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ESCRT-dependent Control of Craniofacial Morphogenesis with Concomitant Perturbation of NOTCH Signaling

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Abstract:

Craniofacial development is orchestrated by transcription factor-driven regulatory networks, epigenetic modifications, and signaling pathways. Signaling molecules and their receptors rely on endo-lysosomal trafficking to prevent accumulation on the plasma membrane. ESCRT (Endosomal Sorting Complexes Required for Transport) machinery is recruited to endosomal membranes enabling degradation of such endosomal cargoes. Studies *in vitro* and in invertebrate models established the requirements of the ESCRT machinery in membrane remodeling, endosomal trafficking, and lysosomal degradation of activated membrane receptors. However, investigations during vertebrate development have been scarce. By ENU-induced mutagenesis, we isolated a mouse line, *Vps25*$^{ENU/ENU}$, carrying a hypomorphic allele of the ESCRT-II component *Vps25,* with craniofacial anomalies resembling features of human congenital syndromes. Here, we assessed the spatiotemporal dynamics of *Vps25* and additional ESCRT-encoding genes during murine development. We show that these genes are ubiquitously expressed although enriched in discrete domains of the craniofacial complex, heart, and limbs. ESCRT-encoding genes, including *Vps25*, are expressed in both the cranial neural crest-derived mesenchyme and epithelium. Unlike constitutive ESCRT mutants, *Vps25*$^{ENU/ENU}$ embryos display late lethality. They exhibit hypoplastic lower jaw, stunted snout, dysmorphic ear pinnae, and secondary palate clefting. Thus, we provide the first evidence for critical roles of ESCRT-II in craniofacial morphogenesis and report perturbation of NOTCH signaling in craniofacial domains of *Vps25*$^{ENU/ENU}$ embryos. Given the known roles of NOTCH signaling in the developing cranium, and notably the lower jaw, we propose that the NOTCH pathway partly mediates the craniofacial defects of *Vps25*$^{ENU/ENU}$ mouse embryos.

Keywords: ESCRT, *Vps25*, Mouse, Craniofacial, Lower Jaw, NOTCH Signaling

Introduction:

Craniofacial morphogenesis is a tightly regulated process characterized by interactions between different germ layers and cell types. Perturbances altering these intricate interplays result in congenital abnormalities affecting the craniofacial complex, accounting for approximately one-third of all birth defects[1,2]. In humans, critical morphogenesis events for craniofacial development occur between week 4 and week 8 of gestation[3], corresponding approximately to gestational day (E)8.5-E15.5 in mice[4]. Craniofacial morphogenesis commences as cranial neural crest cells (CNCCs) delaminate and migrate in defined streams from the dorsal-most portion of the neural tube to populate the ventral regions of the developing head[5,6]. As CNCCs differentiate, distinct structures of the face begin to form. At early week 4 (E8.5 in mice), the frontonasal prominence (FNP) emerges, forming the nose, forehead, and part of the primary palate[5,7,8]. By week 5 (E10.5 in mouse), the FNP is separated into the lateral and medial nasal prominences (LNP and MNP, respectively)[3,8]. In parallel, branchial arch 1 (BA1) emerges, and by E10.5 both BA1 maxillary and mandibular prominences (MxP and MdP, respectively) become distinctly recognizable. The MxP will form the maxilla, incus, malleus and also contribute to the primary palate[5,8], whereas the MdP will form the mandible, lower lip, and portions of the tongue[5,6,9]. Branchial arch 2 (BA2) emerges between week 4-5 (E9.0-9.5 in mouse) and will ultimately form the stapes, the styloid process, and the superior portion of the hyoid body[5].

The above morphogenetic events are in turn orchestrated by a multi-layered code of transcription factor-driven gene regulatory networks, epigenetic modifications, and signaling pathways. Signaling molecules, as well as their cell surface receptors, rely on the cell's endo-lysosomal pathway to prevent their accumulation on the cellular plasma membrane[10,11], which would have detrimental effects on embryonic development. Accordingly, signaling molecules and their receptors are internalized in early endosomes, where they can be either recycled back to the cellular plasma membrane or sorted into late endosomes for further lysosomal degradation[11,12]. To enable degradation of such endosomal cargoes, the early endosome undergoes a process of maturation where the endosomal membrane is bent and severed, giving rise to intraluminal vesicles (ILVs) loaded with protein cargoes that bud away from the cytoplasm into the endosomal lumen. The formation of ILVs requires a highly conserved and ubiquitous protein machinery known as ESCRT (Endosomal Sorting Complexes Required for Transport)[13]. Once ESCRT-dependent endosomal maturation is complete, the late endosome - also known as multivesicular body (MVB) - is ready to fuse with lysosomes for degradation of cargoes, such as signaling molecules[11,14].

The ESCRT machinery is composed of five oligomeric protein complexes that are sequentially recruited to membrane surfaces: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and VPS4[13]. ESCRT-0 is composed of HRS (*HGS*) and STAM[15]. ESCRT-I is a tetrameric complex composed of vacuolar sorting protein 23 (VPS23) (or TSG101), VPS28, MVB12a (MVBB, UPAP1) and a variable paralogue of VPS37 (VPS37A/B/C/D)[16]. ESCRT-II is a tetrameric complex composed of VPS22 (EAP30), VPS36 (EAP45) and 2 subunits of VPS25 (EAP20)[17]. ESCRT-III includes charged multivesicular protein 1A and 1B (CHMP1A/B), CHMP2A/B, CHMP3, CHMP4A/B/C, CHMP5, CHMP6, CHMP7 and IST1 (CHMP8)[18]. Unlike other ESCRT complexes, ESCRT-III subunits are not bound together in the cytoplasm and polymerize into filaments after they are recruited to the membrane surface[19–21]. The VPS4 complex is a heteromeric complex composed of VPS4A-B (SKD1) and VTA1 (LIP5)[22].

Along with degradation of endosomal cargoes such as signaling molecules and their receptors, the ESCRT machinery executes diverse functions within the cell, including cytokinesis[23], plasma membrane (PM) repair[24], endolysosomal repair[25], intraluminal vesicle (ILV) biogenesis[26], nuclear envelope (NE) repair[27], and neuronal pruning[28], among others[13,29–31]. It is important to note that all pathways require both ESCRT-III and VPS4 complexes, but not all pathways require all five core components[32]. In addition to the five core complexes, ESCRT-associated proteins ALIX (*Pdcd6ip*) and LEMD2 nucleate ESCRT-III polymerization in an ESCRT-II-independent manner[27,33].

