Mutations in LZTR1 drive human disease by dysregulating RAS ubiquitination

One Sentence Summary: LZTR1 loss-of-function leads to human disease by dysregulating RAS ubiquitination and its association with the membrane.

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Abstract:

The LZTR1 protein, an adaptor for cullin 3 (CUL3) ubiquitin ligase complex, is implicated in human disease, yet its mechanism of action remains unknown. We found that *Lztr1* haploinsufficiency in mice recapitulates Noonan Syndrome phenotypes; whereas LZTR1 loss in Schwann cells drives dedifferentiation and proliferation. By trapping LZTR1 complexes from intact mammalian cells, we identified the guanosine triphosphatase RAS as a substrate for the LZTR1-CUL3 complex. Ubiquitome analysis showed that loss of *Lztr1* abrogated Ras ubiquitination at lysine 170. LZTR1-mediated ubiquitination inhibited RAS signalling by attenuating its association with the membrane. Disease-associated *LZTR1* mutations disrupted either LZTR1-CUL3 complex formation, or its interaction with RAS proteins. RAS regulation by LZTR1-mediated ubiquitination provides an explanation for the role of LZTR1 in human disease.

Main text: Mutations concurrent with loss of heterozygosity at *LZTR1* are associated with glioblastoma and Schwannomatosis (1-3). LZTR1 mutations predispose for paediatric neoplasms and are increased over background in liver and testicular cancers (4, 5). The most recurrent LZTR1 mutation in cancer is an inactivating splice-site mutation at codon 217 (fig. S1) (4, 6). LZTR1 constitutes to Noonan Syndrome caused by dysregulation of the guanosine triphosphatase RAS (7-9). However, how LZTR1 contributes to human disease is not known.

To uncover *Lztr1* disease mechanisms, we used an *Lztr1* deletion mouse model. We found that loss of *Lztr1* is lethal between E17.5 and birth (fig. S2A). *Lztr1*^{+/-} male mice exhibited decreased weight (fig. S2, B to D) and facial dysmorphia (Fig. 1A). *Lztr1*^{+/-} mice, both male and female, displayed heart malformations, including decreased left ventricular systolic

function, increased diastolic dimensions, eccentric hypertrophy, increased cardiomyocyte area, and reduced longevity (Fig. 1, B to C, and fig. S2, E to F). Collectively, our results show that *Lztr1*^{+/-} mice recapitulates some phenotypes of human Noonan Syndrome patients, indicating that LZTR1 function is evolutionary conserved.

We engineered several cellular models of LZTR1 loss: mouse embryo fibroblasts (MEFs) derived from *Lztr1*^{+/+} and *Lztr1*^{-/-} mouse embryos; primary human Schwann cells expressing shGFP or shLZTR1; immortalized human Schwann cells and HeLa with CRISPR/Cas9-mediated LZTR1-indels (fig. S3). In all tested models, LZTR1 loss increased growth rate (Fig. 1D and fig. S4, A to C). Overexpression of wt-LZTR1, but not of LZTR1 mutants, reduced the enhanced growth rate (Fig. 1E and fig. S4, D and E). Loss of LZTR1 in Schwann cells enhanced 2D colony and anchorage-independent (AI) growth (Fig. 1F and fig. S4, F to H), and overexpression of wt-LZTR1, but not of disease-associated LZTR1 mutants, suppressed AI growth in LZTR1-indel cells (Fig. 1G). Furthermore, depletion of LZTR1 in Schwann cells showed a gene expression signature (Fig. 1H) resembling that of proliferating Schwann cells during nerve regeneration (10). These data suggest that LZTR1 loss drives Schwann cells from quiescent, myelinating cells into proliferating cells.

LZTR1 acts as a substrate adaptor for cullin 3 (CUL3) ubiquitin ligase complexes (11). To identify candidate LZTR1 substrates, we used a mass spectrometry (MS) Virotrap method, which allowed trapping protein complexes from intact mammalian cells (fig. S5A) (12). The screen with LZTR1 as bait detected CUL3, HRAS, and NRAS among the top hits (Fig. 2A). The reciprocal Virotrap screen with HRAS-deltaCAAX mutant lacking the last four amino acids confirmed the complex formation with LZTR1 and identified CUL3 (Fig. 2B). Further, a panRAS antibody that recognizes all RAS isoforms co-immunoprecipitated (co-IP'd) with HA-tagged LZTR1. Similarly, Flag-tagged LZTR1 co-IP'd endogenous RAS proteins, but not RAC1 (fig. S5B). Moreover, we introduced a Halo-tag HiBiT (13) to the LZTR1 locus in HeLa

cells and MEFs (Fig. 2C and fig. S5, C to D). panRAS antibody co-IP'd endogenous RAS and endogenous HiBiT-LZTR1 (Fig. 2C and fig. S5E). Reciprocal co-IPs demonstrated that LZTR1 interacted with each of the three Flag-RAS isoforms (fig. S5F). Together, these results indicate that LZTR1, CUL3, and RAS form a complex.

To test whether the LZTR1-CUL3 complex might control RAS ubiquitination, we performed an *in vitro* ubiquitination reactions. We observed ubiquitination of wt-HRAS specifically in the presence of the LZTR1-CUL3 complex (fig. S6A). Co-expression of LZTR1 and CUL3 in human kidney embryonic (HEK293T) cells increased amounts of ubiquitinated RAS (Fig 2D and fig. S6B). In contrast, treatment with the cullin neddylation inhibitor MLN4924 or loss of LZTR1 led to decreased ubiquitination of all Flag-tagged RAS protein isoforms (fig. S6, C to F). Thus, the LZTR1-CUL3 complex can promote ubiquitination of RAS.

To investigate the role of LZTR1 in ubiquitination of endogenous RAS, we characterized ubiquitination profiles of *Lztr1*^{+/+} and *Lztr1*^{-/-} MEFs by MS (Fig. 2E). Ubiquitome analysis revealed that ubiquitination of Hras at K170 was abrogated in MEFs lacking *Lztr1* (Fig. 2F and fig. S6G), indicating that endogenous Ras may serve as a substrate for the LZTR1-CUL3 complex. We also optimized a proximity ligation assay (PLA) with paired antibodies to ubiquitin (Ub) and panRAS. Consistently to the MS results, Hras-K170R knock-in or depletion of LZTR1 led to a decrease in panRAS-Ub proximity signals (Fig. 2G and fig. S7). MS analyses failed to detect any C-terminal peptides of Nras or Kras, perhaps because these isoforms are not highly expressed in MEFs, and their C-termini are lysine-rich (fig. S8A). However, LZTR1 interacted with (fig. S5F) and ubiquitinated all three RAS isoforms (fig. S6F), consistent with evolutionary conservation of K170 (fig. S8D). Thus, the LZTR1-CUL3 complex appears to mediate ubiquitination of all RAS isoforms.

Although multiple truncating and missense mutations of *LZTR1* have been reported in Noonan Syndrome and Schwannomatosis (1-3, 14), no recurrent germline *LZTR1* mutations have been

identified to date. Additional sequencing analysis of blood samples from Schwannomatosis patients revealed several recurrent germline mutations of *LZTR1* within the BTB (broad-complex, tramtrack and bric a brac)-BACK domains predicted to mediate dimerization and CUL3 binding (11, 15) (Fig. 3A). Concordantly, the BTB-BACK *LZTR1* mutants, except L812P, exhibited reduced binding to CUL3 (Fig. 3B and fig.S9A). Although *LZTR1*-L812P retained interaction with CUL3, it failed to form dimers (Fig. 3C). Oligomerization of BTB domains determines the subcellular distribution of CUL3 adaptors (15, 16). Indeed, both endogenous and ectopically expressed HA-tagged *LZTR1* showed punctate endomembrane immunostaining (fig. S9B), whereas the BTB-BACK domain *LZTR1* mutants, including *LZTR1*-L812P, showed diffuse cytoplasmic staining (Fig. 3D and fig. S9C).

Missense mutations within the *LZTR1* Kelch domain predicted to mediate substrate binding, are also found in human disease. In co-IP assays, Kelch domain *LZTR1* mutants showed decreased binding to RAS (Fig. 3E). The Kelch domain mutants like wt-*LZTR1* displayed punctate immunostaining, but only wt-*LZTR1* led to re-localization of RAS to the *LZTR1*-CUL3-containing puncta, which represent loci of *LZTR1*-CUL3-mediated ubiquitination (Fig. 3F and fig. S9, D and E). Consistently, the *LZTR1*-L812P mutant, which does not form puncta, only weakly ubiquitinated RAS as did the *LZTR1*-Y726* mutant (Fig. 3G). Thus, disease-associated *LZTR1* mutations appear to abrogate RAS ubiquitination by disrupting the formation of the RAS-*LZTR1*-CUL3 complex.

RAS ubiquitination affects RAS- mitogen-activated protein kinase (MAPK) signalling (17). Loss of *LZTR1* led to increased RAS activity and phosphorylation of MEK1/MEK2 and ERK1/ERK2 in all tested model systems; whereas enhanced phosphorylation of AKT was cell-dependent (Fig. 4A and fig. S10). After serum stimulation, *Lztr1*^{-/-} MEFs showed higher MEK1/2 activity at all-time points, whereas *Lztr1*^{+/-} MEFs had higher MEK1/MEK2 phosphorylation only at later time points (fig. S11A). Thus, *Lztr1* abundance may fine-tune

the activation of Ras signalling. Restoration of wt-LZTR1 expression in LZTR1-indel cells decreased MEK1/MEK2 activity (fig. S11B). Finally, LZTR1-mutated Schwannomas showed strong staining of phosphorylated ERK1/ERK2 compared to wt-LZTR1 nerve trunk (fig. S11, C to D). The MEK1 inhibitor pimasertib abolished the colony growth difference between wt-LZTR1 and LZTR1-mutant cells (fig. S11E). Pimasertib treatment also rescued the embryonic lethality of *Lztr1*^{-/-} mice (Fig. 4B). Thus, LZTR1-mediated phenotypes arise, at least in part, from increased RAS signalling.

Although our MS analysis detected Ras ubiquitination at several lysines, loss of *Lztr1* abrogated ubiquitination of Ras only at K170 (Fig. 4C). Thus, ubiquitination of Hras at K170 may specifically require *Lztr1*. Indeed, though LZTR1 depletion hindered ubiquitination of wt-HRAS, it did not affect ubiquitination of the HRAS-K170R mutant (Fig. 4D). Loss of LZTR1 also abolished the difference in Ras ubiquitination between wt-Hras and Hras-K170R MEFs (Fig. 4E and fig. S12A). Nonetheless, the LZTR1-CUL3 complex did ubiquitinate mutant HRAS-K170R in vitro (fig. S6A). The site specificity in vivo could be directed by anchoring of RAS to the membrane. Moreover, overexpression of the HRAS-K170R mutant led to higher activation of ERK1/ERK2 than did overexpression of wt-HRAS, and LZTR1 depletion did not affect ERK1/ERK2 activity in HRAS-K170R overexpressing cells (Fig. 4F and fig. S12B). K170R knock-in MEFs also showed increased MAPK signalling and growth rates (fig. S12, C and D). Collectively, these data indicate that LZTR1-mediated ubiquitination of RAS at K170 suppresses RAS-MAPK signalling.

Ubiquitination of RAS can inhibit its activity by triggering its degradation (18). However, quantitative MS analysis did not reveal an increase in RAS protein abundance in *Lztr1*^{-/-} MEFs (fig. S6G). wt-LZTR1 and LZTR1-indel Schwann cells treated with the protein synthesis inhibitor cycloheximide also showed similar RAS stability (fig. S12E). Thus, LZTR1 regulates RAS by a non-degradative mechanism. Ubiquitination of RAS also induces its re-localization

to endomembranes (19, 20). However, LZTR1 overexpression increased the endomembrane fraction of both wt-RAS and HRAS-K170R mutant (fig. S12F). LZTR1 alone also only slightly increased RAS ubiquitination (Fig. 2D) and did not affect the MAPK pathway (fig. S12G), suggesting that LZTR1 overexpression promotes endomembrane localization of RAS independently of its ability to mediate ubiquitination at K170.

To assess how ubiquitination of RAS at K170 controls its activity, we elucidated modes of the interaction between RAS and conjugated ubiquitin. LZTR1 co-localized with NRAS at RAB11/Transferrin receptor-positive recycling endosomes (fig. S13), suggesting that LZTR1 regulates ubiquitination of farnesylated and palmitoylated RAS. Therefore, we performed molecular simulations on lipidated RAS. In the initial structures of Ub conjugated to K170 of RAS, the hypervariable regions (HVRs) of RAS exposed their anchor portions to the solution. The long-lasting simulations showed that Ub secured the anchor portion of the HVR by sequestering the farnesyl and palmitoyl groups (fig. S14). Concordantly to a rapid kinetics of spontaneous insertion of lipidated RAS into the membrane (21-24), the HVRs of non-ubiquitinated RAS straightforwardly associated with membranes. However, Ub conjugation to K170 of RAS prevented the HVRs from binding to and inserting into membranes (Fig. 4G). Thus, ubiquitination at K170 may disrupt the association of RAS to the membrane. Indeed, loss of LZTR1 or Hras-K170R knock-in increased the fraction of membrane-bound RAS (Fig. 4, H and I, and fig. S15). These results are all consistent with RAS ubiquitination at K170 inhibiting RAS activity by impairing its association with the membrane.

