activation kinetics and actin stress fiber formation in response to EGF treatment in β_2-subunit knockdown cells. While at this point we do not know the molecular mechanisms that inversely regulate Merlin expression in β_2-subunit knockdown cells, it is conceivable that the observed phenotypic changes in these cells are due to a combination of reduced pump function and loss of the adhesion function of the β_2-subunit within Merlin-EGFR signaling microdomains (Figure 7).

Merlin is well known as a tumor suppressor protein. Deletion or loss-of-function mutations in Merlin cause neurofibromatosis 2 (NF2), an autosomal dominant disease characterized by the bilateral formation of vestibular schwannomas and often associated with severe comorbidity and neuropathy. NF2 mutations and Merlin inactivation are further found in spontaneous meningiomas and schwannomas as well as in other types of cancers, including breast, colorectal, skin, kidney, liver and prostate and in glioblastoma [43,44]. In these tumors, Merlin inhibits tumor growth through mechanisms that are commonly associated with a tumor suppressor protein such as regulating cell proliferation, cell survival, apoptosis, cell adhesion, cell motility and invasion [45]. Nevertheless, tumor formation requires the loss of both functional alleles of *Nf2*, whereas loss of only one allele may result in neuronal deficiencies even before tumors develop [25], suggesting that Merlin may have cell-type specific functions in neuronal cells that are not directly associated with its tumor suppressor function. This is particularly intriguing in cerebellar granule cells.

In CGPs, Merlin expression not only decreases during granule cell differentiation at a time when CGPs cease to proliferate and start to migrate from the EGL to the IGL on their way to differentiate into mature neurons (Fig. 5a) [24], but also inversely correlate with AMOG/β_2 expression. With the limited expression pattern of the β_2-subunit in brain, mostly in glial cells and certain types of neurons such as cerebellar granule cells, and β_2-subunit expression regulating Merlin levels, we propose that a functional interaction between the β_2-subunit and Merlin may provide a unique regulatory pathway during the spatio-temporal differentiation of cerebellar granule cells (Figure 7). Such a mechanism may possibly involve the control of granule cell migration along the fibers of Bergmann glial cells [46,36], a process that requires neuron-glia interaction and is regulated by EGFR signaling [47,48], or during the final differentiation of granule cells into mature neurons. However, while the β_2-subunit has been implicated in granule cell migration [6], AMOG$^{0/0}$ mice do not have gross structural anomalies in the layered organization of the cerebellar cortex [15]. At the same time, it has been shown that expression of the β_2-subunit promotes neurite outgrowth [21] while overexpression of Merlin in primary cultures of cerebellar granule cells causes a significant