Although the biochemical and cellular physiology of the ESCRT machinery has been widely studied *in vitro* or in invertebrate animal models[34–38], investigation and validation of ESCRT machinery's roles during mammalian embryonic development have been scarce. Notably, the functions of the ESCRT machinery in mammalian systems are even less defined, which can be mostly attributed to the early embryonic lethality observed when genes encoding ESCRT components are constitutively knocked out[39–41]. Early lethality was circumvented when we isolated a hypomorphic allele for the ESCRT-encoding gene[41] *Vps25*, named $Vps25^{ENU}$, while conducting a forward genetic screen in mice using N-ethyl-N-nitrosourea (ENU) mutagenesis[42]. The $Vps25^{ENU/ENU}$ homozygous hypomorphic mutation resulted in later embryonic lethality (E15.5-E16.5) and provided the first evidence of ESCRT machinery's roles in both limb and craniofacial development. $Vps25^{ENU/ENU}$ limbs displayed preaxial polydactyly, whereas the craniofacial complex exhibited striking morphological deformities. We comprehensively characterized the signaling pathways affected in $Vps25^{ENU/ENU}$ polydactylous limbs and found that the defective degradation of active fibroblast growth factor (FGF) signaling receptors resulted in hyperactivation of the FGF-sonic hedgehog (SHH) feedback loop[41]. While

placing *Vps25*$^{ENU/ENU}$ mutants on a SHH deficient (*Shh*$^{+/-}$) background rescued the polydactyly, the craniofacial phenotype remained unaffected, strongly suggesting that ESCRT machinery operates in a tissue-specific manner[41]. Despite the presence of striking craniofacial defects in the *Vps25*$^{ENU/ENU}$ mouse embryos, the underlying pathways that are perturbed in these mutants have remained, so far, unexplored.

Here, we sought to investigate the spatiotemporal dynamics of ESCRT-encoding genes during key timepoints in murine craniofacial development and to characterize the disrupted pathways underlying the craniofacial phenotypes observed in *Vps25*$^{ENU/ENU}$ mouse mutant embryos. We show that although the ESCRT machinery is ubiquitously distributed in the mouse embryo throughout the developmental timepoints analyzed, there are distinct areas of enrichment in domains involved in heart, limb, and craniofacial morphogenesis. Specifically, ESCRT proteins are preferentially localized to distinct domains of the developing head, including LNP, MNP, and BAs, as well trigeminal stream CNNCs. Lastly, we uncover perturbations of NOTCH signaling in LNP, MNP, and BAs of *Vps25*$^{ENU/ENU}$ mouse embryos. Interestingly, mouse models harboring mutations in the NOTCH pathway exhibit craniofacial, as well as limb and heart, defects that mimic phenotypes of *Vps25*$^{ENU/ENU}$ embryos[43–49].

Results:

Expression of ESCRT-encoding genes during early mouse development

We previously characterized limb phenotypes in *Vps25*$^{ENU/ENU}$ mutant mouse embryos, which exhibit polydactyly[41], and uncovered additional abnormalities affecting the heart and select craniofacial structures. However, we did not determine when and where *Vps25* is expressed during mouse embryonic development. Therefore, here we sought to establish the temporo-spatial expression of *Vps25*, other ESCRT-encoding genes representative of each core ESCRT complex, and the ESCRT-associated gene *Pdcd6ip* (encoding ALIX), at timepoints critical for craniofacial development (E8.25-E11.5).

E8.0-E8.5 is hallmarked by axial rotation of the embryo, the emergence of somites along the anterior-posterior (AP) axis and the formation of the neural folds in the dorsal cephalic region. At this stage, CNCCs delaminate from the dorsal-most neural tube, migrate ventrally, and interact with the surface cephalic epithelium (SCE) to shape primordial structures of the developing face, including the FNP and BA1[50]. At E8.25-E8.75, we examined expression patterns of select ESCRT-encoding and ESCRT-associated genes using whole-mount *in situ* hybridization (WISH) and uncovered that they are all ubiquitously expressed and share overlapping patterns of expression (**Figure 1A**). These genes display high enrichment in the neural folds along the entirety of the dorsal AP-axis, and at the distal tip of BA1 (**Figure 1A**). These results highlight that the examined ESCRT-encoding genes are enriched in early embryonic domains involved in craniofacial morphogenesis.

From E8.5 to E9.5, there is a dramatic increase in cranium size and sharper definition of craniofacial anlagen including FNP, BA1, and BA2. However, it is important to note that although BA1 is discernable, the MdP and the MxP have not yet differentiated into distinct entities. Thus, at E9.5 we observed that the ESCRT-encoding genes under study and the gene encoding ALIX continue to be ubiquitously expressed and share distinct domains of enrichment in the FNP, BA1, BA2 and forelimb buds (**Figure 1B**). In the FNP, high enrichment is present at the rostral tip with attenuated signal caudally; however, signal persists along the dorsal AP-axis. In BA1, expression remains strongest at the rostral tip with reduced expression extending as far as its caudal boundary (**Figure 1B**). Similar to BA1, these genes are expressed also in BA2 (**Figure 1B**). Outside of the cephalic domain, we observed high enrichment in the forelimb bud, which displays a gradient pattern with highest level of expression at the distal tip (**Figure 1B**).

These data demonstrate that ESCRT-encoding genes and *Pdcd6ip* (encoding ALIX) are enriched in craniofacial prominences, BAs, and developing limbs.

There is sustained growth of the facial structures from E9.5-E10.5. Notably, the FNP separates and, as the nasal pit begins to take form, gives rise to the lateral nasal process (LNP) and medial nasal process (MNP). In BA1, a clear boundary between MxP and MdP begins to form as the MxP extends under the LNP and MNP. In parallel, outgrowth of the forelimb bud continues and the hindlimb bud emerges. Analysis of ESCRT-encoding genes and *Pdcd6ip* at E10.5 revealed that they remain ubiquitously expressed with shared domains of enrichment in craniofacial prominences, BAs, and limbs (**Figure 2A & 2B**). Expression in the LNP and MNP is highest at the rim of the nasal pit perimeter (**Figure 2B and Video 1**). In contrast, expression in BA1 is highest in the most posterior edge of the MxP, and in the most anterior edge of the MdP, respectively (**Figure 2B**). In BA2, expression is highest in the posterior edge with lower uniform expression seen throughout the rest of the arch. In the forelimb, the genes examined maintain a gradient-like pattern, as expression is highest in the apical ectodermal ridge (AER) and is reduced towards the center of the developing bud (**Figure 2A**). Similar to what is observed in the forelimb at E9.5 (**Figure 1B**)**,** expression in the emerging hindlimb is most apparent at the most distal tip with reduced expression proximally (**Figure 2A**). Thus, it is further emphasized that for the ESCRT genes examined, expression remains high in facial prominences, BAs, and limbs as the embryo developmentally matures.

