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**ESCRT-II/Vps25 Constrains Digit Number by Endosome-Mediated Selective Modulation of FGF-SHH Signaling**

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**In Brief**
Using a polydactylous mouse line carrying a hypomorphic mutation in the Vps25 subunit of the ESCRT-II complex, Handschuh et al. now establish that ubiquitously expressed machineries that sort signaling proteins preferentially regulate, or are rate limiting for, select signaling pathways in different contexts of the developing embryo.

**Highlights**
- ENU-induced mutation of mouse ESCRT-II/Vps25 causes polydactyly
- Vps25 hypomorphic mutants survive until late gestation unlike ESCRT LOF embryos
- ESCRT-II constrains digit number by endosome-mediated modulation of FGF signaling
- Mutations in ESCRT reveal a mechanism underlying congenital limb defects
ESCRT-II/Vps25 Constrains Digit Number by Endosome-Mediated Selective Modulation of FGF-SHH Signaling

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SUMMARY

Sorting and degradation of receptors and associated signaling molecules maintain homeostasis of conserved signaling pathways during cell specification and tissue development. Yet, whether machineries that sort signaling proteins act preferentially on different receptors and ligands in different contexts remains mysterious. Here, we show that Vacuolar protein sorting 25, Vps25, a component of ESCRT-II (Endosomal Sorting Complex Required for Transport II), directs preferential endosome-mediated modulation of FGF signaling in limbs. By ENU-induced mutagenesis, we isolated a polydactylous mouse line carrying a hypomorphic mutation of Vps25 (Vps25ENU). Unlike Vps25-null embryos we generated, Vps25ENU/ENU mutants survive until late gestation. Their limbs display FGF signaling enhancement and consequent hyperactivation of the FGF-SHH feedback loop causing polydactyly, whereas WNT and BMP signaling remain unperturbed. Notably, Vps25ENU/ENU Mouse Embryonic Fibroblasts exhibit aberrant FGFR trafficking and degradation; however, SHH signaling is unperturbed. These studies establish that the ESCRT-II machinery selectively limits FGF signaling in vertebrate skeletal patterning.

INTRODUCTION

The complexity of development is dependent upon signal transduction pathways, which are critical for cell specification, tissue patterning, organ morphogenesis, and growth. Notably, the embryo constructs markedly different structures by using the same signaling pathways (Wolpert, 1994). The developing vertebrate limb can serve as a tractable model to analyze mechanisms of cell signaling (Zeller, 2010).

In vertebrates, limb development is controlled by two signaling centers, the apical ectodermal ridge (AER) at the distal bud and the zone of polarizing activity (ZPA) in the posterior mesenchyme (Zeller, 2010). Both centers produce instructive signals that direct anterior-posterior (AP) and proximal-distal (PD) limb axis formation. The AER produces multiple fibroblast growth factors (FGF8, FGF4, FGF9, FGF17), whereas the ZPA produces Sonic Hedgehog (SHH) (Tabin and Wolpert, 2007). Throughout limb development, the AER and ZPA are interlinked by a feedback signaling loop, which is also influenced by other signaling molecules and transcription factors (Tabin and Wolpert, 2007; Zakany and Duboule, 2007; Zeller et al., 2009). Genetic mutations that perturb the FGF-SHH loop lead to alterations of the highly conserved pentadactyl pattern (Biesecker, 2011). Therefore, how cells maintain signaling homeostasis is critical for the establishment of digit number and identity.

Homeostasis of signaling proteins is maintained through sorting and degradation via endosome-mediated vesicular trafficking (MacGurn et al., 2012). Mutations of ESCRT (Endosomal Sorting Complex Required for Transport) machinery components compromise their ability to degrade conserved signaling proteins in...
multiple organisms (Henne et al., 2011, 2013). Indeed, ESCRT LOF models display perturbations of signaling (Rusten et al., 2012). However, early lethality of mutants with complete LOF of ESCRT components (Rusten et al., 2012) and the absence of conditional or hypomorphic mutations has prevented understanding how ESCRT members contribute to shaping organismal forms. Furthermore, whether constitutively expressed multincomponent ESCRT machineries act on different receptors and associated signaling proteins in a specialized or preferential manner in different contexts of the developing embryo remains poorly understood.

Here, identification of a hypomorphic mutation in the ESCRT-II complex in a polydactylous mouse line isolated by a N-ethyl-N-nitrosourea (ENU) mutagenesis screen (Anderson and Ingham, 2003; Stottmann and Beier, 2010) allowed deconstruction of the mechanisms by which specific ESCRT components execute patterning and morphogenetic processes. This unique mouse model with a partially functional ESCRT-II allele afforded testing the mechanisms by which specific ESCRT components execute patterning in vertebrate development.