decline in neurite outgrowth [24], suggesting that the increase in β_2-subunit and reduced Merlin expression may be required in later stages of cerebellar granule cell maturation and neuronal wiring. Interestingly, AMOG$^{0/0}$ mice develop motor incoordination at around 15 days of age. However, as AMOG$^{0/0}$ mice do not reach adulthood and die around the age of 17 days before complete maturation of the cerebellum, at this time we do not know whether loss of the β_2-subunit causes a disease phenotype attributable to an altered function of the cerebellum. As of now, no neurological disease have been associated with either the β_1-or β_2-subunit of Na,K-ATPase. Understanding the molecular and cellular roles of these subunits could help to reveal a larger role in the nervous system and neurological diseases. In the case of the β_2-subunit these may mimic at least in part diseases that have been associated with aberrant Merlin function or expression, which beyond its role in NF2, has been implicated as a candidate gene for autism spectrum disorder and dendritic spine abnormalities [25].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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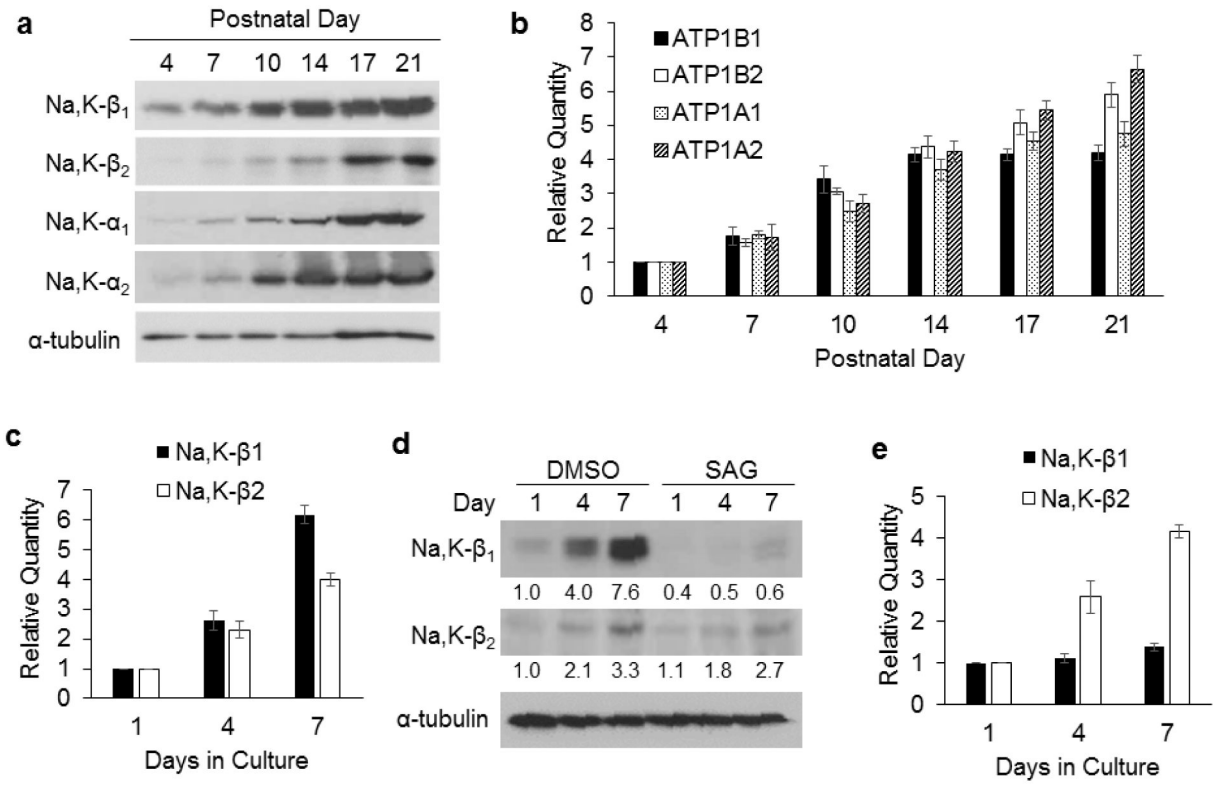

Figure 1. Na,K-ATPase subunit expression during postnatal differentiation of mouse cerebellar granule cells.

(a) Expression of Na,K-ATPase α- and β-subunit isoforms in cerebella of C57BL/6J mice at indicated ages, α-tubulin served as a loading control. **(b)** mRNA levels of Na,K-ATPase α- and β-subunit isoforms in cerebella taken from C57BL/6J mice at indicated ages. mRNA expression was normalized to beta-2-microglobulin. Graph represents averages from three independent experiments ± standard error (SE). **(c)** mRNA levels of Na,K-ATPase β isoforms in primary cultures of cerebellar granule precursor (CGP) cells. Data are averages from three experiments ± SE. mRNA expression was normalized to beta-2-microglobulin. **(d)** Na,K- ATPase β_1 and β_2-subunit protein levels in CGP cells that were srown *in vitro* for indicated time points in the presence of the smoothened agonist SAG (1 μM), Cells were treated with the vehicle DMSO as a control. Values underneath each immunoblot represent average densitometric values taken from three independent experiments. α-tubulin served as the loading control. **(e)** mRNA levels of Na,K-ATPase β isoforms in CGP cells grown *in vitro* and treated with 100 nM SAG. Averages from three experiments ± SE are shown. mRNA expression was normalized to beta-2-microglobulin.