Our results indicate that LZTR1-mediated ubiquitination of RAS on K170 modulates RAS activity, dysregulation of which leads to human disease. An accompanying study shows that LZTR1 dysregulation also confers drug resistance (Bigenzahn et al). Understanding this unconventional mechanism of RAS activation may help to identify patients who might benefit from RAS pathway inhibitors and inform novel therapeutic approaches for these patients.

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Figure legends

Fig. 1. LZTR1 loss recapitulates disease phenotypes.

(A) Morphometric characteristics of skulls of 12-month-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. (B) H&E-stained heart ventricular sections. Scale bar, 0.5 mm. The total cardiac area was quantified by Fiji. Horizontal lines are means $\pm$ SD. (C) A mean area of 200 cardiomyocytes measured in laminin-stained heart sections of $Lztr1^{+/+}$ and $Lztr1^{+/-}$ mice. Horizontal lines are means $\pm$ SD. (D) Growth rate of early-passage MEFs isolated from 3 $Lztr1^{+/+}$ and 3 $Lztr1^{-/-}$ embryos. $n=3$. (E) Growth rate of $Lztr1^{-/-}$ MEFs expressing an empty vector (EV), wt-LZTR1, or LZTR1 mutants. $n=3$. (F) AI growth of Schwann cells expressing Cas9 or Cas9/gLZTR1. $n=3$. (G) AI growth of LZTR1-indel Schwann cells expressing the indicated constructs. $n=3$. (H) qRT-PCR analysis of primary human Schwann cells expressing shGFP or pooled shLZTR1. $n=3$. *D-H*, Values are means $\pm$ SEM. *A-C, F-H*, p values are from by two-sided t test. *D, E*, P values detected by two-way ANOVA.

Fig. 2. The LZTR1-CUL3 complex ubiquitinates RAS proteins.

(A,B) Virotrap screens performed in HEK293T cells using Group-specific antigen (GAG)-LZTR1 (A) or GAG-HRAS-deltaCAAX (B) as baits. Escherichia coli dihydrofolate reductase (eDHFR) fused to GAG was used as a negative control. (C) A scheme for the generation of cells expressing in-frame HiBiT-LZTR1 protein. RAS was IP'd from the HiBiT-LZTR1 edited HeLa cell lysates with panRAS antibody. Luminescent signal was generated by HiBiT incubated with LgBiT. (D) Ubiquitinated RAS was purified from HEK293T cells expressing the indicated constructs by Co^{2+} metal affinity chromatography, and detected by immunoblotting. (E) A workflow for the ubiquitome analysis. (F) A heatmap showing differentially ubiquitinated peptides in $Lztr1^{+/+}$ and $Lztr1^{-/-}$ MEFs. (G) The quantification of the PLA analysis of Schwann cells expressing Cas9 or Cas9/gLZTR1 using antibodies against

panRAS and Ub. Values are mean $\pm$ SEM, n=3. P-value from by two-sided t test. EV, empty vector. Whole cell lysate, WCL.

Fig. 3. Disease-associated LZTR1 mutations are loss of function.

(A) LZTR1 mutations in Schwannomatosis and Noonan Syndrome individuals. Missense LZTR1 mutations identified in our cohort of Schwannomatosis patients are shown in blue. K, Kelch domain. (B) Flag-tagged CUL3 purified from HEK293T cells was incubated with HA-tagged LZTR1 overexpressing cell lysates, and then IP'd using anti-Flag resin. LZTR1 was detected by immunoblotting with anti-HA antibody. (C) Cross-linking reactions were performed using HA-tagged LZTR1 purified from HEK293T cells. LZTR1 was detected by immunoblotting using anti-HA antibody. (D) Immunostaining of HeLa cells expressing HA-tagged wt-LZTR1 or LZTR1 mutants with anti-HA antibody. Scale bar, 10 μ m. (E) RAS proteins were IP'd with antibody against panRAS. LZTR1 was detected by immunoblotting with anti-HA antibody. (F) Co-localization of mCherry-NRAS and HA-tagged LZTR1 expressed in HeLa-Cas9/gLZTR1 cells. Scale bar, 10 μ m. Values are means $\pm$ SEM. P values were detected by two-sided t test. (G) Ubiquitinated NRAS was purified from HEK293T cells expressing the indicated constructs by Co²⁺ metal affinity chromatography and detected by anti-Flag antibody. Whole cell lysate, WCL.

Fig. 4. Ubiquitination at K170 inhibits RAS by impairing its association to the membrane.

(A) MEFs isolated from 3 Lztr1^{+/+} and 3 Lztr1^{-/-} embryos were serum-starved, stimulated with 10% serum, and analyzed by immunoblotting. Values are means of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM. P value are from a two-way ANOVA. (B) Progeny from the indicated Lztr1^{+/-} matings. Pregnant mice were treated with pimasertib starting from E7.5. (C) Quantitative ubiquitome analysis of Lztr1^{+/+} and Lztr1^{-/-} MEFs. (D) Ubiquitinated RAS was purified from HEK293T expressing the indicated constructs by Co²⁺ metal affinity

chromatography, and detected by immunoblotting. **(E)** The PLA analysis of wt-Hras and Hras-K170R MEFs expressing shGFP or shLztr1 using antibodies against panRAS and Ub. Scale bar, 10 μ m. **(F)** HEK293T cells expressing the indicated constructs were serum-starved overnight, stimulated with 10% serum, and analyzed by immunoblotting. Values are means of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM, n=3. P values are from a two-way ANOVA. **(G)** Snapshots of Ub-conjugated RAS at the lipid bilayer composed of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) lipids (3:1 molar ratio). **(H,I)** Immunoblotting of the membrane and cytoplasmic fractions isolated from HeLa cells expressing shGFP and shLZTR1 (*H*); wt-Hras and Hras-K170R MEFs (*I*).

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Author Contribution: MSt, FR, SP, MS performed biochemical experiments; MSt, MFB, SP performed cellular experiments; MFB, TP performed qRT-PCR analysis; MFB performed PLA assay; MFB, PN performed immunofluorescence experiments; SP, MDT performed mice experiments; AB, YC generated CRISPR clones; LAA performed experiments on non-immortalized Schwann cells; ER, PC did the IHC staining and analysis; SE, KG, JT designed and performed the Virotrap screen; FI did the MS analysis; LM, EL, NR performed mutation analysis of Schwannomatosis patients; RN, MZ, HJ, performed protein simulations; PC, SH performed echocardiography analysis; MSt, MFB, SP, AS analysed the data; AS wrote the manuscript. All authors discussed the results and commented on the manuscript. **Competing interests:** No potential conflict of interest was reported by the authors. **Data and materials availability:** We thank the EUCOMM Consortium for providing us *Lztr1^{tm1a(EUCOMM)Wtsi}* embryonic stem cells.

Figure 1

Figure 3

Figure 4

Supplementary Materials for
**Mutations in LZTR1 drive human disease by dysregulating RAS
ubiquitination**

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This PDF file includes:

Materials and Methods
Figs. S1 to S15
References (25-40)

Materials and Methods

Genetically modified mice

All procedures involving animals were performed in accordance with the guidelines of the IACUC of KU Leuven and approved in project application P143/2016.

Lztr1^{tm1a(EUCOMM)Wtsi} embryonic stem cells were purchased from EUCOMM and used for in vitro fertilization of a female CD-1. Primers used for genotyping are:

Sequence		
<i>neo</i>	Forward	5'-AAGATGGATTGCACGCAGGTTCTC-3'
	Reverse	5'-GACGAGATCCTCGCCGTCGGGCATGCGCGCC-3'
<i>Lztr1</i>	Forward	5'-AGGTGAGGGGTTAGAGGAAGC-3'
	Reverse	5'-TTTCTGACCCAACCTCCCAAG-3'

To justify the number of analyzed animals, power analysis was conducted using the findings of (25). The phenotypic characteristics of *Lztr1*^{+/+} and *Lztr1*^{+/-} mice were assessed by observers blind to the genotype. For the rescue experiments, pregnant mice were treated with pimasertib (a daily i.p. injection 1 mg/kg of body weight) starting from E7.5.

Mouse Embryo Fibroblasts (MEFs) were prepared from individual E13.5 embryos and cultured in DMEM containing 10% FBS, 1% Pen-Strep, 50 μ M β -ME. Briefly, embryos were finely minced with razor blade and incubated for 30 minutes at 37°C with 0.05% trypsin.

Skull measurements were performed with a calliper as described in (26).

Cell culture and lentiviral transduction

Mouse Embryonic Fibroblasts (MEFs), HEK293T, and HeLa cells were cultured in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Primary human Schwann cells were purchased from ScienCell Research Laboratories and cultured according to the manufacturer's protocol. Transient transfections were performed using GeneJuice (Millipore). Lentiviral infections were performed as described by the RNAi Consortium (TRC)

(<http://www.broadinstitute.org/rnai/public/resources/protocols>). Infected cells were selected by treatment with 2 μ g/ml puromycin (InvivoGen) for 3 days or with 300 μ g/ml of hygromycin-B (Thermo Fisher Scientific) for 4 days.

Cell growth was determined by CellTiter-Glo luminescent cell viability kit (Promega); or by an Xcelligence; or by an IncuCyte ZOOM system (Essen BioScience) according to the manufacturers' protocols. For growth assay of MEFs, 20000 cells/well were seeded in

12-well plates in DMEM containing 10% FBS and counted by trypan blue exclusion at the indicated time points.

For clonogenic growth assay $3\text{--}5 \times 10^3$ cells were plated in triplicates and grown for 2 weeks before staining in 1% crystal violet, 20% methanol. 2D colony growth was quantified using ImageJ software (NIH). Anchorage-independent growth in soft-agar was performed as previously described in (27). 10^4 cells were plated in triplicates in 0.35% Noble agar over a 0.5% agar bottom layer. Three weeks after plating, several random areas were imaged and colony number was quantified using ImageJ software (NIH).

Virotrap Screening

The pMET7-GAG-HRASd9 and pMET7-GAG-LZTR1 constructs were generated as described (12). Virotrap experiments were performed as described in (28). Briefly, HEK293T cells were transfected with pMET7-GAG-HRAS, pMET7-GAG-LZTR1, or pMET7-GAG-eDHFR together with either untagged, or FLAG-tagged VSV-G using polyethylenimine (Sigma-Aldrich). Particles were harvested after 36 hours and purified from the supernatant by incubation with 20 μ l MyOne paramagnetic streptavidin beads (ThermoFisher Scientific) pre-loaded with 2 μ g FLAG-Bio-M2 (Sigma Aldrich). After a single wash step with TRIS wash buffer (TWB: 20 mM Tris-HCl pH 7.5, 150 mM NaCl), particles were eluted with FLAG peptide (Sigma Aldrich) in TWB and processed by amphipols lysis and precipitation under acidic conditions. After resuspension in triethylammonium bicarbonate (TEAB) buffer and boiling, proteins were overnight digested with sequencing-grade trypsin (Promega). After acidic precipitation of partially or undigested proteins by amphipols and acidification, the sample was analyzed by mass spectrometry.

MAXQUANT searches (version 1.5.6.5) were performed against the human SwissProt database. The searches were performed together with the corresponding control samples to allow matching of MS spectra between runs. Contaminants and identifications against the REVERSE database were removed using the PERSEUS software (version 1.5.5.3). A two-sided t-test was performed with multiple testing correction using 1000 randomizations. FDR was set at 5% with the S0 curve set at 2.

All raw data and MAXQUANT combined files were uploaded to PRIDE through ProteomExchange: **Project accession:** PXD007049

Ubiquitome analysis

Cells were washed twice with PBS and scraped in urea lysis buffer containing 9 M urea, 20 mM HEPES pH 8.0, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate. Samples were sonicated and centrifuged for 15 minutes at $16,000 \times g$ at room temperature. 12 mg of total protein per sample was used for further analysis. Proteins in each sample were reduced by incubating with 4.5 mM DTT for 30 minutes at 55°C. Alkylation of the proteins was done by addition of 10 mM iodoacetamide for 15 minutes at room temperature. The samples were diluted with 20 mM HEPES pH 8.0 to a urea concentration of 4 M and the proteins were digested with lysyl endopeptidase (Wako) for 4 hours at room temperature. All samples were further diluted with 20 mM HEPES pH 8.0 to a final urea concentration of 2 M and proteins were digested with 120 μ g trypsin (Promega) overnight at 37 °C.

Immunocapture of GG-modified peptides was then performed using the PTMScan® Ubiquitin Remnant Motif (K- ϵ -GG) Kit (Cell Signaling Technology) according to the

manufacturer's instructions. Briefly, peptides were purified on Sep-Pak C18 cartridges (Waters), lyophilized for two days and re-dissolved in the immunoprecipitation buffer. Aliquots corresponding to 100 µg of digested protein material were taken for shotgun proteomics analysis. Peptides were incubated with the antibody-bead slurry for 2 hours on a rotator at 4°C. GG-modified peptides were eluted in 100 µl 0.15% TFA and desalted on reversed phase C18 OMIX tips (Agilent). Purified GG-modified peptides were dried under vacuum in HPLC inserts and stored at -20°C until LC-MS/MS analysis. Purified GG-modified peptides were re-dissolved in 20 µl loading solvent A (0.1% TFA in water/ACN (98:2, v/v)) of which 10 µl was injected for LC-MS/MS analysis on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo Scientific). From the aliquots for shotgun proteomics analysis, approximately 3 µg of peptides was injected for LC-MS/MS analysis on an Ultimate 3000 HPLC system in-line connected to an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific). Data analysis was performed with MaxQuant (version 1.5.7.4) using the Andromeda search engine with default search settings including a false discovery rate set at 1% on both the peptide and protein level. Two different searches were performed to analyze the spectra from the GG-enriched samples and the shotgun samples separately. In both searches spectra were interrogated against all mouse proteins in the Uniprot/Swiss-Prot database (www.uniprot.org). The minimum score for modified peptides was set to 30 and for the GG-enriched samples, GlyGly modification of lysine residues was set as an additional variable modification. Further data analysis was performed with the Perseus software (version 1.5.5.3) after loading the GlyGly (K) Sites table from MaxQuant. Reverse database hits were removed as well as potential contaminants. Sites with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution around the detection limit, then t-test was performed ($FDR \leq 0.05$ and $S0=1$) to compare site intensities in *Lztr1*^{+/+} and *Lztr1*^{-/-} samples.