From E10.5-E11.5 the craniofacial complex continues to grow in size. By E11.5 the nasal pit has narrowed to a slit-like structure. In BA1, the MxP and MdP become distinctly separated. Meanwhile, outgrowth of the forelimbs and hindlimbs progresses. At E11.5, expression of ESCRT-encoding genes and *Pdcd6ip* (encoding ALIX) remains ubiquitous and the shared domains of enrichment are maintained (**Figure 3A & 3B**). Expression in the LNP is highest in the lateral edges, whereas in the MNP it is highest in the posterior edge bordering the oral cavity. In the MxP, there is high expression in the lateral edges; in contrast, signal in the MdP is highest in the medial anterior tips. Expression is detectable also in BA2, although overall weaker (**Figure 3B and Video 2**). In fore- and hindlimbs, expression appears highest distally, with attenuated signal at the core of the limb buds (**Figure 3A**). Expression of the ESCRT-encoding genes *Hgs*, *Chmp1a*, *Chmp4b* and *Vps25* in the craniofacial complex was further assessed by RNAScope *in situ* hybridization[51] on E10.5 sections and whole mount ß-galactosidase staining of a $Vps25^{LacZ}$ reporter line[41], confirming enrichment of these transcripts in CNCCs of the trigeminal stream (**Figure 4A-F**). Interestingly, we also observed intense LacZ activity in the neuroepithelium (NE) and the surface cephalic epithelium (SCE) (**Figure 4F**).

Our results show that although there is ubiquitous expression of these ESCRT-encoding genes and *Pdcd6ip*, enrichment is highest in FNP, LNP, MNP, BA1 and BA2 throughout critical stages of craniofacial morphogenesis. We also demonstrate that as the embryo develops the domains of expression are dynamic. Notably, the ESCRT-encoding genes examined here and *Pdcd6ip* share strikingly similar patterns of enrichment in the facial prominences, BAs, and limb buds until at least E11.5.

ESCRT proteins are enriched in heart, limb, and cranial neural crest cells that populate FNP and BAs

In order to determine whether the observed enrichment of ESCRT-encoding genes and *Pdcd6ip* (encoding ALIX) in specific craniofacial domains results in comparable accumulation of ESCRT proteins, we conducted immunofluorescence using antibodies against proteins representative of all ESCRT complexes (0, I, II, III, VPS4-VTA1 complex and ALIX) at E9.5 and E10.5 (**Figure 5 & Figure 6**). By using SOX10 as a CNCC marker[52], we determined that all ESCRT components examined and the ESCRT-associated protein ALIX are strikingly enriched in the trigeminal stream of CNCCs at both gestational days (**Figure 5 & Figure 6**). This stream of cells migrates from the midbrain and hindbrain (rhombomeres 1 and 2) into craniofacial regions that exhibit enrichment for ESCRT-encoding genes by *in situ* hybridization: the upper jaw primordia, the periocular mesenchyme of the FNP, and the lower jaw primordia in BA1[53] (**Figures 1, 2 & 3**). We also observed intense immunodetection of ESCRT proteins in the NE and SCE layers (**Figure 5 & Figure 6**). By bulk RNA-seq from mouse embryonic midface epithelium and mesenchyme at E11.5, we uncovered that most genes encoding ESCRT components and *Pdcd6ip* are similarly expressed in both epithelium and mesenchyme of the midface (LNP, MNP and MxP) (**Figure 7A**). Altogether, our results suggest that the ESCRT machinery plays roles not only in CNCC and CNCC-populated craniofacial domains, but also in the SCE during mammalian development.

Upon identifying domains qualitatively enriched for ESCRT-encoding genes, we examined by qRT-PCR whether there were quantitative differences in gene expression in the FNP, BA1, forelimbs, and hearts at both E10.5 and E11.5 (**Figure 7B & 7C**). We found that all genes examined share a similar level of expression in the FNP, BA1, and forelimbs at both E10.5 and E11.5. However, we observed that select ESCRT genes are expressed at higher levels in the heart at both timepoints (**Figure 7B & 7C**). Moreover, Western blot analysis shows that all ESCRT proteins examined are present in the FNP, BA1, heart, and forelimbs at both

E10.5 and E11.5. More than one isoform is present for some ESCRT proteins under analysis (**Figure 7D**). Our data confirm presence of ESCRT proteins in limb[41] and heart[40,54,55] development, and further establish that they are highly enriched in the forming craniofacial complex, pointing to their potential roles in mammalian head development.

ESCRTII-Vps25 deficiency results in craniofacial defects

Knowledge of the potential functions of ESCRT-encoding genes in the development of the vertebrate craniofacial complex is still rudimentary. Reports have highlighted associations of mutations in ESCRT-encoding genes with craniofacial malformations; specifically, mutations of *Chmp4B* and *Chmp5* in mouse models and *VPS4A* in humans[56–58]. However, no mechanisms or perturbed pathways underlying these mutations have been identified so far.

Previous work aimed at uncovering the functions of ESCRT components in mammals has been challenging due to early lethality of mice with constitutive LOF of ESCRT-encoding genes[39–41,54,55,57,59–61]. Using ENU-induced mutagenesis, we isolated a mouse line carrying a hypomorphic mutant allele for the ESCRT-II encoding gene *Vps25,* named *Vps25*^{ENU} [41]. A G-to-A transition in *Vps25* intron 3 generated an mRNA splice variant containing an in-frame 27-nucleotide insertion encoding nine additional neutral amino acids. Heterozygous animals are undistinguishable from wild-type littermates and survive into adulthood without any noticeable phenotype. Homozygous hypomorphic *Vps25*$^{ENU/ENU}$ mice start to exhibit detectable defects at E12.0 and die at E16.5, allowing for the study of ESCRT deficiency during embryonic development. Consistently, *in situ* hybridization shows lower levels of *Vps25* transcript in *Vps25*$^{ENU/ENU}$ embryos (**Figure 8A**). Gross morphology of *Vps25*$^{ENU/ENU}$ embryos reveals striking edema, which, together with the marked expression of *Vps25* we observed in the developing heart (see **Figure 7B-D**), suggests the presence of severe cardiovascular defects[62]. In addition, mutant embryos display multiple fully penetrant abnormalities affecting the limb[41], the craniofacial complex, and the ear. Notably, in the developing head, *Vps25*$^{ENU/ENU}$ mutants exhibit low set and dysmorphic ear pinnae, hypoplastic lower jaw and extremely stunted snout (**Figure 8B**). Although Meckel's cartilage – the CNCC-derived structure that supports the ossification of the lower jaw[63] – is strikingly shorter in mutant embryos compared to wild-type littermates, overall chondrogenesis does not appear to be affected by the mutation (**Figure 8C**). Micro Computed Tomography (μCT) of wild-type and *Vps25*$^{ENU/ENU}$ mutant embryonic heads at E15.5 (**Figure 8E, Videos 3&4**) not only corroborates the defects seen in ear pinnae, lower jaw, and FNP, but also reveals the presence of fully penetrant cleft secondary palate, which was