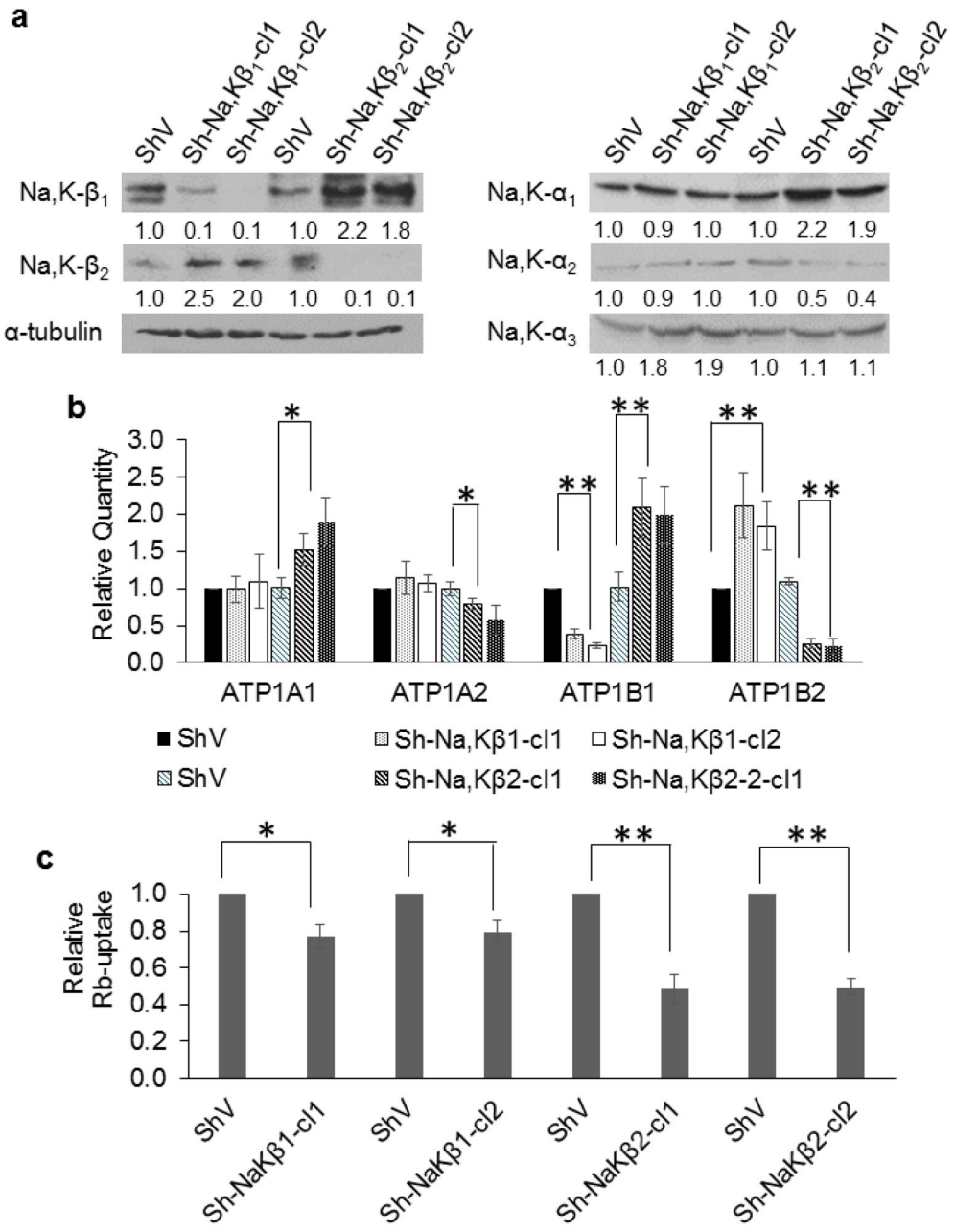

Figure 2. Na,K-ATPase activity and isoform expression in β_1-and β_2-subunit knockdown cell lines.

Na,K-ATPase α- and β-subunit isoform protein (**a**) and mRNA (**b**) expression in two independent clones each of β_1-subunit (Sh-Na,Kβ_1-cl1 and Sh-Na,Kβ_1-cl2) and β_2-subunit (Sh-Na,Kβ_2-cl1 and Sh-Na,Kβ_2-cl2) knockdown cells and their respective control DAOY cell lines (ShV). Values underneath each immunoblot represent average densitometric values taken from three independent experiments. α-tubulin served as a loading control (**a**). (**b**) mRNA expression was normalized to β-actin. Graph represents averages from three experiments ± SE. *p<0.05, **=p<0.001 (**c**) Ouabain-sensitive Na,K-ATPase activity in β_1- and β_2-subunit knockdown and their respective control DAOY cell lines as determined by a

$^{86}Rb^{+}$ uptake assay. Graph represents averages from three replicates ± SE. *p<0.005, **=p<0.0005.