LZTR1 mutation analysis

Individuals with Schwannomatosis were referred for genetic testing. Comprehensive *LZTR1* mutational testing was performed on DNA extracted from peripheral white blood cells. All 21 coding exons and exon-intron boundaries (+20, -20bp) of *LZTR1* were genomically amplified (primer sequences available upon request). All primers were M13 tagged to facilitate downstream sequencing. PCR products were bidirectionally sequenced on an ABI 3730xl genetic analyser. Sequences were analysed using SeqPilot (JSI medical systems) and mutations interpreted with the Alamut software (Interactive Biosoftware). Copy number changes (deletions or duplications) were analysed by multiplex ligation-dependent probe amplification (MLPA) using SALSA MLPA probe mix P455 (MRC Holland). Nucleotide numbering is based on cDNA reference sequence NM_006767.3 with the A of the start codon marked as 1.

Expression vectors and antibodies

Full-length expression constructs were generated by cloning the gBlocks (IDTDNA). Point *LZTR1* mutations were introduced using QuickChange site-directed mutagenesis PCR (Stratagene, La Jolla). SV40 large T genomic DNA was cloned to pLenti-hygro vector. Lentiviral pLA-CMV-N-Flag or pLA-CMV-N-HA vectors were used to generate Flag/HA-tagged constructs. The pMT107-6xHis-ubiquitin plasmid was a generous gift

from Dr. Bohmann (University of Rochester, USA). pLKO.1-puro shGFP and pLKO.1-puro vectors containing shRNAs targeting *LZTR1* (pLKO.1-sh*LZTR1*-3 (TRCN0000240480); pLKO.1-sh*LZTR1*-5 (TRCN0000181026); pLKO.1-sh*Lztr1*-2 (TRCN0000103741); pLKO.1-sh*Lztr1*-3 (TRCN0000103742) were purchased from Sigma Aldrich. pLentiCRISPR v2 empty vector and pLentiCRISPR v2 plasmids with gRNA targeting *LZTR1* were purchased from GeneScript.

The following antibodies were used: mouse monoclonal anti-FLAG (Sigma-Aldrich, M2), anti-RAS (Millipore, Clone 10), anti-vinculin (Sigma-Aldrich, clone hVIN-1), anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, 3A7, #9107), HSP 70/HSC 70 (Santa Cruz Biotechnology, W27, sc-24), anti-MEK1/2 (Cell Signaling, L38C12, #4694), anti-AKT1 (Cell Signaling, 40D4); rat monoclonal anti-HA (Roche, 3F10), anti-GM130 (BD Biosciences, Clone 35), anti-EEA1 (BD Biosciences, #610457), anti-TFR (ThermoFisher, clone H68.4), anti-CD63 (Abcam, ab8219); rabbit monoclonal anti-pMEK1/2 (Ser217/221) (Cell Signaling, #9121), anti-Rab11 (Cell Signaling, Clone D4F5), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, #9101), anti-pAKT1 (Ser473) Cell Signaling, D9E), anti-GST (Cell Signaling Technology, 91G1); rabbit polyclonal anti-DYKDDDDK (Cell Signaling), anti-His (Santa Cruz Biotechnology, H-15), anti-ubiquitin (Abcam, ab7788), anti-laminin (Sigma Aldrich, L9393), anti-Calnexin (Abcam, ab10286), anti-*LZTR1* (Avivas Systems Biology, OAAB18526), anti-CUL3 (Bethyl Laboratories); guinea pig polyclonal anti-GFAP (Synaptic Systems).

CRISPR/Cas9 mediated gene targeting

The modification at the target site of *LZTR1* was validated using Surveyor mutation detection kit according the manufacturer's protocol (IDTDNA). Primers used for Surveyor assay and Sanger sequencing of *LZTR1* clones are 5'-GGGGCTTGTGGAAGGAGTC-3' and 5'-AGGAGGGAGTCTAAGGGCTG-3'. Single clones with wt-*LZTR1* or mutated *LZTR1* from positive pools were isolated as previously described(29).

Genomic DNA of cells expressing Cas9 only or both Cas9 and gRNA targeting *LZTR1* was extracted using QIAamp DNA mini kit (Qiagen). Primers were designed to produce amplicons specifically from wildtype *LZTR1*. qPCR was performed on Light Cycler 480 (Roche).

Primers targeting on genomic DNA:

Gene		Sequence
<i>HMBS</i>	Forward	5'-ACAGACGGGGTCCTTTCATTC-3'
	Reverse	5'-AAGACGTTTGTGTGCAGTTCG-3'
<i>TBP</i>	Forward	5'-TGGTGAAGAACTGATGACTTTAGA-3'
	Reverse	5'-AAAATAAGACTGACGAAGTTTGC-3'

<i>B2M</i>	Forward	5'- AAACGCAAATCCCCTGTCTAC-3'
	Reverse	5'- AAATGGTTGAGTTGGACCCGA-3'
<i>YWHAZ</i>	Forward	5'- AGAGTCATACAAAGACAGCACG-3'
	Reverse	5'- AGAGCCTATGAAATGTGTTGAGA-3'
<i>LZTR1</i>	Forward	5'- TCTCTTTACTCAGGGGGTTACA-3'
	Reverse	5'- TTTCTGCAAAGTTTCTCACCGTC-3'

For the generation of Hras K170R knockin, immortalized MEFs expressing Cas9 were transfected with gRNA targeting Hras (5'-GCAGCCAGGACCACTCTCAT-3') and a single strand DNA repair template (CTCTGTCCCCAGGGCGTGGAGGATGCATTCTATACACTAGT CCGTGAGATTCGCCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAAAGT GGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCTGACACCAGGT, PAGE Ultramer® DNA Oligo, IDT) using Lipofectamine 2000 (Invitrogen). 48 h post transfection single cells were FACS- sorted into 96well plates. gDNA from single cell clones was extracted using QuickExtract DNA Extraction Solution (Epicentre, Illumina) following manufacturer's instructions. Screening of CRISPR clones was done by PCR using 5'-CAGCATAAATTGCGGCGC-3' and 5'-GGGGAGCAAGGACATCAGAG-3'primers and confirmed by Sanger sequencing.

For LZTR1/Lztr1-HiBiT knock-ins Alt-R® CRISPR-Cas9 strategy from IDT was used. Cells were transfected with RNP complexes containing cr:tracrRNA and HiFi Cas9 together with ssoligo (PAGE Ultramer® DNA Oligo, IDT) as a repair template using Lipofectamine 2000 (Invitrogen). 48 h post transfection single cells were FACS sorted into 96well plates. Single cell clones were screened using Nano-Glo® HiBiT Lytic Detection System (Promega). gDNA from positive clones was extracted using QIAamp DNA mini kit (Qiagen) and HiBiT knock-ins were confirmed by Sanger sequencing.

LZTR1-HiBiT repair template

/5Phos/CGGCCCCGGGGCGGTGGCCGCAAGTTGGGCTTACAGCGCGGCGGATCC CGCGTGGACCCGCGATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCGC TGGACCGGGCAGCACGGGGGGGCGAGATCGGGGCTGCGGCCCTG/3Phos/

Lztr1-HiBiT repair template

/5Phos/GCGTACCCCTCCTGTCAAGGCCCGGATCCAATTGGGCCTCCGGAGCC AGCGCTAATCTTCTTGAACAGCCGCCAGCCGCTCACCATCACTTGTTTCAGGCT GGATAGGCCGCCACGTTGCAGGACTCACGGCCACCGCCC/3Phos/

In all CRISPR knockin experiments cutting efficiencies of gRNA's were checked by TIDE online tool (<https://tide.deskgen.com>). The analysis of Sanger sequences from CRISPR clones was performed using CRISP-ID online tool.

Immunoprecipitation, immunoblotting, and electrochemiluminescent immunoassay

Cells were washed twice in cold PBS and scraped on ice in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40) containing protease inhibitor and phosphatase inhibitor cocktails (Roche). Samples were subsequently cleared by centrifugation for 10 min 16,000g at 4°C. Tagged proteins were immunoprecipitated using anti-Flag (M2), anti-HA agarose beads (Sigma-Aldrich), or protein A/G magnetic beads (Thermo Scientific, #88803) for 4 hours at 4°C, washed five times with cold co-immunoprecipitation buffer and finally eluted with 3×Flag, HA peptides according to the manufacturer's protocol or by boiling in 2x NuPAGE LDS sample buffer (Thermo Scientific, #NP0007). Ubiquitinated proteins were purified as described previously(30). Briefly, cells were lysed in co-immunoprecipitation buffer containing EDTA-free protease inhibitor cocktail (Roche). Cell lysates were mixed with His-buffer A (PBS, pH 8.0, 6 M guanidinium-HCl, 0.1% NP-40 and 1 mM β-ME) and added to TALON Co²⁺ coated beads (Clontech). After binding, the resin was washed with His-buffer B (PBS, pH 8.0, 0.1% NP-40, 5% glycerol, 20 mM imidazole), and proteins were eluted with 500 mM imidazole solution.

For immunoblotting, equivalent amounts of cell lysates were separated on 4-12% gradient gels (Invitrogen), transferred to nitrocellulose membranes, and incubated with the indicated antibodies. The signal was visualized by chemiluminescence (Amersham Pharmacia Biotech) using an automated digital developer.

Meso Scale Discovery 96-well multispot plates were used for quantitative phospho/total MEK1/2 (K15129D), ERK1/2(K15107D), and AKT1 (K15100A3) analyses according to the manufacturer's instructions (Meso Scale Diagnostics). Plates were analysed using MESO QuickPlex SQ120 multiplex imager (Meso Scale Diagnostics).

RAS activity assay

RAS activity was assessed using the RAS activation ELISA assay Kit according to manufacturer's protocol (EMD Millipore). Briefly, cells were washed twice with cold TBS and scraped on ice in Lysis/Wash Buffer containing protease and phosphatase inhibitor cocktails (Roche). Samples were subsequently cleared by centrifugation for 10 minutes 16,000 g at 4°C. Glutathione-coated 96-well plate was washed and then blocked with RAF1-GST. After incubation with 10 µg of cell lysate and washing, the plate was incubated with primary anti-RAS antibody followed by secondary HRP-conjugated antibody. The signal was detected after addition of Chemiluminescent Substrate and recorded using Victor X3 Multilabel Plate Reader (PerkinElmer).

The RAS activation assay was conducted according to the manufacturer's protocol (Millipore). Briefly, cells were washed with cold PBS and lysed in Lysis/Wash Buffer (Millipore). Equal amounts of clarified cell lysates were mixed with Raf-1-RBD agarose beads (Millipore) and incubated for 45 minutes at 4°C. The beads were washed with Lysis/Wash Buffer three times and eluted by boiling in 2xSDS buffer. Eluted proteins were separated by SDS-PAGE and detected by immunoblotting.

Subcellular Fractionation

Subcellular Fractionation was performed as described previously (31) with minor modifications. Briefly, cells were washed twice with cold TBS and scraped on ice in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose supplemented with protease inhibitors and homogenized. The homogenate was centrifuged for 5 minutes at 2,500 x g at 4°C to remove unbroken cells and nuclei. The membrane fraction was collected by

centrifugation at 100,000 x *g* for 2 hours at 4°C. The pellet was washed twice with cold TBS and re-suspended in Pierce IP Lysis Buffer (ThermoFisher, #87787) supplemented with protease inhibitor, readjusting the volume of the membrane fraction to that of the cytosolic fraction before resolving by SDS-PAGE.

In vitro cross-linking

Cross-linking was performed as described previously (16) with minor modifications. Briefly, HA-LZTR1 was purified from HEK293T cells using anti-HA agarose beads (Sigma-Aldrich). Protein samples were prepared in the following buffer, 20 mM HEPES pH 7.5, 150 mM NaCl, and 5 mM DTT. The amine cross-linker BS3 bis(sulfosuccinimidyl)suberate, (ThermoFisher, # 21585) was added with a final concentration of 2.5 mM. Reactions were incubated at room temperature for 30 minutes. Then the reactions were quenched by the addition of 100 mM Tris pH 7.6 and incubation at room temperature for 15 minutes.

Cellular and tissue staining

For cell immunostaining, 2×10^4 cells per well were plated on 8-well chamber glass slide (Corning, # 354118) and fixed 24 hours after transfection with 4% PFA.