subsequently confirmed by H&E staining of histological sections (**Figure 8F**). Lastly, superimposition of wild-type and mutant 3D renderings obtained by µCT further confirms the presence of a strikingly hypoplastic lower jaw structure in *Vps25*$^{ENU/ENU}$ embryos (**Figure 8D**). Our results provide additional evidence for the critical roles of the ESCRT machinery, specifically of ESCRT-II, during craniofacial morphogenesis, as shown by malformation of structures that derive from the domains where ESCRT-encoding genes are enriched at early stages of craniofacial development.

Craniofacial defects in ESCRTII-Vps25 mutants are mediated by perturbed NOTCH signaling

Next, we investigated which cellular and molecular pathways are impacted by the hypomorphic mutation of *Vps25*. Since the anatomical structures most affected in mutant embryos are limb[41] and CNCC-derived elements[5], and because we observed enrichment of ESCRT genes and proteins in CNCCs and CNCC-populated domains, we first evaluated migration of CNCCs by WISH of CNCC markers *Sox10* and *Tfap2α*[52]. We found no defects in CNCC migration in *Vps25*$^{ENU/ENU}$ embryos versus controls (**Figure 9A**). We previously reported that *Vps25*$^{ENU/ENU}$ mutant embryos exhibit polydactyly as a result of dysregulation of FGF and SHH signaling pathways in developing limbs[41]. However, while rescue of the polydactylous phenotype could be obtained by reducing SHH levels in *Vps25*$^{ENU/ENU}$ mutants, the craniofacial defects were not ameliorated by said genetic manipulations[41]. Unlike in mutant limb buds, we uncovered that expression of *Fgf8* and *Shh* is not perturbed in the developing head of *Vps25*$^{ENU/ENU}$ embryos, including the FNP and BA1 (**Figure 9B & 9C**), suggesting that other pathways are responsible for the observed mutant craniofacial phenotype.

NOTCH signaling is critical for development of the embryonic head and largely reliant on the endosomal pathway[44–46,64]. NOTCH receptors are activated by cleavage upon binding of surface ligands on adjacent cells. Once cleaved, the intracellular domain (ICD) of the NOTCH receptor translocates into the nucleus to activate transcription of target genes[64,65]. WISH showed overall lower *Notch1* expression in mutant MNP, LNP and maxillary process compared to wild-type embryos (**Figure 9D**). Consistently, mutant BA1 and FNP exhibit significantly lower levels of the active form of NOTCH1 (ICD), but higher levels of the inactive full-length form at E10.5, suggesting defective cleavage of the receptor, leading to decreased activation of signaling (**Figure 9E-H**)**.** Immunofluorescence of the FNP shows lower levels of the NOTCH1 ICD in the nasal epithelium of mutant embryos compared to wild-type littermates, whereas the

extracellular domain (ECD) of the receptor is retained in enlarged LAMP1-positive endosomes, suggesting defective trafficking of the receptor in mutant cells (**Figures 10A &10B**). Accordingly, qPCR analyses of control and mutant FNP corroborated decreased expression of NOTCH receptors (*Notch1-4*), ligands (*Jag1* and *Jag2*), and target genes (*Hes1, Hes5*, but not *Hes6*) (**Figure 10C**). Our results suggest that abnormal trafficking of the NOTCH1 receptor results in downregulation of the pathway in mouse embryos with defective functioning of ESCRT-II (**Figure 10D**). These data provide novel evidence that while perturbation of the *Fgf-Shh* cross regulatory loop by mutant ESCRT-II is responsible for the limb phenotype of $Vps25^{ENU/ENU}$ embryos, disrupted NOTCH signaling mediates at least in part the craniofacial defects observed in mutant embryos.

Altogether, our results demonstrate that ESCRT-encoding genes are not distributed uniformly in the mammalian embryonic body but are enriched in select tissues and structures, such as the developing craniofacial complex. Consistently, mouse embryos harboring a hypomorphic homozygous mutation of the ESCRT-II component *Vps25* exhibit drastic craniofacial defects. In addition, the NOTCH pathway is perturbed in $Vps25^{ENU/ENU}$ embryonic heads, whereas expression of *Fgf8* and *Shh*, which is dysregulated in mutant limb buds, remains unaffected in developing mutant crania. Our results provide new evidence for crucial roles of the ESCRT-II complex in craniofacial morphogenesis and further suggest that ESCRT-dependent regulation of select signaling pathways is executed in a tissue-specific manner during mammalian development.

Discussion:

Despite the critical roles of the ESCRT complex in cell signaling, most of the knowledge on this cellular machine derives from *in vitro* approaches or from studies in invertebrate animals[38,66]. Early gestational lethality in mouse models with loss-of-function (LOF) mutations for ESCRT-encoding genes supports the notion that ESCRT machinery plays essential roles during development[40,41,54,55,57,59–61,67]. However, this early lethality has prevented characterization of organogenesis phenotypes resulting from loss of ESCRT-encoding genes. Using the mouse model, we identified embryonic domains enriched in ESCRT-encoding gene transcripts and protein products. Our results establish that – although most if not all of these genes and proteins are ubiquitously present at early developmental stages – there is greater abundance of ESCRT components in distinct domains of the embryo. These include the developing heart and limb buds, as well as craniofacial domains populated by migrating CNCCs, particularly in the FNP and BAs[5]. Surprisingly, genes encoding ESCRT components are not equally and uniformly expressed in all domains of the mammalian embryo but exhibit organ- and tissue-specific enrichment, strongly suggesting context-dependent functions of ESCRT machine proteins in developing mammals.