RESULTS

Isolation of 04-014 Mouse Line with Polydactyly through an N-ethyl-N-nitrosourea Mutagenesis Screen and Identification of the ENU-Induced Mutation in the Vps25 Gene

By a N-ethyl-N-nitrosourea (ENU) mutagenesis screen, we isolated mouse line 04-014 based on recessive hindlimb polydactyly with variable expressivity (Figure 1A–1D). We located the mutation within the murine ortholog of yeast Vacuolar protein sorting 25, Vps25 (also known as EAP20), encoding a subunit of the ESCRT-II complex (Babst et al., 2002b; Slater and Bishop, 2006) essential for endosomal protein trafficking (Henne et al., 2011; MacGurn et al., 2012; Rusten et al., 2012) (Figures 1E; Table S1). Identified by sequencing of candidate gene cDNA, and confirmed by deep sequencing (Arnold et al., 2011) of the narrowest genomic interval linked to the mutation (Table S1), a G-to-A transition in Vps25 third intron generated an mRNA splice variant containing an in-frame 27-nucleotide insertion encoding nine additional neutral amino acids (Figures 1E–1G and S1A; Table S2). Hereafter, the ENU-induced allele will be designated Vps25ENU and the recessive homozygous mutant embryos Vps25ENU/ENU.

ENU-Induced Mutation of ESCRT-II/Vps25 Results in a Partially Functional Hypomorphic Allele, whereas LOF Causes Early Gestational Lethality

To characterize effects of the ENU-induced mutation on Vps25 transcription, quantitative RT-PCR (qRT-PCR) detected the Vps25 splice variant as well as wild-type (WT) mRNA in the mutants. However, in mutant embryos at various gestational days, both mRNA species and their encoded proteins were significantly less abundant than WT Vps25 in WT littermates (Figures 1H, 1I, and S1B). Thus, the ENU-induced allele is hypomorphic, affording analysis of how ESCRT-II/Vps25 execute patterning and morphogenesis in vertebrate development.

To elucidate the requirements for Vps25 during development, we compared the activity of Vps25ENU to a Vps25 global LOF allele (Vps25lacZ) we generated by replacing Vps25 with a LacZ/Neo cassette (www.komp.org). At E8.5 no Vps25lacZ/LacZ embryos were recovered, only empty deciduae, defining the Vps25 LOF allele as more deleterious than Vps25ENU (Figure S1C). Thus, similar to LOF of other mouse ESCRT components (Komada and Soriano, 1999; Lee et al., 2007; Ruland et al., 2001; Shim et al., 2006; Yamada et al., 2002), global Vps25 LOF causes early lethality in uterus. Consistent with early lethality, RT-PCR and X-gal staining of Vps25lacZ/LacZ embryos corroborated widespread Vps25 expression, including in limbs, from E9.5–E13.5 (Figures 1J–1M and S1D–S1J). Absence of complementation between Vps25lacZ and Vps25ENU in transheterozygous Vps25lacZ/ENU embryos confirmed the G-to-A transition in Vps25 intron 3 as the causative mutation (Figures 1N–1Q; Table S3). As predicted for a hypomorphic allele, Vps25ENU/ENU embryos survived until E15.5–16.5 on a mixed genetic background (Figure 1Q), whereas Vps25lacZ/ENU embryos exhibited developmental delay and hemorrhaging as early as E9.5 (Figure 1P).

ENU-Induced Mutation of ESCRT-II/Vps25 Causes Fully Penetrant Polydactyly

Skeletal preparations and Optical Projection Tomography (Sharpe et al., 2002) (OPT) revealed fully penetrant hindlimb polydactyly (Figures 2A–2D; Movies S1 and S2), whereas in mutant forelimbs polydactyly was less severe and not fully penetrant (Figures S2A–S2F; Movies S3 and S4). Compared to WT, the mutant scapular blade was thinner with a central hole (Figures S2G and S2H). Mutant limbs also exhibited shorter and thicker skeletal elements (Figures 2A, 2B, and S2H–S2L).

To uncover the cellular behaviors underlying the polydactyly, we examined proliferation (Koss et al., 2012) and apoptosis (Ferretti et al., 2011) in WT and Vps25ENU/ENU limbs (Figures 2E–2L). E12.5 WT hindlimb showed a highly proliferative mesenchyme and a quiescent anterior AER (Figures 2E and 2G). In contrast, hindlimb anterior AER of Vps25ENU/ENU littermates appeared strikingly proliferative (Figures 2F and 2H). Additionally, unlike WT (Figures 2I and 2K), Vps25ENU/ENU anterior hindlimb contained no detectable cell death (Figures 2J, 2L, and S2U–S2X). We observed similar, albeit less pronounced, proliferation and apoptosis defects in Vps25ENU/ENU forelimbs (Figures S2M–S2T), Msx2, a marker of apopotic cells (Lallemand et al., 2009), was expressed in the digital spaces and around digit 1 of E13.5 WT hindlimb; however, Vps25ENU/ENU hindlimb bud lacked Msx2 in the domain where polydactyly arises (Figures 2M and 2N). Last, Sox9 expression (Mariani and Martin, 2003) appeared diffuse in E13.5 Vps25ENU/ENU hindlimb anterior mesenchyme, suggesting multiple digit condensations (Figures 2O and 2P). These results implicate perturbations of proliferation and cell death in Vps25ENU/ENU anterior limb domains as the underlying causes of polydactyly or sympolydactyly.

Vps25ENU/ENU Early Limb Buds Exhibit Specific Enhancement of the FGF-SHH Cross-Regulatory Loop Underlying Polydactyly, whereas WNT and BMP Signaling Are Unperturbed

To assess whether Vps25 mutation induced ESCRT-II-dependent perturbation of signaling molecules critical for limb
Figure 1. Vps25 Regulates Normal Eyed Development.

(A) Wild-type (WT) and ENU-mutant E14.5-HL eyes.

(B) 4-O14 ENU mutant E14.5-HL eyes.