Immunofluorescence was performed as described previously (30). Briefly, cells were permeabilized in PBS-0.15% Triton-X100 and blocked with 1% BSA and 10% goat serum. Primary antibodies and goat Alexa-conjugated secondary antibodies were applied diluted in blocking buffer before mounting with Vectashield (Vector Laboratories). To avoid antibody mouse-rat cross-reactivity, secondary anti-mouse antibodies were pre-blocked with 10% rat serum.

For visualizing ubiquitin conjugation to RAS proteins proximity ligation assay (PLA) was performed using the Duolink kit according to manufacturer's protocol (Sigma Aldrich). Briefly, cells were fixed in 4% PFA, permeabilized in PBS-0.15% Triton and incubated with blocking solution 30 minutes at 37°C. Primary antibodies were applied by diluting in antibody diluent solution 1 hour at room temperature (PanRAS 1/300; Ub Rabbit 1/100). Secondary anti-mouse and anti-rabbit antibodies conjugated to PLA probes were incubated 1 hour at 37°C at room temperature, followed by ligation and amplification with far red fluor-labeled oligonucleotides (30 minutes and 100 minutes at 37°C, respectively). Samples were mounted in Duolink mounting medium containing DAPI (Sigma Aldrich).

Images were obtained by using a confocal Nikon C2 microscope (60x magnification). Image analysis was performed using ImageJ (NIH). Quantification of colocalization was done using JACoP (32). For quantification of PLA signals, random images were taken and the number of particles per nuclei was measured.

Schwannomas tissues were immunostained for p-ERK1/2 and GFAP on a Discovery Ventana automated staining platform. For the evaluation of pERK1/2 immunohistochemistry samples were scored based on an intensity scale and subcellular localization of pERK1/2 immunostaining.

Cardiac tissue was fixed in Neutral Buffered Formalin (NBF) 10% overnight, and further processed for paraffin embedding. Hematoxylin and eosin – stained sections (4 µm) were used to assess overall morphology, and Picro Sirius Red staining for overall fibrosis. Myocyte cross-sectional areas were calculated by measuring the inner circumference of 200 myocytes per sample on laminin–stained sections. Microscopic

analyses were performed using a microscope (Axiovert 200M; Zeiss), and Fiji software (33).

MEFs were stained for SA-B-Gal staining using the Senescence β -Galactosidase Staining Kit (Cell Signaling) according to the manufacturer's protocol.

Gene expression analysis

RNA extraction was performed using RNeasy MINI kit (Qiagen). 500ng of total RNA was used to prepare cDNA for mRNA analysis using RT2 First Strand Kit. The quantification of mRNA was performed on Light Cycler 480 (Roche) system using RT2 SYBR Green qPCR Mastermix and RT2 qPCR LZTR1 primer assay (PPH10123A) (Qiagen).

Primers:

Gene		Sequence
<i>LZTR1</i>	Forward	5'- TCCTGCGGTTTCGATGTGAAA -3'
	Reverse	5'- ACATGCTGCTCCCATAGACG -3'
<i>EGR1</i>	Forward	5'- CTTCAACCCTCAGGCGGAC -3'
	Reverse	5'- GTCTCCACCAGCACCTTCTC -3'
<i>SOX2</i>	Forward	5'- GAGCTTTGCAGGAAGTTTGC -3'
	Reverse	5'- GCAAGAAGCCTCTCCTTGAA -3'
<i>EGR2</i>	Forward	5'- AACGGAGTGGCCGGAGAT-3'
	Reverse	5'-ATGGGAGATCCAACGACCTCTT-3'
<i>GAP43</i>	Forward	5'- AACCATGCTGTGCTGTATGAGAA-3'
	Reverse	5'- CCCAGGTGATGCTGTGACTC -3'
<i>PMP22</i>	Forward	5'-TGGAAGAAGGGGTTACGCTG -3'
	Reverse	5'-GTGGACTGGGAGGGAGGTAT -3'
<i>HPRT1</i>	Forward	5'-TGACACTGGCAAAACAATGCA-3'
	Reverse	5'-GGTCCTTTTCACCAGCAAGCT -3'

<i>GAPDH</i>	Forward	5'-ACCACAGTCCATGCCATCAC -3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA -3'
<i>B2M</i>	Forward	5'- AAACGCAAATCCCACCTGTCAC-3'
	Reverse	5'-TCTCTGCTCCCCACCTCTAAGT-3'
<i>ACTB</i>	Forward	5'- CTGGAACGGTGAAGGTGACA -3'
	Reverse	5'- AAGGGACTTCCTGTAACAACGCA-3'
<i>Lztr1</i>	Forward	5'-GATGAAGGAGTTTGAGCGCC-3'
	Reverse	5'-ATGTCACAGAACTCCGAGCC-3'
<i>Cdkn1a</i>	Forward	5'-TTGCACTCTGGTGTCTGAGC-3'
	Reverse	5'- TGATAGAAATCTGTCAGGCTGGT -3'
<i>Hmbis</i>	Forward	5'- GAAACTCTGCTTCGCTGCATT -3'
	Reverse	5'-TGCCCATCTTTCATCACTGTATG -3'
<i>B2m</i>	Forward	5'- GGTGCTTGTCTCACTGACC -3'
	Reverse	5'- CCCGTTCTTCAGCATTTGGA -3'
<i>Rpl19</i>	Forward	5'- CAGGCATATGGGCATAGGGAA -3'
	Reverse	5'- TGCCTTCAGCTTGTGGATGT -3'

Constructing ubiquitin-conjugated RAS models

The initial coordinates of HRAS were obtained from our previous works (24). The NRAS coordinates are based on the crystal structure of H-RAS with residue modifications to reflect the NRAS sequence. All RAS isoforms are in the GTP-bound state. The initial structure of ubiquitin was obtained from the Protein Data Bank (PDB ID: 1UBQ) (34). To simulate RAS ubiquitination, we developed the patch parameter for the isopeptide bond that connects ubiquitin to the lysine residue at the linker portion of the hypervariable region (HVR) of RAS. Ubiquitin was conjugated to K170 of HRAS or NRAS. To determine possible binding modes of the ubiquitin and the RAS catalytic

domain, we performed molecular docking using the PatchDock online server (35). During the docking, we constrained the residue distance (5 Å) between the C-terminal residue of ubiquitin and the target lysine residue of RAS, ensuring that the ubiquitin stayed at the ubiquitination site. The top 50 structures with high docking score were refined by the FireDock server (36, 37). Structures with the highest scores were selected and solvated in a water box. The unit cell containing proteins, waters and ions were constructed by selecting a proper cell dimension with a criterion that the minimal distance between the edge of the box and the protein surface is 15 Å. Na⁺ and Cl⁻ were added to neutralize the system and mimic the 0.15 M ionic strength. Molecular dynamics (MD) simulations in explicit solvent were performed to evaluate the structural and dynamic properties. To observe the effects of ubiquitination on RAS membrane binding ability, the final structures of ubiquitin-conjugated HRAS or NRAS from the water simulations were placed on membranes composed of DOPC and DOPE lipids (3:1 molar ratio). The initial configurations of the lipid bilayers were obtained from the CHARMM-GUI online server (38).

Molecular dynamics simulation protocols

MD simulations with the all-atom model were performed by the NAMD package (39) using the updated CHARMM all-atom additive force field (version C36) (40). The simulations employed the NPT (constant number of atoms, pressure, and temperature) ensemble at a temperature of 310 K and pressure of 1 atm. The RATTLE algorithm was used to constrain the covalent bonds with hydrogen atoms. Short-range van der Waals (VdW) interactions were calculated by the switch function with the twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the shift methods with the cutoff of 14 Å. The velocity verlet integration was used to generate time steps of 2 fs. Analysis was performed using the tools in CHARMM, VMD and in-house TCL codes.

Echocardiography

At the indicated age range, mice were anesthetized (2% isoflurane, ecuphar) via a nose cone at 1.5 l/min oxygen flow and examined by transthoracic echocardiography with a MS400 18–38 MHz transducer (VEVO 2100, FUJIFILM VisualSonics, Inc. Toronto, Canada). Left ventricular function was assessed in both parasternal long-axis and short-axis views at a 300 Hz frame rate. Left ventricular internal diameter (LVID), the overall left ventricular wall thickness including Inter-Ventricular Septum (IVS) and Posterior Wall (PW) were measured in both End-diastole (d) and End-Systole (s); Fractional Shortening (FS) and Ejection Fraction (EF) and Stroke Volume (SV) were calculated. Pulmonary Valve function was examined through Pulse Wave Doppler acquisition in a modified parasternal short-axis view at aortic-level. Pulmonary Velocity Time Integral (PVTI), Pulmonary Acceleration Time (PAT) and Pulmonary Ejection Time (PET) were measured and their ratio (PET/PAT %) calculated.

Statistical analysis

All data were analysed using Graphpad Prism 7.

Fig. S1. LZTR1 mutations in human cancer. Somatic mutations of LZTR1 in the TCGA PanCancer Atlas cohort. Data were obtained from cBioPortal (Cerami et al, 2012; Gao et al, 2013).

Fig. S2. LZTR1 alterations lead to disease phenotypes. (A) Progeny from the intercross of $Lztr1^{+/-}$ mice. **(B)** Growth curves of $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. p value was assessed by two-way ANOVA. **(C)** Weight of 24-week-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. A horizontal line represents a mean $\pm$ SD. p value was determined by two-sided t test. **(D)** Representative image of 12-month-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. **(E)** Echocardiography analysis of 40-64 week-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ mice. IVSd, Interventricular septum in diastole; IVSs, Interventricular septum in systole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; PWd, posterior wall in diastole; PWs, posterior wall in systole; V(d), volume in diastole; V(s), volume in systole; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; FS area, fractional shortening area; LVV, left ventricle volume; HR, heart rate; Pulmonary VTI, pulmonary velocity time integral; PAT, pulmonary artery acceleration time; PET, pulmonary ejection time. p values were assessed by two-sided t test. **(F)** Survival curve of $Lztr1^{+/+}$ and $Lztr1^{+/-}$ mice. p value was assessed using Log-Rank t test.

Fig. S3. LZTR1 loss cellular models. (A) qRT-PCR gene expression analysis of *Lztr1* in MEFs isolated from *Lztr1*^{+/+}, *Lztr1*^{+/-}, and *Lztr1*^{-/-} embryos. *p* value assessed by two-sided *t* test, *n*=3. (B) Immunoblot analysis of LZTR1 expression in primary human Schwann cells expressing shGFP or pooled shLZTR1. (C) Surveyor assay to detect indel formation in HeLa expressing Cas9 or Cas9 together with gRNAs targeting LZTR1 (gLZTR1). (D) Primer design for the detection of indels in the LZTR1 gene. The gLZTR1 target sequence is shown in red. (E) PCR amplification of the indicated loci using genomic DNA purified from HeLa cells expressing only Cas9 or Cas9 together with gRNAs targeting LZTR1. (F,G) CRISPR/Cas9-mediated modification efficiency of the target LZTR1 locus in HeLa (F) or immortalized Schwann (G) estimated by qPCR. *n*=2. (H) Sequence analysis of single-cell Schwann and HeLa clones after overexpression of Cas9 together with gLZTR1. The gRNA target sequence is shown in red. Exon 5 of LZTR1 is highlighted in grey.

Fig. S4. LZTR1 loss increases cell growth. (A) Growth rate of primary human Schwann cells expressing shGFP or pooled shLZTR1. Data are shown as mean $\pm$ SEM. P values were detected by two-way ANOVA, $n=3$. (B) Growth rate of single-cell Schwann clones. p value assessed by two-way ANOVA, $n=3$. (C) Growth rate of single-cell HeLa clones. p value assessed by two-way ANOVA, $n=3$. (D) Representative immunoblot analysis of ectopic expression of HA-tagged wt-LZTR1 and LZTR1 mutants in *Lztr1*^{-/-} MEFs. (E) Immunoblot analysis of LZTR1 expression after overexpression of an empty vector (EV) or the indicated LZTR1 constructs (top). Growth rate of LZTR1-indel HeLa clone 6 expressing an empty vector (EV) or the indicated LZTR1 constructs. Data are presented as mean $\pm$ SEM. P values were detected by two-way ANOVA, $n=3$. (F) 2D colony growth of single-cell immortalized Schwann clones. Data are shown as mean $\pm$ SEM. $n=3$. (G) Soft agar colonies formed by immortalized Schwann cells after overexpression of Cas9 or Cas9/gLZTR1. (H) Anchorage-independent growth of single-cell immortalized Schwann clones. Data are presented as mean $\pm$ SEM. p values were assessed by two-sided t test. $n=3$.

Fig. S5. LZTR1 interacts with RAS proteins. (A) Schematic outline of the Virotrap approach. Briefly, a bait is fused to the Group-specific antigen (GAG) protein and co-expressed with Flag-tagged glycoprotein of the vesicular stomatitis virus (VSV-G); this triggers the formation of virus-like particles that trap protein complexes under native conditions. The Flag-tagged particles were purified, and their contents determined by MS. (B) HEK293T cells expressing LZTR1 were analysed by immunoprecipitation with anti-Flag beads or anti-panRAS antibody bound to protein A/G agarose. Immunoblotting was performed with the indicated antibodies. (C) Sequence analysis of the HiBiT knock-in HeLa clone. The gRNA target sequence is shown in red; PAM sequence in yellow; start codon in green; HiBiT insertion in purple. (D) HeLa and MEF clones expressing in-frame HiBiT-LZTR1 protein. Luminescent signal was generated by a Halo-tag HiBiT incubated with LgBiT. (E) The HiBiT-Lztr1 edited MEFs were analysed by immunoprecipitation with anti-panRAS antibody bound to protein A/G agarose. Luminescent signal was generated by a Halo-tag HiBiT incubated with LgBiT. (F) The indicated tagged proteins overexpressed in HEK293T cells were immunoprecipitated with anti-Flag (M2) or anti-HA beads, followed by immunoblotting using anti-Flag or anti-HA antibodies.