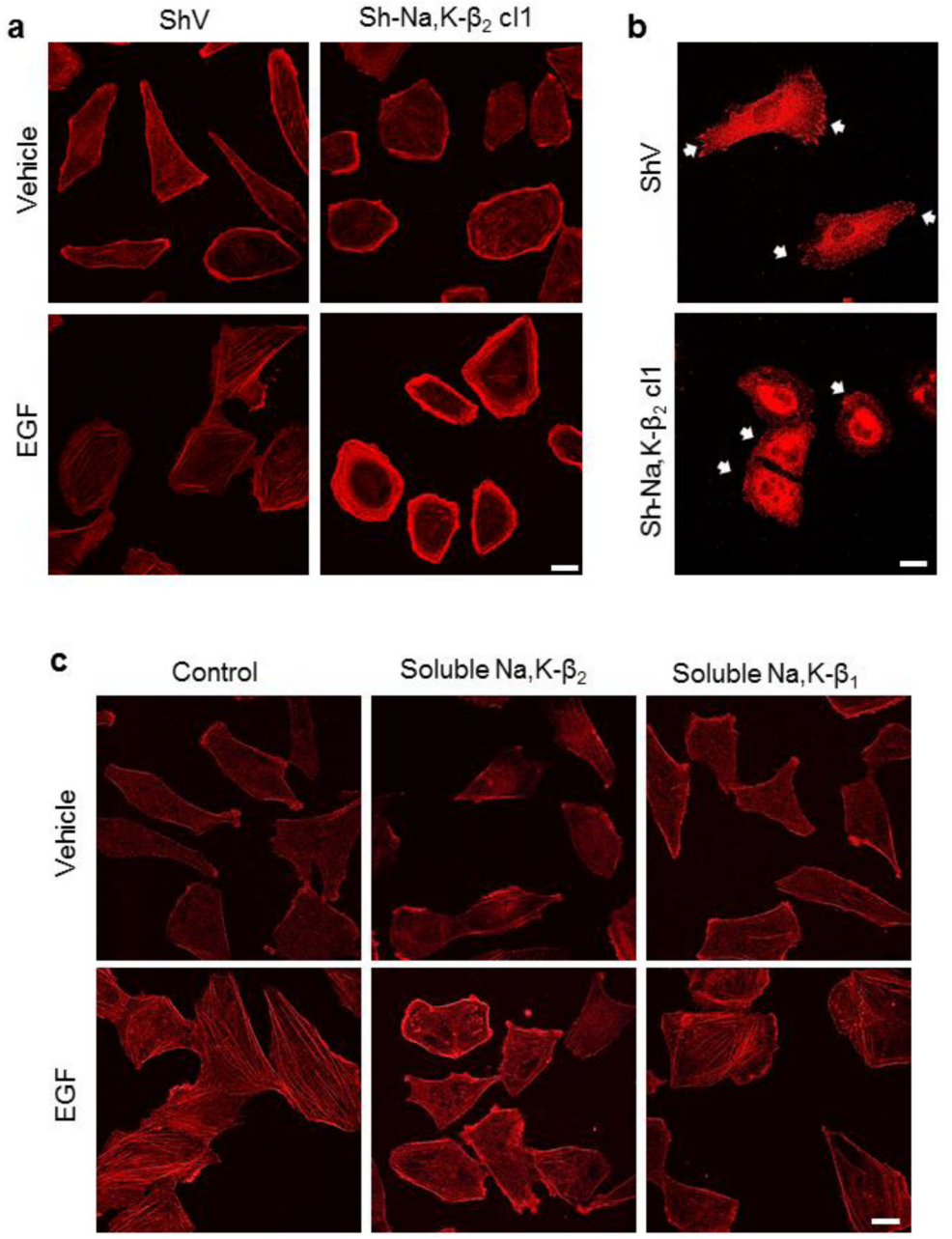

Figure 3. Loss of Na,K-ATPase β_2-subunit expression and cell adhesion function lead to changes in the organization of the actin cytoskeleton.

(a) Phalloidin staining of actin fibers in β_2-subunit knockdown cells treated with or without 10 ng/mL EGF for 15 minutes. Scale bar, 25 μm. **(b)** Control and β_2-subunit knockdown cells were treated with 10 ng/mL EGF for 15 minutes, fixed, and immunostained with an α-vinculin antibody. Arrows pointing to differences in focal adhesion localization in the two cell lines. Scale bar, 25 μm. **(c)** Phalloidin staining of DAOY cells treated with or without 10 ng/ml EGF for 15 minutes following 24 hour treatment with control media or media containing the soluble extracellular domain of human Na,K-ATPase β_1- or β_2-subunit. Scale bar, 25 μm.