By using a mouse line carrying a hypomorphic allele of the ESCRT-II-encoding gene *Vps25* (*Vps25*^{ENU})[41], we established here that the ESCRT-II complex plays critical roles in craniofacial morphogenesis. Unlike other animal models with LOF of ESCRT-encoding genes, *Vps25* hypomorphic embryos survive until late gestation, displaying drastic craniofacial abnormalities, including fully penetrant low-set and dysmorphic ear pinna, hypoplastic lower jaw, stunted snout, and clefting of the secondary palate. The observed phenotypes resemble features of human autosomal dominant congenital syndromes such as DiGeorge (DGS), CHARGE and Treacher-Collins (TCS)[68,69]. Of note, no genetic alterations have been identified in 30% of CHARGE and 10% of DGS patients[31,70,71]. Moreover, some of the features displayed by *Vps25*$^{ENU/ENU}$ embryos overlap with those seen in recessive human disorders known as ciliopathies[72], for many of which the genetic cause is still unknown[73,74]. Together, our findings support preliminary evidence for critical roles of the ESCRT machinery in mammalian craniofacial morphogenesis and specifically establish novel functions of the ESCRT-II complex in head development. In addition, our study highlights ESCRT-II-encoding genes as attractive candidates for the above listed birth defects, which critically alter the craniofacial structures affected in *Vps25*$^{ENU/ENU}$ embryos.

We also demonstrated that signaling pathways are differentially affected in different domains and structures of *Vps25*$^{ENU/ENU}$ mutant embryos, pointing to tissue-specific roles of

ESCRT components during mammalian development. We previously reported that *Vps25*$^{ENU/ENU}$ embryos exhibit polydactyly as a result of dysregulation of FGF and SHH signaling in developing limbs, with concomitantly perturbed *Fgf8* and *Shh* gene expression in mutant limb buds[41]. These findings imply the presence of a potential feedback loop between transcript and protein abundance in the regulation of these signaling pathways in our mouse model system. In contrast, in this study we uncovered that *Fgf8* and *Shh* expression is not perturbed in the developing head of *Vps25*$^{ENU/ENU}$ embryos. These results suggested that other pathways are responsible for the observed mutant craniofacial phenotype of *Vps25* hypomorphic embryos. Interestingly, mouse models harboring mutations in genes encoding NOTCH pathway components exhibit craniofacial, limb, and heart defects that closely resemble features observed in *Vps25*$^{ENU/ENU}$ embryos[43,44]. Furthermore, mutations in human genes encoding proteins of NOTCH signaling pathway components are associated with congenital syndromes, including Adams-Oliver and Alagille, which present with heart, muscle, and skeletal abnormalities similar to those exhibited by *Vps25*$^{ENU/ENU}$ embryos[75], reinforcing the pivotal roles of the NOTCH signaling pathway in mammalian embryogenesis, specifically in skeletal and craniofacial development. Consistently, we uncovered here that deficiency of the ESCRT-II gene *Vps25* results in decreased NOTCH signaling in the embryonic midface and BAs.

The NOTCH signaling pathway is highly conserved in the animal kingdom; however, the number of NOTCH receptors differs among species, with four total receptors in mammals. NOTCH signaling requires direct cell-to-cell contact and is linked to proliferation and differentiation during embryonic development[65]. Both the NOTCH receptor and its ligands, DELTA and JAGGED, are transmembrane proteins with large extracellular domains. Ligand binding promotes two proteolytic cleavage events in the NOTCH receptor. The first cleavage is catalyzed by ADAM metalloproteases[64], whereas the second is mediated by the γ-secretase enzyme complex. The latter cleavage releases the NOTCH intracellular domain (ICD) that translocates into the nucleus for transcriptional activation of target genes[65]. Given that the NOTCH receptor becomes the nuclear signaling effector upon cleavage, endocytosis has an especially critical impact on this pathway[64]. Indeed, recycling and trafficking of NOTCH ligands and receptors through the endosomal pathway play critical roles in NOTCH signaling[65].

Our results demonstrated abnormal trafficking and decreased processing of the NOTCH1 receptor in *Vps25*$^{ENU/ENU}$ mutant cells at E10.5. In addition, there are decreased transcript levels of downstream targets and of receptors and ligands that participate in the pathway. The latter findings support a feedback loop that has been described in T-ALL cells upon inhibition of NOTCH processing or transcriptional activation[76]. Importantly, while transcript

levels of NOTCH pathway targets *Hes1* and *Hes5* are downregulated in *Vps25*$^{ENU/ENU}$ FNP, *Hes6* transcript levels are not. Of note, unlike *Hes1* and *Hes5*, *Hes6* expression is NOTCH-independent[77]. Abnormal trafficking and processing of NOTCH1 also correlates with downregulation of NOTCH receptor- and ligand-encoding genes at E10.5, which further sustains downregulation of the pathway, as seen by decreased overall NOTCH1 protein levels at E11.5.

Interestingly, in *Drosophila* downregulation of ESCRT-II encoding genes, including *vps25,* results in retention of Notch in endosomal compartments and ectopic Notch signaling due to ligand-independent hyperactivation of Notch[38,78]. Similar results have been reported under LOF of *ept*, the ortholog of ESCRT-I gene *Tsg101*[79]. However, recent work has demonstrated that *vps25* loss does not impact Notch signaling in *Drosophila* wing imaginal disc[80]*.* Additionally, depletion of the ESCRT-0 component *Hrs,* or the *Drosophila* orthologs of mammalian ESCRT-III components *Chmp4* and *Chmp6,* suppresses ectopic Notch signaling in the fly[78,80,81]. The conflicting results related to NOTCH dysregulation from loss of ESCRT-encoding genes may be attributed to differences in the techniques utilized to assess NOTCH activity, mosaicism in the fly, the type of mutation, the organism, the tissue analyzed, and the gene affected by LOF. Notably, abnormal intracellular accumulation of NOTCH1 receptor has been observed in all the abovementioned studies regardless of the mutation, supporting critical roles for the ESCRT machinery in the regulation of NOTCH1 signaling.

A possible explanation for the observed downregulation of NOTCH signaling in mouse *Vps25* hypomorphic embryos may reside in the decreased availability of NOTCH1 in the plasma membrane due to abnormal trafficking of the full-length receptor. Evidence in *Drosophila* and mammalian cells suggest that ligand-dependent activation of NOTCH receptors by γ-secretase takes place in the plasma membrane[82–84]. Moreover, in mammalian cells, endocytosis impairs activation of NOTCH1 in the plasma membrane by γ-secretase[82], suggesting that endosomal internalization downregulates NOTCH signaling by decreasing NOTCH availability in the plasma membrane for cleavage upon ligand binding. In *Vps25*$^{ENU/ENU}$ embryonic FNP, we observed the NOTCH1 receptor in enlarged LAMP1-positive late endosomes, suggesting potential endosomal receptor trapping in said compartments. Diminished availability of the receptor in the plasma membrane may result, in turn, in decreased cleavage upon ligand binding, leading to downregulation of the signaling pathway. In addition, decreased expression of NOTCH ligands in the signaling cell could also negatively impact γ-secretase-dependent cleavage in the plasma membrane of the receiver cell (see **Figure 10E**).