(C) 04-014 ENU mutant E14.5-HL eyes.

(D) 04-014 ENU mutant E14.5-HL eyes.

(E) Vps25 gene structure with exons 1-6 indicated. Open reading frame (ORF) start and stop codons are indicated by red lines. Splicing sites are indicated by arrows.

(F) WT and Vps25<sup>ENU/ENU</sup> E14.5-HL eyes.

(G) Comparison of WT and Vps25<sup>ENU/ENU</sup> amino acid sequences. Amino acids identical in WT and Vps25<sup>ENU</sup> are indicated in black. Amino acids identical in Vps25<sup>ENU</sup> and Vps25<sup>ENU/ENU</sup> are indicated in blue.

(H) Relative expression of Vps25<sup>WT</sup>, Vps25<sup>ENU/ENU</sup>, and Vps25<sup>ENU</sup> in E10.5 embryos.

(I) Western blot analysis of Vps25<sup>WT</sup>, Vps25<sup>ENU/ENU</sup>, and Vps25<sup>ENU</sup> expression in E10.5 embryos.

(J) Comparison of WT and Vps25<sup>LacZ/4</sup> lacZ<sup>+/-</sup> embryos.

(K) Comparison of WT and Vps25<sup>ENU/+</sup> lacZ<sup>+/-</sup> embryos.

(L) Comparison of WT and Vps25<sup>acZ/ENU</sup> lacZ<sup>+/-</sup> embryos.

(M) Comparison of WT and Vps25<sup>ENU/ENU</sup> lacZ<sup>+/-</sup> embryos.

(legend on next page)
patterning, we analyzed their expression by whole-mount in situ hybridization (Figure 3). We examined the signaling loop between AER-FGF and ZPA-Shh. SHH activation requires not only FGFs, but also Hand2 and 5’ HoxD transcription factors (Zakany and Duboule, 2007). Notably, perturbations of 5’ Hox genes on Gli3-null background (Lopez-Rios et al., 2012) yield more severe polydactyly than LOF of Gli3 alone (Sheft et al., 2012). In E10.5 Vps25ENU/ENU hindlimb, AER-Fgf4 was expanded anteriorly (Figures 3A), whereas ZPA-Shh expression was not perturbed (Figure 3B). At E11–11.5, Vps25ENU/ENU hindlimb Fgf4 and mesenchymal Gremlin (Verheyden and Sun, 2008) were expanded anteriorly (Figures 3C, 3D, and S3A) as was Shh expression, whereas Fgf8 was unperturbed (Figure 3E). Consistent with ZPA-Shh expansion, Patched1 was upregulated in E11.5 Vps25ENU/ENU hindlimb (Figure 3F). Moreover, significantly increased Shh and Patched1 mRNA levels were detected by qRT-PCR in E11.5 Vps25ENU/ENU versus WT hindlimbs (Figure 3G and 3H). In addition, western blot on whole-limb lysates demonstrated greater reduction of Gli3 repressor (Gli3R) (Wang et al., 2000) levels in Vps25ENU/ENU hindlimbs (Figures S3I and S3B) versus forelimb (Figure S3C). Furthermore, in E11.5 Vps25ENU/ENU anterior hindlimb, ectopic expression of Hand2, HoxD13, and Gli1, as well as downregulation of Gli3, were observed before Shh ectopic anterior expression could be detected at E12–12.5 (Figures 3J–3N). Of note, in E12.5 mutant hindlimb, Shh persisted in the ZPA (Figure 3N), Fgf4 and Fgf8 were still present in anterior AER (Figures S3O and 3P), and Sprouty2 was therein upregulated (Figure 3Q). Also, ectopic Gli1 and HoxD13 were still manifest in E12.5 Vps25ENU/ENU hindlimb (Figures S3D and S3E). Intriguingly, whereas bone morphogenetic protein (BMP) activity is required to regulate digit number (Selever et al., 2004), early BMP as well as WNT signaling components (Zeller, 2010) appeared unperturbed in E11 mutant hindlimbs (Figures S3F–S3K). These results establish selective enhancement of the FGF-Shh cross-signaling loop in Vps25ENU/ENU limb patterning.

The WT ESCRT-II/Vps25-ESCRT-III/Vps20 Complex Is Mostly Detected within Late Endosomes, whereas the Mutant Vps25ENU/Vps20 Complex Is Diffuse throughout the Cytoplasm

To test whether the nine amino acid insertion into the Vps25ENU protein in mutant embryos led to perturbed function, we assessed cellular localization of transfected FLAG- or HA-tagged WT and Vps25ENU cDNA constructs expressed alone or in combination. Regardless of the tag, WT and Vps25ENU proteins colocalized within the cytoplasm, also within distinct puncta (Figures 4A–4G). Coimmunoprecipitation of WT and Vps25ENU proteins by tag-specific antibodies followed by western blotting with an anti-Vps25 antibody (Langelier et al., 2006) showed that Vps25 ENU-induced mutation does not inhibit heterodimerization of Vps25 mutant protein with WT (Figure 4H). Furthermore, cotransfected WT or Vps25ENU proteins colocalized within the cytoplasm and coimmunoprecipitated with Vps20, a component of the ESCRT-III complex that binds to ESCRT-II via its Vps25 subunit (Babst et al., 2002a; Im et al., 2009), suggesting that both WT and Vps25ENU proteins can recruit Vps20 (Figures 4I–4O). Given colocalization with the late endosome marker Lamp1 (Shim et al., 2006), but not EEA1, which stains early endosomes, cytoplasmic puncta containing either WT or mutant Vps25-Vps20 complex are late endosomes (Figure S4). However, whereas the WT Vps25-Vps20 protein complex was predominantly localized within late endosomes, the mutant Vps25ENU·Vps20 complex appeared mostly diffuse throughout the cytoplasm (Figures 4I–4N). Because interactions between ESCRT-II and ESCRT-III are critical for scission of cargo-filled vesicles into the developing endosome (Im et al., 2009), mislocalization of the mutant complex likely contributes to disruption of vesicular trafficking.