Fig. S6. The LZTR1-CUL3 ubiquitin ligase controls RAS ubiquitination. (A) wt-HRAS or HRAS-K170R purified from HEK293T cells was incubated with UBE1 (E1), UBE2T (2), the Flag-LZTR1/Flag-CUL3 complex (E3), recombinant HA-tagged ubiquitin and in the presence of Mg-ATP and ubiquitin aldehyde. (B) Flag-tagged NRAS and 6xHis-ubiquitin were introduced into HEK293T cells expressing HA-LZTR1/V5-CUL3 or an empty vector (EV). Whole cell lysates were analyzed by immunoblotting using the indicated antibodies. (C) Flag-tagged NRAS was overexpressed together with 6xHis-Ubiquitin in HEK293T cells. The cells were treated with the indicated doses of MLN4924 for 20 hours. Ubiquitinated NRAS was purified by Co^{2+} metal affinity chromatography and detected by anti-Flag antibody. (D) 6xHis-ubiquitin and Flag-tagged NRAS were introduced into HeLa-Cas9 or HeLa-Cas9/gLZTR1 cells. Ubiquitinated NRAS was purified by Co^{2+} metal affinity chromatography and detected by anti-Flag antibody. Pull-down of His-tagged ubiquitinated proteins, His-Ub. Immunoprecipitates, IP. Whole cell lysate, WCL. (E) qRT-PCR gene expression analysis of LZTR1 in HEK293T cells expressing the indicated shRNAs. Data are present as mean $\pm$ SEM, $n=3$. (F) The indicated RAS constructs were overexpressed together with 6xHis-Ubiquitin in HEK293T cells expressing shGFP or shRNAs against LZTR1. Ubiquitinated RAS was purified by Co^{2+} metal affinity chromatography and detected by anti-Flag antibody. (G) MS analysis of protein expression in MEFs isolated from 3 $Lztr1^{+/+}$ and 3 $Lztr1^{-/-}$ embryos.

A

WT Hras sequence: TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT

Hras WT/KO single-cell clones:

Hras-p.S177VfsX6 clone 1

TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT wt-HRAS
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGA-AGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 1nt deletion

Hras-p.S177WfsX104 clone 2

TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT wt-HRAS
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGA-TGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 2nt deletion

Hras K170R/KO single-cell clones:

Hras-p.K170R; p.E176VfsX6 clone 6

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATG-TGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 4nt deletion

Hras-p.K170R; p.E176TfsX18 clone 47

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATACACGGATGCAATGGCAATAGCATATTGGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 32nt insertion

Hras-p.K170R; p.D175AfsX2 clone 65

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACC-----TGCATGAGCTGCAAATGTGTGCTGTCCT 19nt deletion

B **C** **D** **E**

Fig. S7. LZTR1 controls RAS ubiquitination. (A) Sequence analysis of single-cell Hras-K170R knockin MEFs clones. The gRNA target sequence is shown in red. Triplet coding K170 is highlighted in green. (B,C,D,E) The PLA assays in single-cell Hras-K170R knockin MEFs clones (B), HeLa cells expressing Cas9 or Cas9/gLZTR1 (C), Schwann cells expressing Cas9 or Cas9/gLZTR1 (D), and single-cell clones of Schwann cells (E). The PLA assay was performed using a pair of anti-panRAS and anti-ubiquitin antibodies. Quantification of the PLA staining is presented as mean $\pm$ SEM, n=3. p values were calculated by two-sided t test. Scale bar, 10 μ m.

Fig. S8. The LZTR1-CUL3 ubiquitin ligase controls ubiquitination of all RAS isoforms. (A,B,C) Relative abundance of RAS isoforms compared with expression of all other detected proteins in MEFs (A), HeLa (B), and Schwann cells (C). Data are present as mean $\pm$ SD, n=3. (D) Multiple sequence alignment of the amino acids in the RAS hypervariable regions.

Fig. S10. Loss of LZTR1 triggers hyperactivation of the RAS/MAPK pathway. Cell were serum-starved overnight, and then stimulated with 10% serum for the indicated time periods. **(A)** The amount of GTP-bound RAS in single-cell Schwann clones was determined by RAS GTPase Activation ELISA kit. **(B)** The amount of GTP-bound RAS in HeLa cells expressing shGFP or shLZTR1 was determined by pull down with RAF1-RBD conjugated to agarose beads followed by immunoblotting with anti-RAS antibody. **(C)** Whole cell lysates of *Lztr1*^{+/+} and *Lztr1*^{-/-} MEFs were analysed by immunoblotting using the indicated antibodies. **(D)** Whole cell lysates of primary human Schwann cells expressing shGFP or pooled shLZTR were also analysed by immunoblotting using the indicated antibodies. **(E)** The levels of phosphorylated and non-phosphorylated proteins in primary human Schwann cells expressing shGFP or pooled shLZTR1 were analysed by Meso Scale assay. **(F)** Whole cell lysates of single-cell Schwann clones were analysed by immunoblotting using the indicated antibodies. **(G)** Whole cell lysates of single-cell HeLa clones were analysed by immunoblotting using the indicated antibodies. Data are present as a mean of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM. p values were evaluated by a two-way ANOVA. n>3.

Fig. S11. Loss of LZTR1 triggers hyperactivation of the RAS/MAPK pathway. (A) *Lztr1*^{+/+}, *Lztr1*^{+/-}, and *Lztr1*^{-/-} MEFs were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies. Representative immunoblots are shown. **(B)** An empty vector (EV) or wt-LZTR1 was overexpressed in LZTR1-indel HeLa clone 6. The cells were then starved overnight and then stimulated with 10% serum for the indicated time periods. The levels of phosphorylated and total MEK1 were analysed by immunoblotting. Quantification of the immunoblots is presented as a mean of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM. *p* values were calculated by a two-way ANOVA. *n*=3. **(C)** Representative examples of phosphorylated-ERK1/2 immunostaining of wt-LZTR1 nerve trunk and LZTR1-mutated Schwannoma tissues. Scale bar, 100 μ m. **(D)** Fluorescent IHC detection of GFAP and phosphorylated ERK1/2 in LZTR1-mutated Schwannomas. Scale bar, 100 μ m. **(E)** 2D colony growth of single-cell HeLa clones in the presence of DMSO or pimasertib. Data are presented as mean $\pm$ SEM. *p* values were assessed by two-sided *t* test.

Fig. S12. LZTR1 controls RAS ubiquitination at K170. (A) qRT-PCR gene expression analysis of *Lztr1* in Hras-K170R knock-in MEFs expressing shGFP or shLztr1 (left). The PLA analysis of single-cell MEFs clones expressing shGFP or shLztr1 using a pair of anti-panRAS and anti-Ub antibodies. Representative images (center) and quantification (right) of the PLA analysis are shown. Scale bar, 10 μ m. Data are presented as mean $\pm$ SEM. *p* values were assessed by two-sided *t* test. *n*=3. (B) Flag-tagged wt-HRAS and HRAS-K170R were overexpressed in HEK293T expressing shGFP or pooled shRNAs against LZTR1. The cells were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies. Representative immunoblots are shown. (C) wt-Hras and Hras-K170R MEFs were serum-starved overnight and then stimulated with 10% serum. The levels of the indicated phosphorylated and total proteins were analysed by immunoblotting. (D) Growth rate of wt-Hras and Hras-K170R MEFs. Data are shown as mean $\pm$ SEM. *P* values were detected by two-way ANOVA, *n*=3. (E) Schwann cells expressing Cas9 or Cas9/gLZTR1 were treated with Cycloheximide (CHX, 100 μ g/mL) for the indicated time periods. RAS levels were analysed by immunoblotting using anti-panRAS antibody. Data are presented as a mean of panRAS levels relative to vinculin levels $\pm$ SD. *n*=2. (F) Confocal images of HeLa cells expressing HA-tagged LZTR1 and the indicated mCherry-HRAS constructs, immunostained for HA. Scale bar, 10 μ m. (G) HeLa cells expressing an empty vector (EV) or HA-tagged LZTR1 were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies.

A **B**

Fig. S13. LZTR1 and RAS co-localize at recycling endosomes. (A) Confocal images of HeLa cells expressing HA-tagged LZTR1 and mCherry-NRAS constructs (red), immunostained with antibodies against HA (blue) and RAB11, TFR, EEA1, or CD63 (green). Scale bar, 10 μ m. (B) Quantification of colocalization of mCherry-NRAS in HeLa cells overexpressing HA-tagged LZTR1 with endosomal or Golgi markers. Data are presented as mean $\pm$ SEM. n=3.

Fig. S14. LZTR1-mediated ubiquitination results in dissociation of RAS from the membrane. The final structures of HRAS and NRAS proteins ubiquitinated at K170 in solution.

Fig. S15. LZTR1-mediated ubiquitination increases the fraction of membrane-bound RAS. (A) Quantification of RAS distribution between membrane and cytoplasmic fractions isolated from HeLa cells overexpressing shGFP or shLZTR1. Data are presented as mean $\pm$ SEM. P value was detected by two-sided t test, $n=4$. (B) Immunoblot analysis of panRAS, HSP70, and Calnexin in the membrane and cytoplasmic fractions isolated from single-cell HeLa clones. Quantification of RAS distribution between membrane and cytoplasmic fractions is presented as mean $\pm$ SEM. P value was detected by two-sided t test, $n=4$.

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Supplementary Materials for
**Mutations in LZTR1 drive human disease by dysregulating RAS
ubiquitination**

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This PDF file includes:

Materials and Methods
Figs. S1 to S15
References (26-41)

Materials and Methods

Genetically modified mice

All procedures involving animals were performed in accordance with the guidelines of the IACUC of KU Leuven and approved in project application P143/2016.

Lztr1^{tm1a(EUCOMM)Wtsi} embryonic stem cells were purchased from EUCOMM and used for in vitro fertilization of a female CD-1. Primers used for genotyping are:

Sequence		
<i>neo</i>	Forward	5'-AAGATGGATTGCACGCAGGTTCTC-3'
	Reverse	5'-GACGAGATCCTCGCCGTCGGGCATGCGCGCC-3'
<i>Lztr1</i>	Forward	5'-AGGTGAGGGGTAGAGGAAGC-3'
	Reverse	5'-TTTCTGACCCAAC TCCCAAG-3'

To justify the number of analyzed animals, power analysis was conducted using the findings of (26). The phenotypic characteristics of *Lztr1*^{+/+} and *Lztr1*^{+/-} mice were assessed by observers blind to the genotype. For the rescue experiments, pregnant mice were treated with pimasertib (a daily i.p. injection 1 mg/kg of body weight) starting from E7.5.

Mouse Embryo Fibroblasts (MEFs) were prepared from individual E13.5 embryos and cultured in DMEM containing 10% FBS, 1% Pen-Strep, 50 μ M β -ME. Briefly, embryos were finely minced with razor blade and incubated for 30 minutes at 37°C with 0.05% trypsin.

Skull measurements were performed with a calliper as described in (27).

Cell culture and lentiviral transduction

Mouse Embryonic Fibroblasts (MEFs), HEK293T, and HeLa cells were cultured in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Primary human Schwann cells were purchased from Sciencell Research Laboratories and cultured according to the manufacturer's protocol. Transient transfections were performed using GeneJuice (Millipore). Lentiviral infections were performed as described by the RNAi Consortium (TRC) (<http://www.broadinstitute.org/rnai/public/resources/protocols>). Infected cells were selected by treatment with 2 μ g/ml puromycin (InvivoGen) for 3 days or with 300 μ g/ml of hygromycin-B (Thermo Fisher Scientific) for 4 days. Cell growth was determined by CellTiter-Glo luminescent cell viability kit (Promega); or by an Xcelligence; or by an IncuCyte ZOOM system (Essen BioScience) according to the manufacturers' protocols. For growth assay of MEFs, 20000 cells/well were seeded in

12-well plates in DMEM containing 10% FBS and counted by trypan blue exclusion at the indicated time points.

For clonogenic growth assay $3\text{--}5 \times 10^3$ cells were plated in triplicates and grown for 2 weeks before staining in 1% crystal violet, 20% methanol. 2D colony growth was quantified using ImageJ software (NIH). Anchorage-independent growth in soft-agar was performed as previously described in (28). 10^4 cells were plated in triplicates in 0.35% Noble agar over a 0.5% agar bottom layer. Three weeks after plating, several random areas were imaged and colony number was quantified using ImageJ software (NIH).