Alternatively, ligand-independent activation of NOTCH may occur in the limiting membrane of lysosomes where γ-secretase displays optimal enzymatic activity in an acidic

environment and cleaves NOTCH to release the ICD[85]. Decreased acidification of endolysosomes by genetic or pharmacological downregulation of V-ATPase leads to the accumulation of NOTCH in enlarged late endosomes and a decrease in NOTCH signaling in *Drosophila*, zebrafish, and mammalian cells[86–88]. Interestingly, we previously reported both a decrease of lysosomal activity and engorged late endosomes that fail to fuse with lysosomes in *Vps25*$^{ENU/ENU}$ mutant embryonic limb tissue[41]. Here, we observed enlarged LAMP1-positive endosomal compartments that colocalize with NOTCH ECDs in FNPs of *Vps25*$^{ENU/ENU}$ embryos, suggesting abnormal trafficking of the full-length receptor. Hence, it is plausible that NOTCH receptors remain sequestered within enlarged endosomes that will not encounter lysosomal γ-secretase for ligand-independent activation in *Vps25* mutant FNP.

In support of ligand-dependent activation, phenotypes caused by LOF mutations of NOTCH ligands overlap with distinct phenotypes observed in *Vps25* hypomorphic embryos. For instance, abnormal palatogenesis has been reported in embryos with LOF mutations in *Jag2*, one of the five cell surface ligands for NOTCH receptors in mice[45]. A missense mutation of *Jag1* that recapitulates a mutation found in human Alagille Syndrome causes eye abnormalities and altered snout proportions, in addition to heart and liver defects[89]. Similarly, CNCC-specific *Jag1* loss leads to midfacial hypoplasia, jaw misalignment, and delayed palatal shelf elongation and fusion[46]. Notably, both *Jag1* and *Jag2* are significantly downregulated in *Vps25* hypomorphic embryos. Lastly, LOF mutations of *Delta1* (*Dll1*) and *Delta3* (*Dll3*) result in muscle and bone abnormalities in the murine embryonic trunk[47,90,91]. Given that NOTCH ligands exhibit differential expression throughout the embryonic body, downregulation of NOTCH signaling in *Vps25* hypomorphic embryos may differentially impact distinct tissues and cellular processes.

In summary, here we have demonstrated that the ESCRT-encoding genes analyzed and *Pdcd6ip* (encoding ALIX) are enriched in CNCCs and murine craniofacial structures populated by CNCCs, pointing to relevant roles of the ESCRT machinery in craniofacial development. Accordingly, we have uncovered that a hypomorphic mutation in the ESCRT-II encoding gene *Vps25* causes striking craniofacial abnormalities that resemble features observed in patients with congenital syndromes such as DGS, CHARGE and TCS. We have also shown that the genes encoding FGF8 and SHH signaling molecules – which are dysregulated in *Vps25*$^{ENU/ENU}$ limb buds[41] – remain unperturbed in the *Vps25*$^{ENU/ENU}$ developing cranium. By aiming to identify aberrant signaling pathways underlying the observed craniofacial phenotype of *Vps25* hypomorphic embryos, we have uncovered that the NOTCH pathway is severely perturbed in *Vps25*$^{ENU/ENU}$ embryos, supporting a conserved ESCRT-dependent regulation of this signaling pathway during development. Additional research will further address whether the mechanisms

underlying NOTCH dysregulation are causative of the craniofacial phenotype exhibited by *Vps25* mutant embryos. This study and future work stemming from it will enhance our understanding of these mechanisms, paving the way towards the early diagnosis, treatment and potential repair of human congenital syndromes that present with craniofacial defects[92].

Materials and Methods

Experimental Animals

The *Vps25*$^{ENU/ENU}$ mutant allele used in this study was previously reported in Handschuh *et al*., 2014[41]. The *Vps25*$^{tm1(KOMP)Vlcg}$ reporter-tagged deletion allele (named *Vps25*^{LacZ} in this study) was obtained from the International Mouse Phenotyping Consortium (IMPC)[93]. Both *Vps25* mutant lines were maintained on a C3H/HeJ background. Swiss Webster wild-type mice were purchased from Charles River Laboratories. UCSF IACUC guidelines and experimental procedures concerning housing, husbandry, and welfare were followed for all experiments conducted on mice.

Whole mount in situ hybridization (WISH)

WISH was conducted using established protocols on wild-type and *Vps25*$^{ENU/ENU}$ embryos harvested at E8.5, E9.5, E10.5 and E11.5[8,94]. Probes for *Fgf8*, *Shh*, and *Tfap2α* are described elsewhere[8,95]. All the other probes for WISH used in this study were synthesized from cDNA derived from E8.5-E16.5 whole C57Bl6J embryo RNA using the oligoprimers listed in **Supplementary Table I**. The produced cDNA was used as a template for PCR amplification of select genes. Each purified PCR product was ligated to the TOPO™ TA Cloning™ Kit for Sequencing (Invitrogen Ref#45-0030) vector, and subsequently used to transform One Shot™ TOP10 Chemically Competent Cells (Invitrogen Ref#C404003). Plasmids where then isolated using NucleoSpin® Plasmid (Takara Ref#740588.250) and sent to Primordium Labs (www.primordiumlabs.com) for sequencing to determine ligated product's orientation. Plasmids were linearized using relevant restriction enzymes and linearized products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Takara Ref#740609.250). Antisense DIG labeled riboprobes were then synthesized by *in vitro* transcription using DIG RNA Labeling Mix 10x (Roche Ref#11277073910), and either the T3 or T7 RNA Polymerase (Roche Sku#11031163001 & Sku#10881767001, respectively). All products were used according to manufacturer guidelines. After WISH, mouse embryos were imaged using a Leica M205 FA with an attached Leica DFC 7000 T camera. All images were processed using Fiji-ImageJ software. At least three embryos for each probe and timepoint were analyzed, unless otherwise stated.