Structural Rigidification of the Vps25ENU Protein Underlies Abnormal Subcellular Localization of the Mutant ESCRT-II/ESCRT-III Complex with Perturbed Lysosome-Mediated Trafficking and Impaired Degradation of Cargo

To determine whether the nine amino acid insertion (Figure 1G) affects the conformation and dynamics of the mutant protein, we used computational modeling of the Vps25ENU protein structure. In three separate simulations of Vps25 WT and mutant proteins, the WH2 domain (Im and Hurley, 2008) in Vps25ENU was found to be less flexible than in WT (Figures 5A and S3A–S3C). Analyses of conformational changes and fluctuations identified a stabilizing hydrogen bond in Vps25ENU between a residue from the ENU-induced insertion, Ser (IV), and Gln (105+9) (Figures 5A and S3D). The structural context observed from
the simulations attributes the greater conformational stability of the Vps25ENU protein to the presence of stronger hydrogen bonds, one of which cannot form in the Vps25WT. The increased rigidity of the Vps25ENU framework might be unable to conform to the characteristic Y-shaped structure typical of ESCRT-II (Teis et al., 2010). Such a structural rigidification of the Vps25ENU protein could potentially change its overall interaction with other ESCRT-II components like Vps22 and Vps36, as well as interactions with ESCRT-III (Im et al., 2009). Although Vps20/ESCRT-III is recruited to Vps25ENU/ESCRT-II, the subcellular localization of the mutant Vps25ENU-Vps20 complex is altered (Figures 4 and S4), suggesting perturbed degradation of signaling molecules.

To evaluate whether the Vps25 ENU-induced mutation caused abnormal lysosome-mediated trafficking and degradation of cargo within the cell, we isolated mouse embryonic fibroblasts (MEFs) from WT and Vps25ENU/ENU embryos. Transmission electron microscopy (TEM) revealed significantly enlarged multivesicular bodies (MVBs, a form of late endosomes [Henne et al., 2011, 2013]) in mutant versus WT MEFs (Figures 5B–5D). Horseradish peroxidase (HRP) uptake experiments (Shim et al., 2006) followed by TEM showed that in Vps25ENU/ENU MEFs there are significantly higher numbers of HRP-positive MVBs than HRP-positive lysosomes (Figure 5E). Accordingly, in Vps25ENU/ENU MEFs HRP-positive lysosomes were negligible (Figure 5F). HRP pulse-chase experiments followed by western blotting with an anti-HRP antibody (Ab) confirmed HRP degradation defects in Vps25ENU/ENU MEFs (Figure 5G). Importantly, TEM revealed significantly enlarged MVBs also in vivo in limb bud AER as well as mesenchyme of E11.5 Vps25ENU/ENU embryos versus WT littermates (Figures 5H–5K). Altogether, these results indicate that in WT MEFs exogenous HRP is trafficked to lysosomes via MVBs, whereas in Vps25ENU/ENU MEFs HRP does not reach lysosomes for degradation and is trapped in engorged MVBs. The latter are significantly enlarged in cultured mutant MEFs and also in mutant limb bud compartments, demonstrating that similar abnormalities occur in vivo as a result of Vps25 mutation.

In Vps25ENU/ENU Mouse Embryonic Fibroblasts, pFGFR Retained within Late Endosomes Leads to Increased Levels of FGF Second Messengers, whereas SHH Signaling Remains Unperturbed