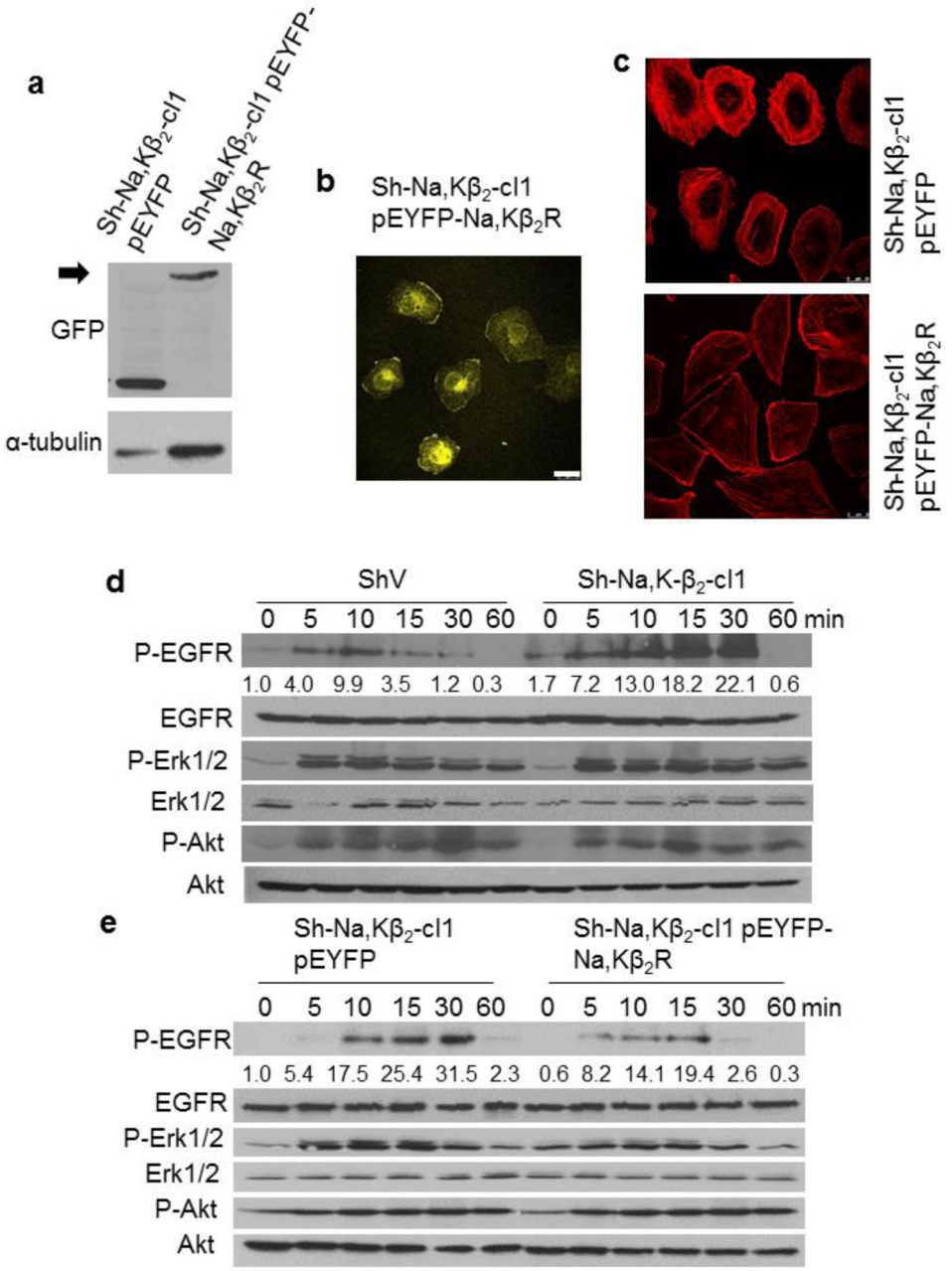

Figure 4. Na,K-ATPase β_2-subunit knockdown alters EGFR phosphorylation kinetics.

(a) Sh-Na,Kβ_2-cl1 cells were stably transfected with empty pEYFP vector as a control β_2 knockdown cell line (Sh-Na,Kβ_2-cl1 pEYFP) or with a YFP-β_2-subunit fusion plasmid (Sh-Na,Kβ_2-cl1 pEYFP- Na,Kβ_2R) to rescue β_2-subunit expression in knockdown cells. An immunoblot with anti-GFP antibody that cross-reacts with YFP shows expression of Na,K-β_2-tagged YFP in the rescue cell line. α-tubulin served as a loading control. More rescue cell protein was loaded than control cell protein because the cytoplasmic YFP in the control cells gave a much stronger band than the membrane-bound β_2 fusion protein. (**b**) Confocal microscopy images of YFP expression in the β_2-subunit rescue cell line. Scale bar, 25 μm. (c) β_2-subunit knockdown and rescue cell lines were treated with 10 ng/mL EGF for 15

minutes, fixed, and stained with phalloidin to visualize F-actin stress fibers. Scale bar, 25 μm. (**d**) Control and β_2-subunit knockdown cells were treated with 10 ng/mL EGF for indicated time points. Immunoblots were done using anti-phospho- and total EGFR, anti-phospho- and total Erk1/2, and anti-phospho- and total Akt antibodies. (**e**) EGF time courses experiments were performed in control β_2-subunit knockdown and β_2-subunit rescue cell lines. Cell treatment and immunoblots were performed as in (**d**). Values underneath the P-EGFR immunoblots in (**d**) and (**e**) represent average densitometric values taken from three independent experiments.