RNAScope in situ hybridization

RNAScope was conducted as previously described[51]. Briefly, wild-type Swiss Webster E10.5 embryos were collected and fixed with 4% paraformaldehyde in 1X PBS O/N at 4 °C. Embryos were then immersed in 30% sucrose and embedded in Epredia™ Neg-50™ Frozen Section Medium. Frozen blocks were cut to 12-µm-thick cryosections and stored at −80 °C. Slides were thawed, washed with 1X PBS, and assayed using an RNAscope™ Multiplex Fluorescent Reagent Kit V2 following the manufacturer's instructions with some modifications. By skipping target retrieval steps and treating slides with Protease Plus for no longer than 15 minutes, damage to fragile embryonic tissues was avoided. Probe mixes were hybridized for 2 h at 40 °C in a HybEZ™ II Oven (Advanced Cell Diagnostics, Newark, CA). The following probes were used: *Hgs* (ACD # 1215751-C2, ESCRT-I), *Chmp1a* (ACD # 456301, ESCRT-III), *Chmp4b* (ACD # 418331, ESCRT-III). The appropriate HRP channels were developed with TSA™ Cy5 Plus (PerkinElmer) dye. Sections were then assays for SOX10 immunofluorescence as described below. Following DAPI staining and mounting with ProLong™ Gold (Invitrogen), sections were imaged using the ZEISS AXIO Observer.Z1 Inverted Fluorescence Motorized Microscope Image processing was conducted in ZEISS ZEN lite software.

Whole mount β-galactosidase staining (LacZ activity)

LacZ activity was detected following standard procedures[96] with minor modifications. Briefly, whole litters comprised of *Vps25*$^{+/+}$ and *Vps25*$^{LacZ/+}$ embryos were collected at E8.5-E10.5 and fixed in 4% paraformaldehyde in 1X PBS at room temperature for 20 to 60 minutes depending on the developmental stage. Embryos were rinsed four times during 30 minutes at room temperature in rinse solution (0.1 M Phosphate Buffer pH 7.3; 2 mM $MgCl_2$, 0.01% w/v Sodium Deoxycholate, 0.008% NP-40). Next, embryos were incubated overnight in staining solution (rinse solution supplemented with Potassium Ferricyanide and Potassium Ferrocyanide at a final concentration of 5 mM each, plus 1 mg/mL of X-Gal chromogenic substrate diluted in dimethylformamide). After staining, embryos were washed in 1X PBS. For section imaging, stained embryos were dehydrated in in serial alcohols (70%, 80%, 90%, 2 x 96%, and 2 x 100% ethanol followed by 1 x Isopropanol; 20 minutes each) and cleared twice for 30 minutes with Histo-Clear II (National Diagnostics) for paraffin wax embedding, as previously described[94]. 10 µm thickness transverse sections were obtained with a Leica Biosystems RM2245 Semi-Automated Rotary Microtome. Sections were de-waxed, rehydrated, and stained with Nuclear Fast Red (Ricca Chemical) for two minutes. After staining, sections were dehydrated and

mounted with Omnimount (National Diagnostics). Images were obtained with the CX-43 microscope and the CellSens standard software from Olympus (Evident).

Immunofluorescence

Wild-type and *Vps25*$^{ENU/ENU}$ mouse embryos were harvested at E9.5 and E10.5, fixed, embedded, and sectioned as described above for RNAScope. Immunofluorescence was conducted using the Mouse on Mouse immunodetection kit (Vector Laboratories, # BMK-2202) following the manufacturer's recommendations and adding DyLight488-conjugated Streptavidin (Rockland #S000-4) for the final step of detection. Primary antibodies used for this work are listed in **Supplementary Table II**. Antibodies against LAMP1 (1D4B-s, developed by Dr. J.T. August at Johns Hopkins School of Medicine, Pharmacology & Molecular Sciences[97]), NOTCH1 ECD (C458.2H-s, developed by Dr. S. Artavanis-Tsakonas, Harvard Medical School[98]), and EpCAM (deposited by Dr. A. G. Farr, University of Washington[99]) were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Anti-Rat Alexa Fluor 555 and Anti-Rabbit Alexa Fluor 647 (Invitrogen #A21434 and #31573) were used as secondary antibodies. DNA was stained with DAPI (4',6-diamidino-2-phenylindole). Slides were imaged with ZEISS AXIO Observer.Z1 Inverted Fluorescence Motorized Microscope or with Leica STELLARIS 5 laser scanning confocal microscope. Image processing was conducted in ZEISS ZEN lite and LAS X microscope software, respectively.

mRNA isolation and qRT-PCR

RNA was isolated from micro-dissected FNP, BA1, heart and forelimbs of E10.5 and E11.5 wild-type and *Vps25*$^{ENU/ENU}$ mouse embryos, using the RNeasy Plus Micro kit (Qiagen Ref#74034). Synthesis of cDNA was performed using the SuperScript® III kit (Invitrogen Ref#11752-050). qRT-PCR was conducted using PowerUp™ SYBR™ Master Mix (Applied Biosystems Ref#A25742) and relevant primers listed in **Supplementary Table III**, using a QuantStudio 6 Flex machine (Applied Biosystems). Comparative analysis was performed using Microsoft® Excel® and visualized using GraphPrism. Experiments were performed in triplicate with n=2 of each domain per replicate.

Western Blot

Western blot analyses were conducted on heart, forelimbs, FNP, and/or BA1 of E10.5 and E11.5 wild-type and *Vps25*$^{ENU/ENU}$ mouse embryos. Samples were lysed with Triton buffer (50 mM Tris-HCl pH 7.5; 1% v/v Triton X-100; 150 mM NaCl; 1 mM EDTA; 0.2% w/v Sodium Deoxycholate; 2.5 mM $MgCl_2$; 10% v/v Glycerol) supplemented with protease and phosphatase inhibitor cocktails (Millipore Sigma # P8340, #P5726, # P0044). 50 µg of total protein were separated on a 4-12% precast gel (Invitrogen Ref# NW04122BOX). and transferred to a PVDF membrane (Immobilon-P Cat.#I PVH00010). Membranes were blocked using 5% Milk (BioRad Cat.#1706404) for 1 hour at room temperature (RT). Membranes were then incubated with primary antibodies, described in **Supplementary Table II**, overnight at 4°C and incubated with HRP-conjugated secondary antibodies for 1 hour at RT. Image J was used for quantification of immunoblots and GraphPrism was used to generate graphical representation.

Cartilage staining

Whole mount cartilage staining was performed on E14.5 wild-type and *Vps25*$^{ENU/ENU}$ mouse embryos using alcian blue as previously reported[100].