To assess whether perturbed degradation generated enhancement of FGF and/or SHH signaling in a cellular system, as in...
Vps25\textsubscript{ENU}/\textsubscript{ENU} mutant limbs, we analyzed components of both pathways in WT and mutant MEFs. Pearson’s coefficient analysis (Teis et al., 2008) of FGF4 pulse-chase experiments showed a 1.3-fold increase in colocalization of pFGFR and Lamp1 in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs versus WT (Figures 6A–6G). Additionally, LysoTracker staining (Shim et al., 2006), which measures vesicular pH, was decreased by approximately 50% in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs versus WT after FGF4 pulse-chase (Figures 6H–6J), supporting a defect in lysosomal functionality. Also, in WT MEFs, phospho-ERK (pERK) decreased within 1 hr of cycloheximide (CHX) treatment, whereas in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs, which were already higher than in WT before treatment, remained elevated for 2.5 hr, indicating abnormal endosome-mediated degradation (Figures 6K and 6L). Consistent with accumulation of pFGFR in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs, we observed higher pERK and pSTAT3 (Hart et al., 2000) levels in E13.5 mutant whole embryos and E11.5 mutant versus WT limbs (Figure 6M). Kinetics of pSTAT3 activation versus degradation, in FGF pulse-chase experiments, revealed higher pSTAT3 levels in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs versus WT (Figures 6N and 6O). In contrast, relative expression of SHH pathway components Gli1 and Patched1 was not significantly perturbed in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs untreated or stimulated with the SHH agonist SAG (Goetz et al., 2009) versus WT (Figures 7A and 7B). Accordingly, Smoothened, a transducer of SHH signaling, did not accumulate in the engorged late endosomes of Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs (Figure 7C). Pearson’s coefficient analysis of Smoothened trafficking in MEFs showed no colocalization of Smoothened and engorged Lamp1-positive endosomes in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs untreated or stimulated with SAG (Figure 7D). Together, these results demonstrate that in Vps25\textsubscript{ENU}/\textsubscript{ENU} MEFs pFGFR is retained in late endosomes and does not reach lysosomes for degradation.
Furthermore, FGF second messengers pERK and pSTAT3 are increased in Vps25ENU/ENU embryos and MEFs, highlighting FGF signaling upregulation in both systems. Unexpectedly, SHH signaling is not intrinsically upregulated in Vps25ENU/ENU MEFs and SHH pathway components do not accumulate within late endosomes, suggesting that SHH enhancement in mutant limbs results from selective hyperactive FGF signaling within the FGF-SHH feedback loop.

**Inhibition of the FGF-SHH Signaling Loop via Reduction of SHH Dosage Rescues Polydactyly in Vps25ENU/ENU Limbs**

To determine whether polydactyly could be specifically ameliorated by limiting FGF-SHH cross-signaling in Vps25ENU/ENU embryos, we reduced the dosage of SHH (Chiang et al., 2001). Genetic reduction of SHH in Vps25ENU/ENU,Shh+/− mutants partially rescued polydactyly (Figures 7E and S7A) by preventing Fgf4 expansion present in Vps25ENU/ENU anterior hindlimb AER (Figure 7E; see also Figures 3A, 3C, and 3D). Seven digit hindlimbs decreased from 39% in Vps25ENU/ENU mutant embryos to 14% in Vps25ENU/ENU,Shh+/− mutants, and pentadactyly, albeit with abnormally shaped digits, appeared in 27% of Vps25ENU/ENU,Shh+/− hindlimbs (Figure S7B). Partial genetic rescue of polydactyly in Vps25ENU/ENU,Shh+/− limbs demonstrates that ESCRT-II/Vps25 constrains digit number by specific modulation of the FGF-SHH cross-regulatory loop in the limb.

Remarkably, loss of one allele of Shh did not partially rescue the craniofacial defects of Vps25ENU/ENU embryos, consisting of hypoplastic jaw, stunted snout, and malformed ear pinna (Figure S7C). These findings strongly underscore that the Vps25 ENU-induced mutation preferentially and primarily affects FGF signaling in the limb bud and that SHH enhancement in Vps25ENU/ENU mutant limbs results from hyperactivation of FGF signaling within the FGF-SHH feedback loop (Figures 7F and 7G).

**DISCUSSION**

ESCRRT complex components, first identified in yeast, control multiple cellular functions, including receptor signaling, cytokinesis, autophagy, cell migration/motility, repair of plasma membrane wounds, miRNA activity, and mRNA localization/transport in metazoans (Henne et al., 2013; Jimenez et al., 2014; Rusten et al., 2012). Evidence supports requirements of ESCRT proteins in promoting endosome-mediated degradation of signaling receptors across the animal kingdom (MacGurn et al., 2012; Rusten et al., 2012). However, despite their reported roles in signal attenuation, Vps4 acts as a positive regulator of WNT signaling (Taelman et al., 2010). This suggests a yet unexplored diversity in the regulation of signaling by ESCRT components (Tognon et al., 2014). Consistent with constitutive expression of ESCRT subunits and with their pleiotropic roles in trafficking of signaling proteins, LOF mutations of mouse ESCRT components result in early lethality in utero (E8–11) (Rusten et al., 2012). This has precluded dissection of specific ESCRT-dependent morphogenetic processes during...
organ formation. Here, the ENU-induced hypomorphic mutation of murine ESCRT-II/Vps25 results in late embryonic lethality, thus affording deconstruction of unexplored ESCRT-II requirements in the control of signal transduction underlying mammalian tissue patterning. Using this unique model, we propose that ESCRT-II/Vps25 constrains digit number by exerting selective attenuation of FGF signaling and by consequently maintaining homeostasis of the FGF-SHH feedback loop in the developing limb. Interestingly, together with hyperactivation of FGF-SHH signaling in mutant Vps25ENU/ENU limbs, Fgf and Shh mRNAs are also upregulated suggesting a direct or indirect positive feedback whereby the increased protein levels cause upregulation of their own mRNAs. Alternatively, because ESCRT-II can directly affect mRNA localization acting as RNA binding proteins, ESCRT-II/Vps25 mutations might yield accumulation of select mRNAs within the cell (Iriion and St Johnston, 2007). Last, mutations of Vps25/ESCRT-II might directly affect miRNA-mediated degradation of specific mRNAs (Gibbings et al., 2009), potentially including Fgf and Shh, in mutant limbs.