Virotrap Screening

The pMET7-GAG-HRASd9 and pMET7-GAG-LZTR1 constructs were generated as described (12). Virotrap experiments were performed as described in (29). Briefly, HEK293T cells were transfected with pMET7-GAG-HRAS, pMET7-GAG-LZTR1, or pMET7-GAG-eDHFR together with either untagged, or FLAG-tagged VSV-G using polyethylenimine (Sigma-Aldrich). Particles were harvested after 36 hours and purified from the supernatant by incubation with 20 μ l MyOne paramagnetic streptavidin beads (ThermoFisher Scientific) pre-loaded with 2 μ g FLAG-Bio-M2 (Sigma Aldrich). After a single wash step with TRIS wash buffer (TWB: 20 mM Tris-HCl pH 7.5, 150 mM NaCl), particles were eluted with FLAG peptide (Sigma Aldrich) in TWB and processed by amphipols lysis and precipitation under acidic conditions. After resuspension in triethylammonium bicarbonate (TEAB) buffer and boiling, proteins were overnight digested with sequencing-grade trypsin (Promega). After acidic precipitation of partially or undigested proteins by amphipols and acidification, the sample was analyzed by mass spectrometry.

MAXQUANT searches (version 1.5.6.5) were performed against the human SwissProt database. The searches were performed together with the corresponding control samples to allow matching of MS spectra between runs. Contaminants and identifications against the REVERSE database were removed using the PERSEUS software (version 1.5.5.3). A two-sided t-test was performed with multiple testing correction using 1000 randomizations. FDR was set at 5% with the S0 curve set at 2.

All raw data and MAXQUANT combined files were uploaded to PRIDE through ProteomExchange: **Project accession:** PXD007049

Ubiquitome analysis

Cells were washed twice with PBS and scraped in urea lysis buffer containing 9 M urea, 20 mM HEPES pH 8.0, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate. Samples were sonicated and centrifuged for 15 minutes at $16,000 \times g$ at room temperature. 12 mg of total protein per sample was used for further analysis. Proteins in each sample were reduced by incubating with 4.5 mM DTT for 30 minutes at 55°C . Alkylation of the proteins was done by addition of 10 mM iodoacetamide for 15 minutes at room temperature. The samples were diluted with 20 mM HEPES pH 8.0 to a urea concentration of 4 M and the proteins were digested with lysyl endopeptidase (Wako) for 4 hours at room temperature. All samples were further diluted with 20 mM HEPES pH 8.0 to a final urea concentration of 2 M and proteins were digested with 120 μ g trypsin (Promega) overnight at 37°C .

Immunocapture of GG-modified peptides was then performed using the PTMScan® Ubiquitin Remnant Motif (K- ϵ -GG) Kit (Cell Signaling Technology) according to the

manufacturer's instructions. Briefly, peptides were purified on Sep-Pak C18 cartridges (Waters), lyophilized for two days and re-dissolved in the immunoprecipitation buffer. Aliquots corresponding to 100 µg of digested protein material were taken for shotgun proteomics analysis. Peptides were incubated with the antibody-bead slurry for 2 hours on a rotator at 4°C. GG-modified peptides were eluted in 100 µl 0.15% TFA and desalted on reversed phase C18 OMIX tips (Agilent). Purified GG-modified peptides were dried under vacuum in HPLC inserts and stored at -20°C until LC-MS/MS analysis. Purified GG-modified peptides were re-dissolved in 20 µl loading solvent A (0.1% TFA in water/ACN (98:2, v/v)) of which 10 µl was injected for LC-MS/MS analysis on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo Scientific). From the aliquots for shotgun proteomics analysis, approximately 3 µg of peptides was injected for LC-MS/MS analysis on an Ultimate 3000 HPLC system in-line connected to an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific). Data analysis was performed with MaxQuant (version 1.5.7.4) using the Andromeda search engine with default search settings including a false discovery rate set at 1% on both the peptide and protein level. Two different searches were performed to analyze the spectra from the GG-enriched samples and the shotgun samples separately. In both searches spectra were interrogated against all mouse proteins in the Uniprot/Swiss-Prot database (www.uniprot.org). The minimum score for modified peptides was set to 30 and for the GG-enriched samples, GlyGly modification of lysine residues was set as an additional variable modification. Further data analysis was performed with the Perseus software (version 1.5.5.3) after loading the GlyGly (K) Sites table from MaxQuant. Reverse database hits were removed as well as potential contaminants. Sites with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution around the detection limit, then t-test was performed ($FDR \leq 0.05$ and $S0=1$) to compare site intensities in *Lztr1*^{+/+} and *Lztr1*^{-/-} samples.

LZTR1 mutation analysis

Individuals with Schwannomatosis were referred for genetic testing. Comprehensive *LZTR1* mutational testing was performed on DNA extracted from peripheral white blood cells. All 21 coding exons and exon-intron boundaries (+20, -20bp) of *LZTR1* were genomically amplified (primer sequences available upon request). All primers were M13 tagged to facilitate downstream sequencing. PCR products were bidirectionally sequenced on an ABI 3730xl genetic analyser. Sequences were analysed using SeqPilot (JSI medical systems) and mutations interpreted with the Alamut software (Interactive Biosoftware). Copy number changes (deletions or duplications) were analysed by multiplex ligation-dependent probe amplification (MLPA) using SALSA MLPA probe mix P455 (MRC Holland). Nucleotide numbering is based on cDNA reference sequence NM_006767.3 with the A of the start codon marked as 1.

Expression vectors and antibodies

Full-length expression constructs were generated by cloning the gBlocks (IDTDNA). Point *LZTR1* mutations were introduced using QuickChange site-directed mutagenesis PCR (Stratagene, La Jolla). SV40 large T genomic DNA was cloned to pLenti-hygro vector. Lentiviral pLA-CMV-N-Flag or pLA-CMV-N-HA vectors were used to generate Flag/HA-tagged constructs. The pMT107-6xHis-ubiquitin plasmid was a generous gift

from Dr. Bohmann (University of Rochester, USA). pLKO.1-puro shGFP and pLKO.1-puro vectors containing shRNAs targeting *LZTR1* (pLKO.1-sh*LZTR1*-3 (TRCN0000240480); pLKO.1-sh*LZTR1*-5 (TRCN0000181026); pLKO.1-sh*Lztr1*-2 (TRCN0000103741); pLKO.1-sh*Lztr1*-3 (TRCN0000103742) were purchased from Sigma Aldrich. pLentiCRISPR v2 empty vector and pLentiCRISPR v2 plasmids with gRNA targeting *LZTR1* were purchased from GeneScript.

The following antibodies were used: mouse monoclonal anti-FLAG (Sigma-Aldrich, M2), anti-RAS (Millipore, Clone 10), anti-vinculin (Sigma-Aldrich, clone hVIN-1), anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, 3A7, #9107), HSP 70/HSC 70 (Santa Cruz Biotechnology, W27, sc-24), anti-MEK1/2 (Cell Signaling, L38C12, #4694), anti-AKT1 (Cell Signaling, 40D4); rat monoclonal anti-HA (Roche, 3F10), anti-GM130 (BD Biosciences, Clone 35), anti-EEA1 (BD Biosciences, #610457), anti-TFR (ThermoFisher, clone H68.4), anti-CD63 (Abcam, ab8219); rabbit monoclonal anti-pMEK1/2 (Ser217/221) (Cell Signaling, #9121), anti-Rab11 (Cell Signaling, Clone D4F5), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, #9101), anti-pAKT1 (Ser473) Cell Signaling, D9E), anti-GST (Cell Signaling Technology, 91G1); rabbit polyclonal anti-DYKDDDDK (Cell Signaling), anti-His (Santa Cruz Biotechnology, H-15), anti-ubiquitin (Abcam, ab7788), anti-laminin (Sigma Aldrich, L9393), anti-Calnexin (Abcam, ab10286), anti-*LZTR1* (Avivas Systems Biology, OAAB18526), anti-CUL3 (Bethyl Laboratories); guinea pig polyclonal anti-GFAP (Synaptic Systems).

CRISPR/Cas9 mediated gene targeting

The modification at the target site of *LZTR1* was validated using Surveyor mutation detection kit according the manufacturer's protocol (IDTDNA). Primers used for Surveyor assay and Sanger sequencing of *LZTR1* clones are 5'-GGGGCTTGTGGAAGGAGTC-3' and 5'-AGGAGGGAGTCTAAGGGCTG-3'. Single clones with wt-*LZTR1* or mutated *LZTR1* from positive pools were isolated as previously described(30).

Genomic DNA of cells expressing Cas9 only or both Cas9 and gRNA targeting *LZTR1* was extracted using QIAamp DNA mini kit (Qiagen). Primers were designed to produce amplicons specifically from wildtype *LZTR1*. qPCR was performed on Light Cycler 480 (Roche).

Primers targeting on genomic DNA:

Gene		Sequence
<i>HMBS</i>	Forward	5'-ACAGACGGGGTCCTTTCATTC-3'
	Reverse	5'-AAGACGTTTGTGTGCAGTTCG-3'
<i>TBP</i>	Forward	5'-TGGTGAAGAACTGATGACTTTAGA-3'
	Reverse	5'-AAAATAAGACTGACGAAGTTTGC-3'

<i>B2M</i>	Forward	5'- AAACGCAAATCCCCTGTCTAC-3'
	Reverse	5'- AAATGGTTGAGTTGGACCCGA-3'
<i>YWHAZ</i>	Forward	5'- AGAGTCATACAAAGACAGCACG-3'
	Reverse	5'- AGAGCCTATGAAATGTGTTGAGA-3'
<i>LZTR1</i>	Forward	5'- TCTCTTTACTCAGGGGGTTACA-3'
	Reverse	5'- TTTCTGCAAAGTTTCTCACCGTC-3'

For the generation of Hras K170R knockin, immortalized MEFs expressing Cas9 were transfected with gRNA targeting Hras (5'-GCAGCCAGGACCACTCTCAT-3') and a single strand DNA repair template (CTCTGTCCCCAGGGCGTGGAGGATGCATTCTATACACTAGT CCGTGAGATTCGCCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAAAGT GGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCTGACACCAGGT, PAGE Ultramer® DNA Oligo, IDT) using Lipofectamine 2000 (Invitrogen). 48 h post transfection single cells were FACS- sorted into 96well plates. gDNA from single cell clones was extracted using QuickExtract DNA Extraction Solution (Epicentre, Illumina) following manufacturer's instructions. Screening of CRISPR clones was done by PCR using 5'-CAGCATAAATTGCGGCGC-3' and 5'-GGGGAGCAAGGACATCAGAG-3'primers and confirmed by Sanger sequencing.

For LZTR1/Lztr1-HiBiT knock-ins Alt-R® CRISPR-Cas9 strategy from IDT was used. Cells were transfected with RNP complexes containing cr:tracrRNA and HiFi Cas9 together with ssoligo (PAGE Ultramer® DNA Oligo, IDT) as a repair template using Lipofectamine 2000 (Invitrogen). 48 h post transfection single cells were FACS sorted into 96well plates. Single cell clones were screened using Nano-Glo® HiBiT Lytic Detection System (Promega). gDNA from positive clones was extracted using QIAamp DNA mini kit (Qiagen) and HiBiT knock-ins were confirmed by Sanger sequencing.

LZTR1-HiBiT repair template

/5Phos/CGGCCCCGGGGCGGTGGCCGCAAGTTGGGCTTACAGCGCGGCGGATCC CGCGTGGACCCGCGATGGTGAGCGGCTGGCGGCTGTTCAAGAAGATTAGCGC TGGACCGGGCAGCACGGGGGGGCGAGATCGGGGCTGCGGCCCTG/3Phos/

Lztr1-HiBiT repair template

/5Phos/GCGTACCCCTCCTGTCAAGGCCCGGATCCAATTGGGCCTCCGGAGCC AGCGCTAATCTTCTTGAACAGCCGCCAGCCGCTCACCATCACTTGTTTCAGGCT GGATAGGCCGCCACGTTGCAGGACTCACGGCCACCGCCC/3Phos/

In all CRISPR knockin experiments cutting efficiencies of gRNA's were checked by TIDE online tool (<https://tide.deskgen.com>). The analysis of Sanger sequences from CRISPR clones was performed using CRISP-ID online tool.

Immunoprecipitation, immunoblotting, and electrochemiluminescent immunoassay

Cells were washed twice in cold PBS and scraped on ice in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40) containing protease inhibitor and phosphatase inhibitor cocktails (Roche). Samples were subsequently cleared by centrifugation for 10 min 16,000g at 4°C. Tagged proteins were immunoprecipitated using anti-Flag (M2), anti-HA agarose beads (Sigma-Aldrich), or protein A/G magnetic beads (Thermo Scientific, #88803) for 4 hours at 4°C, washed five times with cold co-immunoprecipitation buffer and finally eluted with 3×Flag, HA peptides according to the manufacturer's protocol or by boiling in 2x NuPAGE LDS sample buffer (Thermo Scientific, #NP0007). Ubiquitinated proteins were purified as described previously(31). Briefly, cells were lysed in co-immunoprecipitation buffer containing EDTA-free protease inhibitor cocktail (Roche). Cell lysates were mixed with His-buffer A (PBS, pH 8.0, 6 M guanidinium-HCl, 0.1% NP-40 and 1 mM β-ME) and added to TALON Co²⁺ coated beads (Clontech). After binding, the resin was washed with His-buffer B (PBS, pH 8.0, 0.1% NP-40, 5% glycerol, 20 mM imidazole), and proteins were eluted with 500 mM imidazole solution.

For immunoblotting, equivalent amounts of cell lysates were separated on 4-12% gradient gels (Invitrogen), transferred to nitrocellulose membranes, and incubated with the indicated antibodies. The signal was visualized by chemiluminescence (Amersham Pharmacia Biotech) using an automated digital developer.