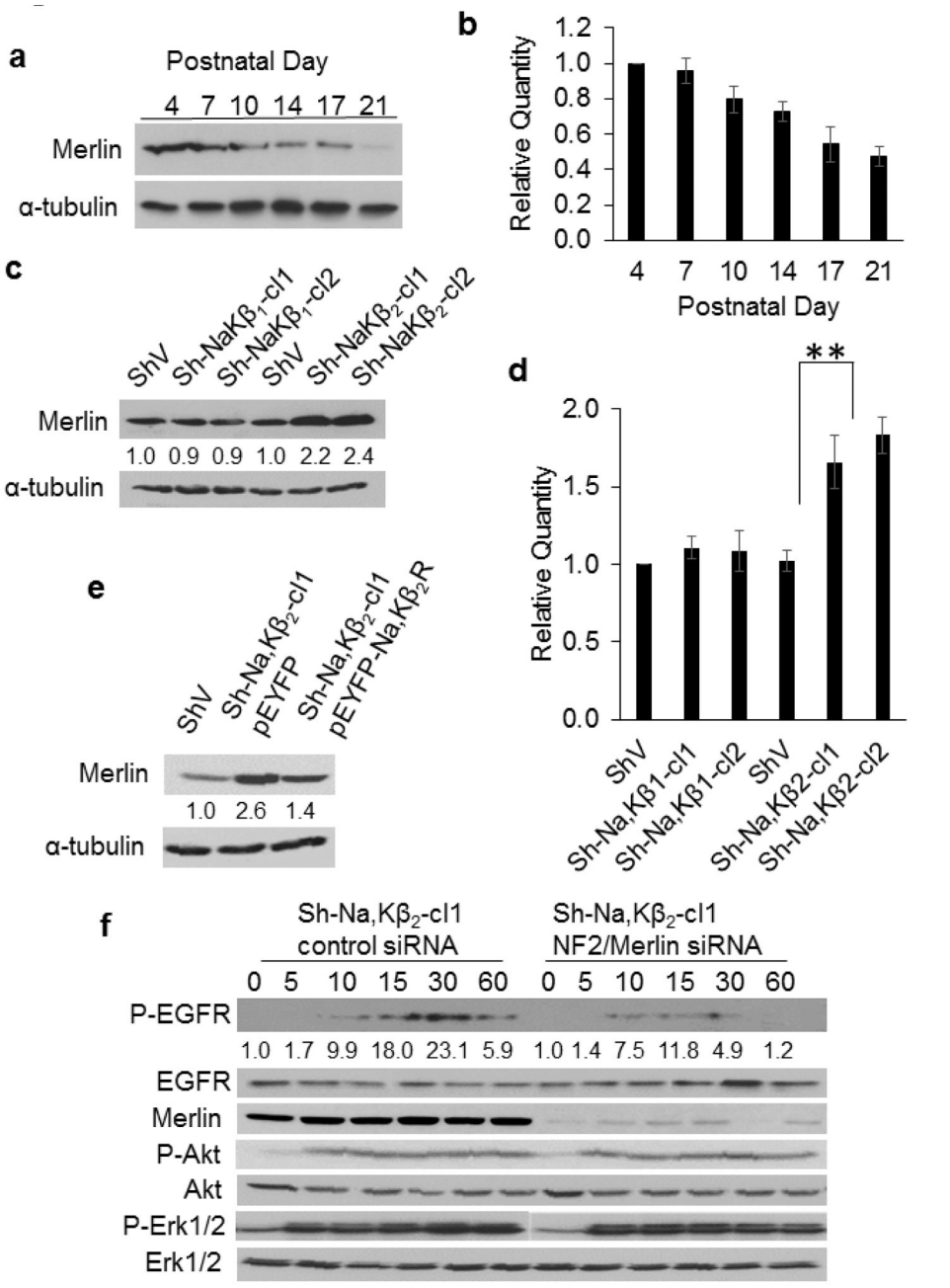

Figure 5. NF2/Merlin expression is upregulated upon loss of Na,K-ATPase β_2-subunit expression.