FGF signaling mediates multiple functions in embryonic development (Goetz and Mohammadi, 2013; Itoh and Ornitz, 2004). In limb bud mesenchyme, FGF4 stimulates proliferation (Niswander and Martin, 1993) and, together with FGF8, is required for cell survival (Sun et al., 2002). Although multiple reports link polydactyly to ectopic or enhanced SHH activity (Anderson et al., 2012), increased Fgf4 expression in early mouse limbs causes polydactyly (Lu et al., 2006) and ectopic Fgf4 expression in spontaneous mutant chickens initiates polydactyly independent of SHH activity (Bouldin and Harfe, 2009). Interestingly, Vps25ENU/ENU mutant hindlimbs exhibit Fgf4 expansion before Shh perturbations arise, which alone could result in enhanced proliferation and lack of cell death causing polydactyly. FGF signaling has also critical roles in the control of endochondral bone development (Chen et al., 2014; Ornitz and Marie, 2002). Notably, limbs of Vps25ENU/ENU embryos display abnormally short and thick skeletal elements (Figures 2A, 2B, and S2F–S2L), similar to humans with achondroplasia (dwarfism) (Ornitz and Marie, 2002). Achondroplastic patients and mouse models harboring activating mutations of the FGF pathway exhibit skeletal hypoplasia and epiphyseal growth plate dysmorphology (Naski et al., 1998). Due to edema and hemorrhaging in Vps25ENU/ENU embryos already at E14.0, it is impossible to determine whether their skeletal hypoplasia is due to cell-autonomous FGF activation in the growth plate until a mouse with Vps25 conditional LOF is available. Nonetheless, the striking similarities of the skeletal phenotypes in these mouse mutants support a scenario whereby Vps25 mutation disrupts bone development by perturbing FGF signaling, leading to achondroplastic phenotypes.

Intriguingly, in the developing limb not all signaling pathways are hyperactive, or otherwise altered, as a result of ESCRT-II complex impairment. For example, in Vps25ENU/ENU limbs at E11, when FGF signaling is hyperactive, early WNT and BMP signaling components appear unperturbed. This is worthy of note, because proper BMP activity is required to regulate digit number (Selever et al., 2004). In addition, in Vps25ENU/ENU mouse embryonic fibroblasts FGF signaling is upregulated, whereas SHH signaling is not intrinsically enhanced. Last, in Vps25ENU/ENU embryos, loss of one allele of Shh partially rescues polydactyly by preventing Fgf4 expansion, whereas it does not partially rescue the craniofacial defects. Together, the findings in this model underscore preferential hyperactivation of FGF signaling as the main culprit of polydactyly in ESCRT-II/Vps25 mutant embryos. Alternatively, these results could suggest that during early limb patterning maintenance of homeostasis of most signaling pathways requires relatively low levels of ESCRT-II function, with a few exceptions such as the FGF pathway for which ESCRT function would become rate limiting in a context-specific manner. On the other hand, because Vps25 has been shown to control Notch and other signaling pathways in Drosophila (Thompson et al., 2005; Vaccari and Bilder, 2005), signaling cascades other than FGF might be affected in other domains of Vps25ENU/ENU embryos. In addition, within the limb bud proper, signaling mediated by other tyrosine kinase receptors might be concomitantly enhanced in Vps25ENU/ENU embryos and might contribute to polydactyly. In particular, expression of constitutively active EGFR in chick limbs in ovo causes polydactyly (Omi et al., 2005). Yet, in contrast to Vps25ENU/ENU embryos, developing limb buds with activated EGFR exhibit multiple, fragmented, or bifurcating ectopic AERs expressing Fgf8, as well as downregulation of Bmp4, resulting in pre- and postaxial polydactyly.

In summary, we isolated an ENU-induced hypomorphic mutation of ESCRT-II/Vps25 in a polydactylous mouse line, which allowed dissection of the mechanisms whereby ESCRT components execute mammalian limb patterning. The availability of this unique mouse model led us to establish that in the developing embryo ubiquitously expressed ESCRT machineries act on different receptors and associated signaling proteins in a preferential manner. In addition, our study demonstrates that ESCRT-mediated downregulation of specific signaling pathways appears more critical in certain embryonic contexts than in others. Although limb, craniofacial, and heart development are severely affected by the ESCRT-II/Vps25 mutation, development of other organ systems appears unperturbed. Our research demonstrates that ESCRT-II/Vps25 effects preferential modulation of FGF signaling in the mammalian limb, which, in turn, controls cellular proliferation and cell death establishing normal digit number and identity, as well as controlling skeletal maturation, relevant to congenital limb defects. Broadly, our study proposes a mechanism for signaling homeostasis in the vertebrate embryo highlighting that endosomal sorting by specific components of the ESCRT machinery preferentially regulates, or is rate limiting for, select conserved signaling pathways in distinct tissue patterning processes.

EXPERIMENTAL PROCEDURES

A detailed description of reagents and protocols is in the Supplemental Experimental Procedures. See the following experimental procedures for brief descriptions.

Generation of Mutant Line 04-014 by an N-ethyl-N-nitrosourea Mutagenesis Screen

Mutant mouse line 04-014 was generated by ENU mutagenesis of C57BL/6J males, as previously described (Anderson and Ingham, 2003; Stottmann and Beier, 2010).
Figure 5. Vps25 ENU-Induced Mutation Yields Low Levels of Both WT and Structurally Altered Mutant Protein and Is Associated with Engorged MVBs in Cultured Cells, Limb AER, and Mesenchyme In Vivo, Resulting in Perturbed Endosomal Trafficking

(A) Structural representation of WT and Vps25ENU proteins reveals a conserved Glu88-Arg151 hydrogen bond between WH1 (green) and WH2 (magenta) domains. In the Vps25ENU protein, a new strong hydrogen bond (<2 Å) Ser(IV)-Glu(105+9) is formed, due to the nine amino acid insertion (orange). See also Figure S5.