Meso Scale Discovery 96-well multispot plates were used for quantitative phospho/total MEK1/2 (K15129D), ERK1/2(K15107D), and AKT1 (K15100A3) analyses according to the manufacturer's instructions (Meso Scale Diagnostics). Plates were analysed using MESO QuickPlex SQ120 multiplex imager (Meso Scale Diagnostics).

RAS activity assay

RAS activity was assessed using the RAS activation ELISA assay Kit according to manufacturer's protocol (EMD Millipore). Briefly, cells were washed twice with cold TBS and scraped on ice in Lysis/Wash Buffer containing protease and phosphatase inhibitor cocktails (Roche). Samples were subsequently cleared by centrifugation for 10 minutes 16,000 g at 4°C. Glutathione-coated 96-well plate was washed and then blocked with RAF1-GST. After incubation with 10 µg of cell lysate and washing, the plate was incubated with primary anti-RAS antibody followed by secondary HRP-conjugated antibody. The signal was detected after addition of Chemiluminescent Substrate and recorded using Victor X3 Multilabel Plate Reader (PerkinElmer).

The RAS activation assay was conducted according to the manufacturer's protocol (Millipore). Briefly, cells were washed with cold PBS and lysed in Lysis/Wash Buffer (Millipore). Equal amounts of clarified cell lysates were mixed with Raf-1-RBD agarose beads (Millipore) and incubated for 45 minutes at 4°C. The beads were washed with Lysis/Wash Buffer three times and eluted by boiling in 2xSDS buffer. Eluted proteins were separated by SDS-PAGE and detected by immunoblotting.

Subcellular Fractionation

Subcellular Fractionation was performed as described previously (32) with minor modifications. Briefly, cells were washed twice with cold TBS and scraped on ice in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose supplemented with protease inhibitors and homogenized. The homogenate was centrifuged for 5 minutes at 2,500 x g at 4°C to remove unbroken cells and nuclei. The membrane fraction was collected by

centrifugation at 100,000 x *g* for 2 hours at 4°C. The pellet was washed twice with cold TBS and re-suspended in Pierce IP Lysis Buffer (ThermoFisher, #87787) supplemented with protease inhibitor, readjusting the volume of the membrane fraction to that of the cytosolic fraction before resolving by SDS-PAGE.

In vitro cross-linking

Cross-linking was performed as described previously (16) with minor modifications. Briefly, HA-LZTR1 was purified from HEK293T cells using anti-HA agarose beads (Sigma-Aldrich). Protein samples were prepared in the following buffer, 20 mM HEPES pH 7.5, 150 mM NaCl, and 5 mM DTT. The amine cross-linker BS3 bis(sulfosuccinimidyl)suberate, (ThermoFisher, # 21585) was added with a final concentration of 2.5 mM. Reactions were incubated at room temperature for 30 minutes. Then the reactions were quenched by the addition of 100 mM Tris pH 7.6 and incubation at room temperature for 15 minutes.

Cellular and tissue staining

For cell immunostaining, 2×10^4 cells per well were plated on 8-well chamber glass slide (Corning, # 354118) and fixed 24 hours after transfection with 4% PFA.

Immunofluorescence was performed as described previously (31). Briefly, cells were permeabilized in PBS-0.15% Triton-X100 and blocked with 1% BSA and 10% goat serum. Primary antibodies and goat Alexa-conjugated secondary antibodies were applied diluted in blocking buffer before mounting with Vectashield (Vector Laboratories). To avoid antibody mouse-rat cross-reactivity, secondary anti-mouse antibodies were pre-blocked with 10% rat serum.

For visualizing ubiquitin conjugation to RAS proteins proximity ligation assay (PLA) was performed using the Duolink kit according to manufacturer's protocol (Sigma Aldrich). Briefly, cells were fixed in 4% PFA, permeabilized in PBS-0.15% Triton and incubated with blocking solution 30 minutes at 37°C. Primary antibodies were applied by diluting in antibody diluent solution 1 hour at room temperature (PanRAS 1/300; Ub Rabbit 1/100). Secondary anti-mouse and anti-rabbit antibodies conjugated to PLA probes were incubated 1 hour at 37°C at room temperature, followed by ligation and amplification with far red fluor-labeled oligonucleotides (30 minutes and 100 minutes at 37°C, respectively). Samples were mounted in Duolink mounting medium containing DAPI (Sigma Aldrich).

Images were obtained by using a confocal Nikon C2 microscope (60x magnification). Image analysis was performed using ImageJ (NIH). Quantification of colocalization was done using JACoP (33). For quantification of PLA signals, random images were taken and the number of particles per nuclei was measured.

Schwannomas tissues were immunostained for p-ERK1/2 and GFAP on a Discovery Ventana automated staining platform. For the evaluation of pERK1/2 immunohistochemistry samples were scored based on an intensity scale and subcellular localization of pERK1/2 immunostaining.

Cardiac tissue was fixed in Neutral Buffered Formalin (NBF) 10% overnight, and further processed for paraffin embedding. Hematoxylin and eosin – stained sections (4 µm) were used to assess overall morphology, and Picro Sirius Red staining for overall fibrosis. Myocyte cross-sectional areas were calculated by measuring the inner circumference of 200 myocytes per sample on laminin–stained sections. Microscopic

analyses were performed using a microscope (Axiovert 200M; Zeiss), and Fiji software (34).

MEFs were stained for SA-B-Gal staining using the Senescence β -Galactosidase Staining Kit (Cell Signaling) according to the manufacturer's protocol.

Gene expression analysis

RNA extraction was performed using RNeasy MINI kit (Qiagen). 500ng of total RNA was used to prepare cDNA for mRNA analysis using RT2 First Strand Kit. The quantification of mRNA was performed on Light Cycler 480 (Roche) system using RT2 SYBR Green qPCR Mastermix and RT2 qPCR LZTR1 primer assay (PPH10123A) (Qiagen).

Primers:

Gene		Sequence
<i>LZTR1</i>	Forward	5'- TCCTGCGGTTTCGATGTGAAA -3'
	Reverse	5'- ACATGCTGCTCCCATAGACG -3'
<i>EGR1</i>	Forward	5'- CTTCAACCCTCAGGCGGAC -3'
	Reverse	5'- GTCTCCACCAGCACCTTCTC -3'
<i>SOX2</i>	Forward	5'- GAGCTTTGCAGGAAGTTTGC -3'
	Reverse	5'- GCAAGAAGCCTCTCCTTGAA -3'
<i>EGR2</i>	Forward	5'- AACGGAGTGGCCGGAGAT-3'
	Reverse	5'-ATGGGAGATCCAACGACCTCTT-3'
<i>GAP43</i>	Forward	5'- AACCATGCTGTGCTGTATGAGAA-3'
	Reverse	5'- CCCAGGTGATGCTGTGACTC -3'
<i>PMP22</i>	Forward	5'-TGGAAGAAGGGGTTACGCTG -3'
	Reverse	5'-GTGGACTGGGAGGGAGGTAT -3'
<i>HPRT1</i>	Forward	5'-TGACACTGGCAAAACAATGCA-3'
	Reverse	5'-GGTCCTTTTCACCAGCAAGCT -3'

<i>GAPDH</i>	Forward	5'-ACCACAGTCCATGCCATCAC -3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA -3'
<i>B2M</i>	Forward	5'- AAACGCAAATCCCACCTGTCAC-3'
	Reverse	5'-TCTCTGCTCCCCACCTCTAAGT-3'
<i>ACTB</i>	Forward	5'- CTGGAACGGTGAAGGTGACA -3'
	Reverse	5'- AAGGGACTTCCTGTAACAACGCA-3'
<i>Lztr1</i>	Forward	5'-GATGAAGGAGTTTGAGCGCC-3'
	Reverse	5'-ATGTCACAGAACTCCGAGCC-3'
<i>Cdkn1a</i>	Forward	5'-TTGCACTCTGGTGTCTGAGC-3'
	Reverse	5'- TGATAGAAATCTGTCAGGCTGGT -3'
<i>Hmbis</i>	Forward	5'- GAAACTCTGCTTCGCTGCATT -3'
	Reverse	5'-TGCCCATCTTTCATCACTGTATG -3'
<i>B2m</i>	Forward	5'- GGTGCTTGTCTCACTGACC -3'
	Reverse	5'- CCCGTTCTTCAGCATTTGGA -3'
<i>Rpl19</i>	Forward	5'- CAGGCATATGGGCATAGGGAA -3'
	Reverse	5'- TGCCTTCAGCTTGTGGATGT -3'

Constructing ubiquitin-conjugated RAS models

The initial coordinates of HRAS were obtained from our previous works (24). The NRAS coordinates are based on the crystal structure of H-RAS with residue modifications to reflect the NRAS sequence. All RAS isoforms are in the GTP-bound state. The initial structure of ubiquitin was obtained from the Protein Data Bank (PDB ID: 1UBQ) (35). To simulate RAS ubiquitination, we developed the patch parameter for the isopeptide bond that connects ubiquitin to the lysine residue at the linker portion of the hypervariable region (HVR) of RAS. Ubiquitin was conjugated to K170 of HRAS or NRAS. To determine possible binding modes of the ubiquitin and the RAS catalytic

domain, we performed molecular docking using the PatchDock online server (36). During the docking, we constrained the residue distance (5 Å) between the C-terminal residue of ubiquitin and the target lysine residue of RAS, ensuring that the ubiquitin stayed at the ubiquitination site. The top 50 structures with high docking score were refined by the FireDock server (37, 38). Structures with the highest scores were selected and solvated in a water box. The unit cell containing proteins, waters and ions were constructed by selecting a proper cell dimension with a criterion that the minimal distance between the edge of the box and the protein surface is 15 Å. Na⁺ and Cl⁻ were added to neutralize the system and mimic the 0.15 M ionic strength. Molecular dynamics (MD) simulations in explicit solvent were performed to evaluate the structural and dynamic properties. To observe the effects of ubiquitination on RAS membrane binding ability, the final structures of ubiquitin-conjugated HRAS or NRAS from the water simulations were placed on membranes composed of DOPC and DOPE lipids (3:1 molar ratio). The initial configurations of the lipid bilayers were obtained from the CHARMM-GUI online server (39).

Molecular dynamics simulation protocols

MD simulations with the all-atom model were performed by the NAMD package (40) using the updated CHARMM all-atom additive force field (version C36) (41). The simulations employed the NPT (constant number of atoms, pressure, and temperature) ensemble at a temperature of 310 K and pressure of 1 atm. The RATTLE algorithm was used to constrain the covalent bonds with hydrogen atoms. Short-range van der Waals (VdW) interactions were calculated by the switch function with the twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the shift methods with the cutoff of 14 Å. The velocity verlet integration was used to generate time steps of 2 fs. Analysis was performed using the tools in CHARMM, VMD and in-house TCL codes.

Echocardiography

At the indicated age range, mice were anesthetized (2% isoflurane, ecuphar) via a nose cone at 1.5 l/min oxygen flow and examined by transthoracic echocardiography with a MS400 18–38 MHz transducer (VEVO 2100, FUJIFILM VisualSonics, Inc. Toronto, Canada). Left ventricular function was assessed in both parasternal long-axis and short-axis views at a 300 Hz frame rate. Left ventricular internal diameter (LVID), the overall left ventricular wall thickness including Inter-Ventricular Septum (IVS) and Posterior Wall (PW) were measured in both End-diastole (d) and End-Systole (s); Fractional Shortening (FS) and Ejection Fraction (EF) and Stroke Volume (SV) were calculated. Pulmonary Valve function was examined through Pulse Wave Doppler acquisition in a modified parasternal short-axis view at aortic-level. Pulmonary Velocity Time Integral (PVTI), Pulmonary Acceleration Time (PAT) and Pulmonary Ejection Time (PET) were measured and their ratio (PET/PAT %) calculated.

Statistical analysis

All data were analysed using Graphpad Prism 7.

Fig. S1. LZTR1 mutations in human cancer. Somatic mutations of LZTR1 in the TCGA PanCancer Atlas cohort. Data were obtained from cBioPortal (Cerami et al, 2012; Gao et al, 2013).

Fig. S2. LZTR1 alterations lead to disease phenotypes. (A) Progeny from the intercross of $Lztr1^{+/-}$ mice. **(B)** Growth curves of $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. p value was assessed by two-way ANOVA. **(C)** Weight of 24-week-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. A horizontal line represents a mean±SD. p value was determined by two-sided t test. **(D)** Representative image of 12-month-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ male mice. **(E)** Echocardiography analysis of 40–64 week-old $Lztr1^{+/+}$ and $Lztr1^{+/-}$ mice. IVSd, Interventricular septum in diastole; IVSs, Interventricular septum in systole; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; PWd, posterior wall in diastole; PWs, posterior wall in systole; V(d), volume in diastole; V(s), volume in systole; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; FS area, fractional shortening area; LVV, left ventricle volume; HR, heart rate; Pulmonary VTI, pulmonary velocity time integral; PAT, pulmonary artery acceleration time; PET, pulmonary ejection time. p values were assessed by two-sided t test. **(F)** Survival curve of $Lztr1^{+/+}$ and $Lztr1^{+/-}$ mice. p value was assessed using Log-Rank t test.