Merlin protein **(a)** and mRNA **(b)** expression in cerebella dissected from C57BL/6J mice at postnatal days 4, 7, 10, 14, 17, and 21. α-tubulin served as a loading control **(a),** and mRNA expression was normalized to beta-2-microglobulin (**b**). Graph represents averages from three experiments ± SE. (**c**) Merlin protein expression in β_1-and β_2-subunit knockdown cell lines and their respective control cell lines. Values underneath the Merlin immunoblot represent densitometric values. α-tubulin served as a loading control. (**d**). Merlin mRNA levels in β_1-and β_2-subunit knockdown cell lines and their respective control cell lines. mRNA expression was normalized to β-actin. Graph represents averages from three

experiments ± SE. **p<0.05. (**e**) An immunoblot for Merlin in control (ShV), β_2 knockdown (Sh-Na,Kβ_2-cl1 pEYFP), and β_2 rescue (Sh-Na,Kβ_2-cl1 pEYFP-Na,Kβ_2R)cell lines. Values underneath the Merlin immunoblot represent densitometric values. α-tubulin served as a loading control. (**f**) β_2-subunit knockdown cells transfected with either non-targeting SMARTpool siRNA or NF2 SMARTpool siRNA were treated with 10 ng/mL EGF for 0-60 minutes as described in figure 4. Immunoblots were done using anti-phospho- and total EGFR, anti-phospho- and total Erk1/2, and anti-phospho- and total Akt antibodies as well as anti-Merlin antibody to verify the knockdown. Values underneath the P-EGFR immunoblot represent average densitometric values taken from three independent experiments.

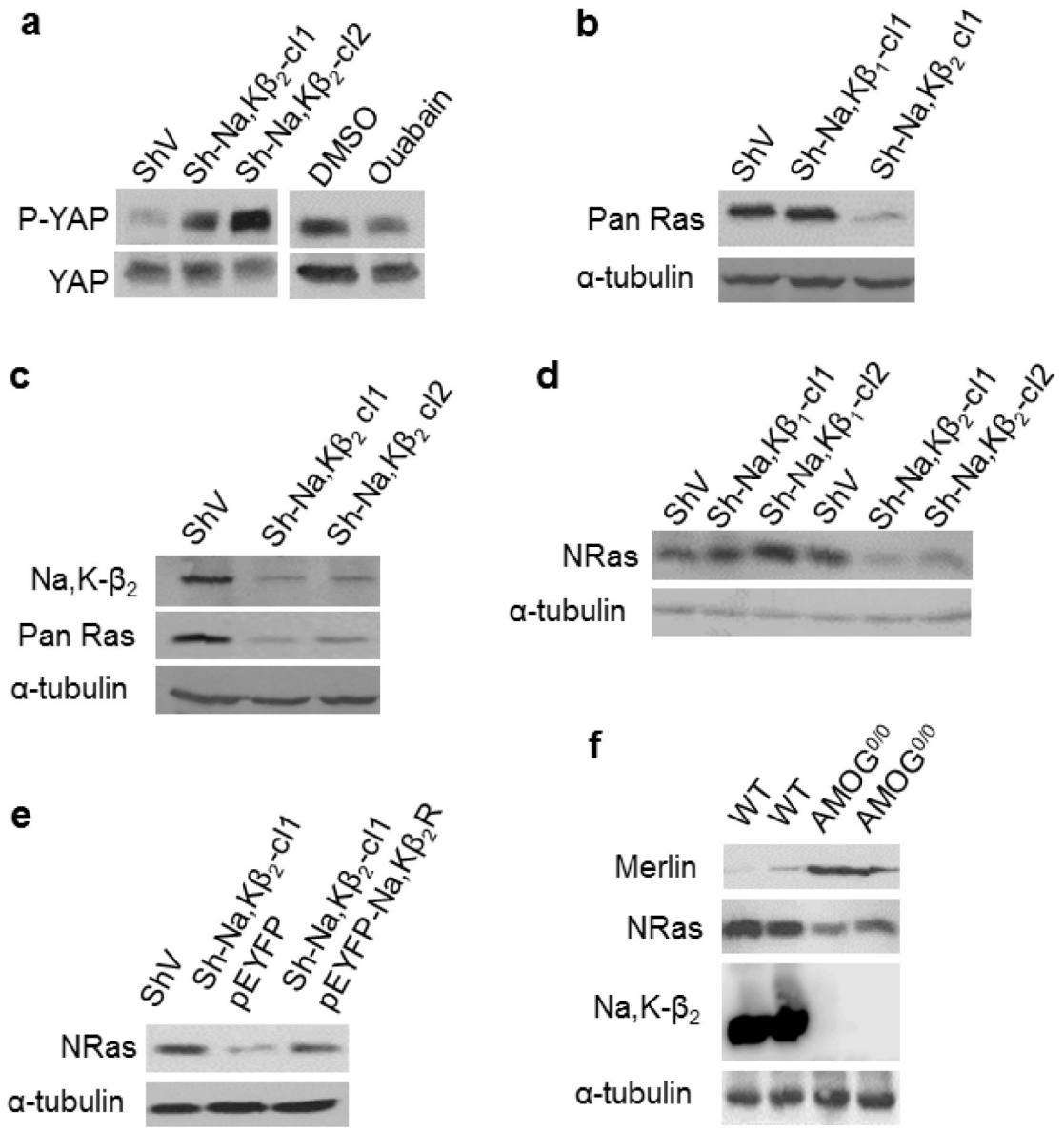

Figure 6. Na,K-ATPase β_2-subunit knockdown leads to decereased N-Ras protein expression.