(B–D) Transmission Electron Microscopy (TEM) reveals a significant increase in the diameters of multivesicular bodies (MVBs) in E13.5 mutant versus WT MEFs treated with HRP. The box and whisker plot (D) depicts MVB diameters (in nanometers, nm) on the y axis and WT and Vps25ENU/ENU MVB sample populations on the x axis. Blue boxes represent the middle 50% of the values of the sample range; red lines represent the value of the median sample; legs and bars represent upper and lower limits of the diameter sample. Red cross indicates an outlier. Diameters measured in 16 WT and 12 mutant MVBs (**p < 0.0001).

(E) One hundred MVBs and 100 lysosomes counted in HRP-treated WT and mutant MEFs. In WT MEFs, HRP-positive MVBs (green) and lysosomes (blue) are present in approximately equal numbers, whereas in Vps25ENU/ENU MEFs HRP-positive lysosomes are negligible.

(F) Numbers of total (purple) and HRP-positive (red) lysosomes (100) counted and reported per surface areas (mm²) in both WT and Vps25ENU/ENU MEFs, showing that in mutant MEFs HRP is not escorted to the lysosomes.

(G) WB analysis of MEFs treated with a 30 min HRP pulse and chased for different times (hours, hrs), demonstrates abnormal HRP degradation in mutant MEFs.

(legend continued on next page)
High-Resolution Mapping and Transcriptome Analysis for Gene Cloning

To clone the gene responsible for the ENU-induced mutation, classic procedures combining whole-genome SNP (Moran et al., 2006) mapping with gene expression microarray analysis for gene finding were used. Total RNA from E12.5 WT and homozygous mutant embryos from line 04-014 was purified using the RNeasy kit (QIAGEN). Roche NimbleGen 12plex HD2 gene expression microarrays (design number 100718_MM9 _exp_HX12) were used.

(H–K) TEM reveals a significant increase in MVB diameters within the limb AER and mesenchyme compartments of E11.5 Vps25ENU/ENU (n = 5) versus WT (n = 2) embryos. The box-and-whisker plots (J and K), as detailed above (in D), depict MVB diameters (in nanometers, nm) on the y axis and WT and Vps25ENU/ENU MVB sample populations on the x axis. Diameters measured in 13 WT and 12 mutant MVBs, in limb AER (p < 0.05). In limb mesenchyme, diameters measured in 15 WT and 21 mutant MVBs (p < 0.05).

(M–O) WB of whole embryos (E13.5) and limbs (E11.5), as well as FGF4 pulse-chase experiment in MEFs, show increase of FGF second messengers and decreased pSTAT3 degradation, quantified by ImageJ64, in Vps25ENU/ENU (red) versus WT (purple) MEFs. Actin as control. min, minutes; ns, nonspecific band.

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used to analyze expression of all genes within the narrowest critical interval (500 kb) linked to the ENU-induced mutation. cDNA from total RNA was amplified/labeled using Cy3-coupled random nonamers. Technical triplicate hybridizations were performed per sample. Hybridizations were conducted using 4 μg of labeled cDNA per subarray, as per the Roche NimbleGen Gene Expression Protocol. The array data were analyzed using ArrayStar software v.4.0.2 (DNASTAR). Both the mutant and control technical replicates were normalized together, and p values were generated for all expression changes.

Pairwise comparisons of global gene expression calls between any two of the technical triplicates per sample correlated with R-squared values of greater than 0.97.

Custom SureSelect Capture and SOLiD Sequencing
DNA capture was performed on 3 μg of high-quality genomic DNA using a custom SureSelect Target Enrichment kit (protocol by Agilent). Chromosome 11 enriched DNA libraries were sequenced on a SOLiD 3plus system (Life Technologies). Twenty-two million reads were generated; 79.7% of the reads were on target, and 95.3% of the target was covered at 10x.

Phenotypic Analysis of 04-014 Embryos
Skeletal preparations, in situ hybridizations, X-Gal stainings, and OPT imaging were carried out as described previously (Ferretti et al., 2011; Sharpe et al., 2002).

Proliferation and Apoptosis Assays
Protocols were as described previously (Ferretti et al., 2011).

mRNA Isolation, RT-PCR, and qRT-PCR
Total RNA was purified from embryos, embryonic limbs, or mouse embryonic fibroblasts (MEFs) at E10.5–13.5 using standard procedures. Gene expression was determined by quantitative real-time PCR using QuantiTect SYBR Green PCR master mix (QIAGEN) or predesigned TaqMan Gene expression Assays and the 7500 Real-Time PCR System (Applied Biosystems).

Generation of Vps25 Global LOF Allele
Vps25 global LOF was obtained by insertion of a LacZ cassette into the gene locus (Vps25<sup>enu/enu</sup>) (trans-NIH Knock-Out Mouse Project [KOMP]).
animal experiments were performed following protocols approved by the WCMC IACUC.