Fig. S3. LZTR1 loss cellular models. (A) qRT-PCR gene expression analysis of *Lztr1* in MEFs isolated from *Lztr1*^{+/+}, *Lztr1*^{+/-}, and *Lztr1*^{-/-} embryos. *p* value assessed by two-sided *t* test, *n*=3. (B) Immunoblot analysis of LZTR1 expression in primary human Schwann cells expressing shGFP or pooled shLZTR1. (C) Surveyor assay to detect indel formation in HeLa expressing Cas9 or Cas9 together with gRNAs targeting LZTR1 (gLZTR1). (D) Primer design for the detection of indels in the LZTR1 gene. The gLZTR1 target sequence is shown in red. (E) PCR amplification of the indicated loci using genomic DNA purified from HeLa cells expressing only Cas9 or Cas9 together with gRNAs targeting LZTR1. (F,G) CRISPR/Cas9-mediated modification efficiency of the target LZTR1 locus in HeLa (F) or immortalized Schwann (G) estimated by qPCR. *n*=2. (H) Sequence analysis of single-cell Schwann and HeLa clones after overexpression of Cas9 together with gLZTR1. The gRNA target sequence is shown in red. Exon 5 of LZTR1 is highlighted in grey.

Fig. S4. LZTR1 loss increases cell growth. (A) Growth rate of primary human Schwann cells expressing shGFP or pooled shLZTR1. Data are shown as mean $\pm$ SEM. P values were detected by two-way ANOVA, $n=3$. (B) Growth rate of single-cell Schwann clones. p value assessed by two-way ANOVA, $n=3$. (C) Growth rate of single-cell HeLa clones. p value assessed by two-way ANOVA, $n=3$. (D) Representative immunoblot analysis of ectopic expression of HA-tagged wt-LZTR1 and LZTR1 mutants in *Lztr1*^{-/-} MEFs. (E) Immunoblot analysis of LZTR1 expression after overexpression of an empty vector (EV) or the indicated LZTR1 constructs (top). Growth rate of LZTR1-indel HeLa clone 6 expressing an empty vector (EV) or the indicated LZTR1 constructs. Data are presented as mean $\pm$ SEM. P values were detected by two-way ANOVA, $n=3$. (F) 2D colony growth of single-cell immortalized Schwann clones. Data are shown as mean $\pm$ SEM. $n=3$. (G) Soft agar colonies formed by immortalized Schwann cells after overexpression of Cas9 or Cas9/gLZTR1. (H) Anchorage-independent growth of single-cell immortalized Schwann clones. Data are presented as mean $\pm$ SEM. p values were assessed by two-sided t test. $n=3$.

Fig. S5. LZTR1 interacts with RAS proteins. (A) Schematic outline of the Virotrap approach. Briefly, a bait is fused to the Group-specific antigen (GAG) protein and co-expressed with Flag-tagged glycoprotein of the vesicular stomatitis virus (VSV-G); this triggers the formation of virus-like particles that trap protein complexes under native conditions. The Flag-tagged particles were purified, and their contents determined by MS. (B) HEK293T cells expressing LZTR1 were analysed by immunoprecipitation with anti-Flag beads or anti-panRAS antibody bound to protein A/G agarose. Immunoblotting was performed with the indicated antibodies. (C) Sequence analysis of the HiBiT knock-in HeLa clone. The gRNA target sequence is shown in red; PAM sequence in yellow; start codon in green; HiBiT insertion in purple. (D) HeLa and MEF clones expressing in-frame HiBiT-LZTR1 protein. Luminescent signal was generated by a Halo-tag HiBiT incubated with LgBiT. (E) The HiBiT-Lztr1 edited MEFs were analysed by immunoprecipitation with anti-panRAS antibody bound to protein A/G agarose. Luminescent signal was generated by a Halo-tag HiBiT incubated with LgBiT. (F) The indicated tagged proteins overexpressed in HEK293T cells were immunoprecipitated with anti-Flag (M2) or anti-HA beads, followed by immunoblotting using anti-Flag or anti-HA antibodies.

Fig. S6. The LZTR1-CUL3 ubiquitin ligase controls RAS ubiquitination. (A) wt-HRAS or HRAS-K170R purified from HEK293T cells was incubated with UBE1 (E1), UBE2T (2), the Flag-LZTR1/Flag-CUL3 complex (E3), recombinant HA-tagged ubiquitin and in the presence of Mg-ATP and ubiquitin aldehyde. (B) Flag-tagged NRAS and 6xHis-ubiquitin were introduced into HEK293T cells expressing HA-LZTR1/V5-CUL3 or an empty vector (EV). Whole cell lysates were analyzed by immunoblotting using the indicated antibodies. (C) Flag-tagged NRAS was overexpressed together with 6xHis-Ubiquitin in HEK293T cells. The cells were treated with the indicated doses of MLN4924 for 20 hours. Ubiquitinated NRAS was purified by Co²⁺ metal affinity chromatography and detected by anti-Flag antibody. (D) 6xHis-ubiquitin and Flag-tagged NRAS were introduced into HeLa-Cas9 or HeLa-Cas9/gLZTR1 cells. Ubiquitinated NRAS was purified by Co²⁺ metal affinity chromatography and detected by anti-Flag antibody. Pull-down of His-tagged ubiquitinated proteins, His-Ub. Immunoprecipitates, IP. Whole cell lysate, WCL. (E) qRT-PCR gene expression analysis of LZTR1 in HEK293T cells expressing the indicated shRNAs. Data are present as mean $\pm$ SEM, n=3. (F) The indicated RAS constructs were overexpressed together with 6xHis-Ubiquitin in HEK293T cells expressing shGFP or shRNAs against LZTR1. Ubiquitinated RAS was purified by Co²⁺ metal affinity chromatography and detected by anti-Flag antibody. (G) MS analysis of protein expression in MEFs isolated from 3 Lztr1^{+/+} and 3 Lztr1^{-/-} embryos.

A

WT Hras sequence: TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT

Hras WT/KO single-cell clones:

Hras-p.S177VfsX6 clone 1

TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT wt-HRAS
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGA-AGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 1nt deletion

Hras-p.S177WfsX104 clone 2

TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT wt-HRAS
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATGA-TGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 2nt deletion

Hras K170R/KO single-cell clones:

Hras-p.K170R; p.E176VfsX6 clone 6

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATG-TGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 4nt deletion

Hras-p.K170R; p.E176TfsX18 clone 47

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACCCGATACACGGATGCAATGGCAATAGCATATTGGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT 32nt insertion

Hras-p.K170R; p.D175AfsX2 clone 65

TCGGCAGCATAAATTGCGGCGCTTGAATCCACCAGATGAGAGTGGTCTGGCTGCATGAGCTGCAAATGTGTGCTGTCCT K170R
TCGGCAGCATAAATTGCGGAACTGAACCCACC-----TGCATGAGCTGCAAATGTGTGCTGTCCT 19nt deletion

B **C** **D** **E**

Fig. S7. LZTR1 controls RAS ubiquitination. (A) Sequence analysis of single-cell Hras-K170R knockin MEFs clones. The gRNA target sequence is shown in red. Triplet coding K170 is highlighted in green. (B,C,D,E) The PLA assays in single-cell Hras-K170R knockin MEFs clones (B), HeLa cells expressing Cas9 or Cas9/gLZTR1 (C), Schwann cells expressing Cas9 or Cas9/gLZTR1 (D), and single-cell clones of Schwann cells (E). The PLA assay was performed using a pair of anti-panRAS and anti-ubiquitin antibodies. Quantification of the PLA staining is presented as mean $\pm$ SEM, $n=3$. p values were calculated by two-sided t test. Scale bar, 10 μ m.

Fig. S8. The LZTR1-CUL3 ubiquitin ligase controls ubiquitination of all RAS isoforms. (A,B,C) Relative abundance of RAS isoforms compared with expression of all other detected proteins in MEFs (A), HeLa (B), and Schwann cells (C). Data are present as mean $\pm$ SD, n=3. (D) Multiple sequence alignment of the amino acids in the RAS hypervariable regions.

Fig. S10. Loss of LZTR1 triggers hyperactivation of the RAS/MAPK pathway. Cell were serum-starved overnight, and then stimulated with 10% serum for the indicated time periods. **(A)** The amount of GTP-bound RAS in single-cell Schwann clones was determined by RAS GTPase Activation ELISA kit. **(B)** The amount of GTP-bound RAS in HeLa cells expressing shGFP or shLZTR1 was determined by pull down with RAF1-RBD conjugated to agarose beads followed by immunoblotting with anti-RAS antibody. **(C)** Whole cell lysates of *Lztr1*^{+/+} and *Lztr1*^{-/-} MEFs were analysed by immunoblotting using the indicated antibodies. **(D)** Whole cell lysates of primary human Schwann cells expressing shGFP or pooled shLZTR were also analysed by immunoblotting using the indicated antibodies. **(E)** The levels of phosphorylated and non-phosphorylated proteins in primary human Schwann cells expressing shGFP or pooled shLZTR1 were analysed by Meso Scale assay. **(F)** Whole cell lysates of single-cell Schwann clones were analysed by immunoblotting using the indicated antibodies. **(G)** Whole cell lysates of single-cell HeLa clones were analysed by immunoblotting using the indicated antibodies. Data are present as a mean of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM. p values were evaluated by a two-way ANOVA. $n \geq 3$.

Fig. S11. Loss of LZTR1 triggers hyperactivation of the RAS/MAPK pathway. (A) *Lztr1*^{+/+}, *Lztr1*^{+/-}, and *Lztr1*^{-/-} MEFs were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies. Representative immunoblots are shown. (B) An empty vector (EV) or wt-LZTR1 was overexpressed in LZTR1-indel HeLa clone 6. The cells were then starved overnight and then stimulated with 10% serum for the indicated time periods. The levels of phosphorylated and total MEK1 were analysed by immunoblotting. Quantification of the immunoblots is presented as a mean of phosphorylated relative to non-phosphorylated protein levels $\pm$ SEM. *p* values were calculated by a two-way ANOVA. *n*=3. (C) Representative examples of phosphorylated-ERK1/2 immunostaining of wt-LZTR1 nerve trunk and LZTR1-mutated Schwannoma tissues. Scale bar, 100 μ m. (D) Fluorescent IHC detection of GFAP and phosphorylated ERK1/2 in LZTR1-mutated Schwannomas. Scale bar, 100 μ m. (E) 2D colony growth of single-cell HeLa clones in the presence of DMSO or pimasertib. Data are presented as mean $\pm$ SEM. *p* values were assessed by two-sided *t* test.

Fig. S12. LZTR1 controls RAS ubiquitination at K170. (A) qRT-PCR gene expression analysis of *Lztr1* in Hras-K170R knock-in MEFs expressing shGFP or shLztr1 (left). The PLA analysis of single-cell MEFs clones expressing shGFP or shLztr1 using a pair of anti-panRAS and anti-Ub antibodies. Representative images (center) and quantification (right) of the PLA analysis are shown. Scale bar, 10 μ m. Data are presented as mean $\pm$ SEM. *p* values were assessed by two-sided *t* test. *n*=3. (B) Flag-tagged wt-HRAS and HRAS-K170R were overexpressed in HEK293T expressing shGFP or pooled shRNAs against LZTR1. The cells were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies. Representative immunoblots are shown. (C) wt-Hras and Hras-K170R MEFs were serum-starved overnight and then stimulated with 10% serum. The levels of the indicated phosphorylated and total proteins were analysed by immunoblotting. (D) Growth rate of wt-Hras and Hras-K170R MEFs. Data are shown as mean $\pm$ SEM. *P* values were detected by two-way ANOVA, *n*=3. (E) Schwann cells expressing Cas9 or Cas9/gLZTR1 were treated with Cycloheximide (CHX, 100 μ g/mL) for the indicated time periods. RAS levels were analysed by immunoblotting using anti-panRAS antibody. Data are presented as a mean of panRAS levels relative to vinculin levels $\pm$ SD. *n*=2. (F) Confocal images of HeLa cells expressing HA-tagged LZTR1 and the indicated mCherry-HRAS constructs, immunostained for HA. Scale bar, 10 μ m. (G) HeLa cells expressing an empty vector (EV) or HA-tagged LZTR1 were starved overnight and then stimulated with 10% serum for the indicated time periods. Whole cell lysates were analysed by immunoblotting using the indicated antibodies.

A **B**

Fig. S13. LZTR1 and RAS co-localize at recycling endosomes. (A) Confocal images of HeLa cells expressing HA-tagged LZTR1 and mCherry-NRAS constructs (red), immunostained with antibodies against HA (blue) and RAB11, TFR, EEA1, or CD63 (green). Scale bar, 10 μ m. (B) Quantification of colocalization of mCherry-NRAS in HeLa cells overexpressing HA-tagged LZTR1 with endosomal or Golgi markers. Data are presented as mean $\pm$ SEM. n=3.

Fig. S14. LZTR1-mediated ubiquitination results in dissociation of RAS from the membrane. The final structures of HRAS and NRAS proteins ubiquitinated at K170 in solution.

Fig. S15. LZTR1-mediated ubiquitination increases the fraction of membrane-bound RAS. (A) Quantification of RAS distribution between membrane and cytoplasmic fractions isolated from HeLa cells overexpressing shGFP or shLZTR1. Data are presented as mean $\pm$ SEM. P value was detected by two-sided t test, $n=4$. **(B)** Immunoblot analysis of panRAS, HSP70, and Calnexin in the membrane and cytoplasmic fractions isolated from single-cell HeLa clones. Quantification of RAS distribution between membrane and cytoplasmic fractions is presented as mean $\pm$ SEM. P value was detected by two-sided t test, $n=4$.

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