(**a**) Immunoblots for phospho- and total YAP in control and β_2-subunit knockdown cell lines, and in DAOY cells treated with vehicle or 50 μM ouabain for 4 hours. (**b**) Immunoblot with pan-Ras antibody in control, β_1-and β_2-subunit knockdown cell lines. α-tubulin served as a loading control. (**c**) Immunoblots for the β_2-subunit and pan-Ras in control and both β_2-subunit knockdown cell clones. α-tubulin served as a loading control. (**d**) An immunoblot for N-Ras in β_1-and β_2-subunit knockdown cell lines and their respective control cell lines. α-tubulin served as a loading control. (**e**) An immunoblot for N-Ras in control, β_2-subunit knockdown, and β_2-subunit rescue cell lines. α-tubulin served as a loading control. (**f**) Merlin and N-Ras protein expression in tissues from wild-type (WT) and β_2-subunit knockout ($AMOG^{0/0}$) mouse cerebellum. α-tubulin served as a loading control.

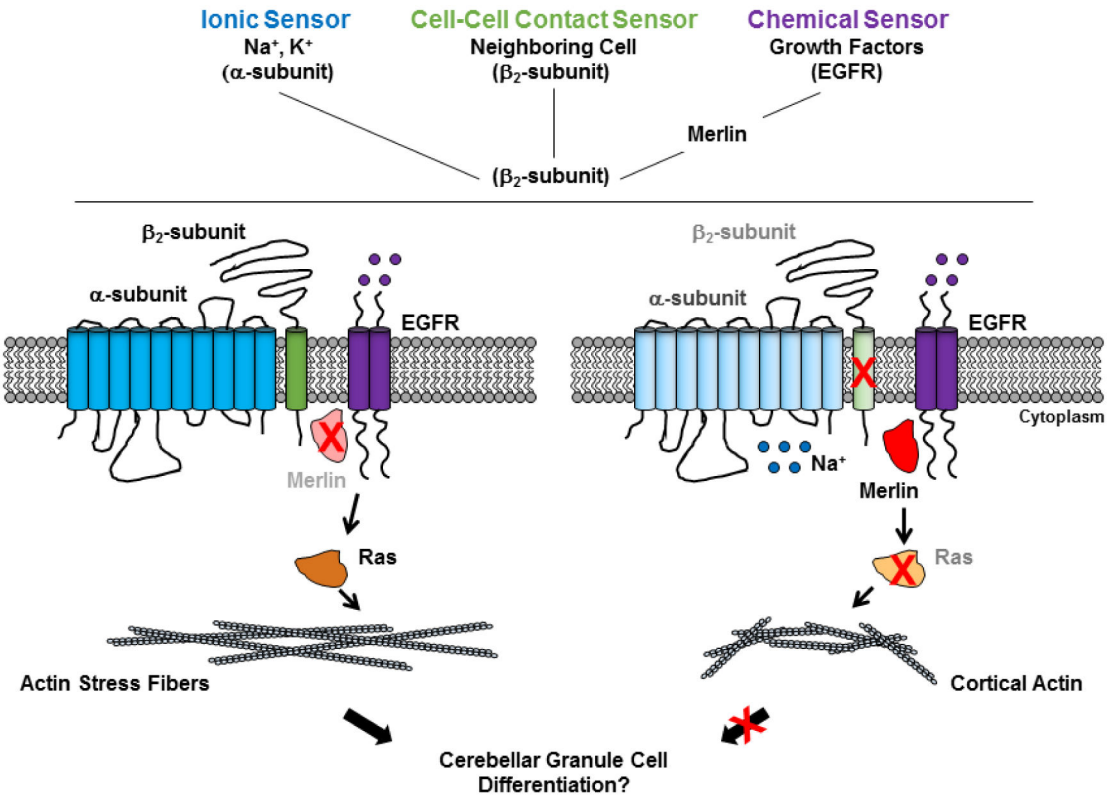

Figure 7. Schematic model.

By serving as an ionic sensor through its association with the catalytic α-subunit a structural sensor through its cell-cell adhesion function and a chemical sensor through its regulation of EGF signaling through NF2/Merlin, the β_2-subunit may function as a molecular switch in cerebellar granule cells by controlling the organization of the actin cytoskeleton during neuronal differentiation.