**Immunohistofluorescence and Whole-Mount Lysotracker**

Embyos were cryosectioned for immunofluorescence (IF) experiments or incubated with Lysotrocker (Naiche and Papaioannou, 2007) to detect whole-mount staining.

**Cell Culture**

MEFs were isolated according to standard protocols from E11.5–13.5 WT and Vps25ENU/ENU embryos. MEFs were pulse-chased with FGFr4 for various time points as indicated or treated for 24 hr with SAG (Goetz et al., 2009) and tested for different markers by IF and qRT-PCR. Cell trafficking was investigated by Lysotracker staining and horseradish peroxidase (HRP) uptake experiments (Shim et al., 2006). For HRP uptake experiments, cells were incubated with 100 μg/ml HRP for 1 hr and then chased for different times as indicated. For HRP uptake experiments followed by transmission electron microscopy (TEM), cells were serum starved prior to stimulation with 5 mg/ml HRP for 30 min and then fixed and crosslinked with 3,3'-diaminobenzidine (DAB) and prepared for TEM. The protein synthesis inhibitor cyclohexamide (CHX) was used to treat MEFs (50 μg/ml) as indicated, in order to examine pERK protein degradation, after which cells were lysed for western blotting (WB).

**In Vitro Transient Transfection Assays**

HEK293 were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine (Invitrogen), in humidified 5% CO2. Transient transfections were performed using LT1 reagent (Minus) at a 5:2 ratio. Cells were fixed for immunostaining or harvested in NP40 buffer for immunoprecipitation (IP) 36 hr after transfection.

**Immunoprecipitations and Western Blot Analysis**

HEK293 cells were transfected with HA- or FLAG-tagged constructs to conduct IP and WB using HA (mouse; Covance; goat, Santa Cruz Biotechnology) or FLAG (Sigma-Aldrich) Ab. For IP and coIP, cells were lysed in NP40 and incubated overnight with appropriate primary Ab. magnetic beads were applied for 2 hr, and then complexes were dissociated using DTT before WB analysis. WB was performed using whole embryos, limbs, MEFs, or HEK293 cells. Samples were lysed in RIPA buffer, and proteins were separated on a 4%–12% polyacrylamide gel and transferred to a nitrocellulose membrane. Primary Abs were incubated in blocking solution overnight at 4°C and blotted with the relevant secondary Abs.

**Computational Approach to 3D Modeling of Vps25WT and Vps25ENU Mutant Proteins**

For computational modeling and simulation studies designed to distinguish differences in structure and dynamics between Vps25WT and Vps25ENU, the molecular models of the two constructs were immersed in a restrained water sphere (Phillips et al., 2005) for extensive dynamics simulations. Six separate simulations were done, consisting of replicate repeats for Vps25WT and Vps25ENU.

**Transmission Electron Microscopy**

MEFs and isolated limb buds were processed following standard procedures (Venable and Coggshall, 1965). Two WT and five Vps25WT/ENU and Vps25WT/ENU mutant embryos at E11.5 were imaged to visualize MVbs in limb AER and mesenchyme compartments. MVb diameters measured using ImageJ64 and MATLAB (MathWorks).

**Statistical Analysis**

Results are given as the average ±SD or SEM. Statistical analyses were performed with Excel (Microsoft) applying the two-tailed t test. p values < 0.05 were considered significant.

**Calculation of Pearson’s Coefficient**

MATLAB (MathWorks) was used to calculate colocalization of IF signals.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.09.019.

**AUTHOR CONTRIBUTIONS**

K.H. and J.F. conducted high-resolution mapping; identified Vps25 mutation; completed genetic complementation; performed skeletal preparations, ISH, IF, TEM, IP, WB, and genetic rescue experiments; and assisted L.S. in manuscript writing. M.K. participated in the ENU screen, conducted high-resolution mapping, cloned tagged Vps20 and Vps25 WT and mutant expression constructs, and performed qRT-PCR for Vps25 expression levels. E.F. participated in the ENU screen, performed skeletal preparations, and conducted initial high-resolution mapping. M.R. performed qRT-PCR for Shh, Patched1, and Gli1 in limb buds and MEFs; performed statistical analyses; and contributed to manuscript writing. R.Z. established that the Vps25 mutation is hypomorphic by WB, generated MEF lines, and contributed to manuscript writing. M.A.S. and H.W. conducted 3D modeling of WT and Vps25ENU proteins and contributed to manuscript writing. D.-B. provided qRT-PCR primers for Shh, Patched1, and Gli1 and assisted the interpretation of limb ISH data. X.P.P. identified involvement of pSTAT3 as second messenger of FGF hyperactivation. M.D. analyzed skeletal preparations of all ENU-mutagenized lines. L.Q. and J.S. performed OPT and 3D analysis of limbs for Vps25 mutant line. B.W. provided a Gii3 antibody. H.A. assisted in deep sequencing. R.R. conducted embryo dissections throughout ENU screen. S.B. and J.R.M. conducted microarray experiments and analyses for gene finding. T.V. performed analyses of MVs and lysosomal diameters and provided suggestions to explore abnormal ESCRT function. K.V.A. initiated the ENU screen and gave input for high-resolution analysis of the SHH pathway. E.L. participated in the ENU screen and in analysis and interpretation of all data. L.S. conducted embryo dissections throughout ENU screen; designed the project; and wrote the manuscript.

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