Histological analysis

Embryos were harvested and fixed overnight in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS). Embryonic heads were dehydrated in serial alcohols and paraffin-embedded as described above for β-galactosidase staining[94]. 10 µm sections were de-waxed, rehydrated, and stained using the H&E Staining Kit from Abcam (ab245880), following the manufacturer's recommendations. Sections were dehydrated in alcohol series for mounting with Omnimount mounting media (National Diagnostics #HS-110). Images were taken with the CX-43 microscope and the CellSens standard software from Olympus (Evident).

Micro-CT

E15.5 wild-type and Vps25$^{ENU/ENU}$ mutant embryos were fixed overnight in 4% paraformaldehyde (w/v) in PBS. The next day, embryos were washed in water twice for 15 minutes and dehydrated in an alcohol series until 70% Ethanol. Embryos were stained for seven days in a solution of 1% (w/v) phosphotungstic acid (PTA) in 70% Ethanol in rotation at 4° C, rinsed twice

in 70% Ethanol and embedded in 0.5% low melting point agarose (NuSieve™ GTG™ Agarose #50081 Lonza) in PBS as reported[101]. Micro-computed tomography (μCT) was conducted at the UCSF Biomaterials and Bioengineering Correlative Microscopy Core. MicroCT images were acquired using the Micro-XCT 200 from Zeiss Xradia with an anode voltage of 70 kV and a power level of 7.44 W. The data were collected with 2X magnification at a voxel resolution of 10.4 μm.

3D model reconstructions

Model reconstructions to visualize the expression patterns of various ESCRT-encoding genes assessed by *in situ* hybridization were rendered using mouse embryonic head images available through FaceBase[102]. The models were produced using 3D Slicer[103,104]. Images from wild-type and *Vps25*$^{ENU/ENU}$ specimens were reconstructed using 3D Slicer[103,104]. The specimen images were registered and aligned in 3D Slicer / SlicerMorph / ALPACA to create the point cloud rendering[103–105]. Gray scale models were created for both wild-type and *Vps25*$^{ENU/ENU}$ specimens, landmarks were placed on the wild-type specimen, and ALPACA was run to generate the current images[105].

Bulk RNA-seq

Embryonic heads from E11.5 wild-type mouse embryos were dissected individually and subjected to fluorescence activated cell sorting (FACS) to separate the epithelium from the cranial neural crest-derived mesenchyme of the midface. After tissue enzymatic dissociation, cell suspensions were filtered through a cell strainer for FACS analysis. DAPI staining allowed discarding non-viable cells and EPCAM[99] staining enabled the positive selection of epithelial cells. Of the live sorted cells obtained from dissected E11.5 midfaces, approximately 10% are epithelial. Each biological replicate consisted of midface epithelium or mesenchyme from one individual embryo, with a total of 2 biological replicates. RNA was extracted from the FACS-sorted cells using the RNeasy Plus Micro kit (Qiagen, #74034) and RNA quantification performed using the Qubit RNA HS Assay Kit (Invitrogen, #Q32852). Quality control of input RNA was performed using the RNA 6000 Pico kit (Agilent, #5067–1513) on a 2100 Bioanalyzer (Agilent) or the Fragment Analyzer (Advanced Analytical) High Sensitivity RNA kit. All RNA samples for library preparation had RIN>9. RNA sequencing libraries were prepared from 50 ng of input RNA using the non-directional kit NEBNext Ultra™ II RNA Library Prep Kit for Illumina

(NEB, #E7775) with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, #E7490) to capture polyA RNAs. Library size and quality was checked using an Agilent 2100 Bioanalyzer with the High Sensitivity DNA kit (Agilent, #5067–4626) or the Fragment Analyzer CRISPR discovery kit. Concentration of the libraries was determined with the Qubit dsDNA HS Assay kit (Invitrogen, #Q32854). Libraries were sequenced in an Illumina HiSeq 4000 to generate 50 base pair single-end reads. Bulk RNAseq datasets were deposited and are available at FaceBase: https://doi.org/10.25550/S-33G0.

Statistical Analyses

All statistical analyses were conducted using GraphPrism 9.5.1. Unpaired t-Test was used to compare the means between two groups, whereas Two-way Analysis of Variance (ANOVA) with Tukey post-test was applied to compare three or more groups. At least three biological replicates were analyzed per genotype per stage in every experiment.

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Figure Legends:

Figure 1: Ubiquitously expressed ESCRT-encoding genes are enriched in select embryonic domains during murine early development. A) Whole-mount *in situ* hybridization (WISH) shows that at E8.25-8.75 representative ESCRT-encoding genes are expressed in the neural folds (pink arrow) and frontonasal process (FNP, teal arrow). Scale bar=500 µm. **B)** By E9.5, representative ESCRT-encoding genes are enriched in craniofacial domains populated by cranial neural crest cells (CNCCs) in branchial arch 1 (BA1, pink arrow) and FNP (teal arrow). Scale bar=500 µm.

Figure 2: Representative ESCRT-encoding genes are enriched in the murine midface and limb buds at E10.5. **A)** WISH shows that at E10.5 the ESCRT-encoding genes under analysis are enriched in the forelimb (yellow arrow) and hindlimb (orange arrow) bud. Scale bar=500 µm. **B)** Top left panel: schematic representation of the expression patterns of the same ESCRT-encoding genes in the face at E10.5. All other panels: at E10.5 enrichment of these ESCRT-encoding genes is visible by WISH in the lateral nasal process (LNP, teal arrow); medial nasal process (MNP, black arrowhead); maxillary process (MxP, light pink arrow) of branchial arch 1 (BA1); mandibular process (MdP, dark pink arrow) of BA1; and branchial arch 2 (BA2, blue arrow). Scale bar=500 µm.

Figure 3: Enrichment of representative ESCRT-encoding genes in midface and limb buds is maintained at E11.5. A) WISH shows that at E11.5 expression of the ESCRT-encoding genes under analysis persists in discrete domains of the forelimb (yellow arrow) and hindlimb (orange arrow) buds. Scale bar=500 µm. **B)** Top left panel: schematic representation of the expression patterns of the same ESCRT-encoding genes in the face at E11.5. All other panels: at E11.5, enrichment of these ESCRT-encoding genes is visible by WISH in the lateral nasal process (LNP, teal arrow); medial nasal process (MNP, black arrowhead); maxillary process (MxP, light pink arrow) of branchial arch 1 (BA1); mandibular process (MdP, dark pink arrow) of BA1); and branchial arch 2 (BA2, blue arrow). Scale bar=500 µm.

Figure 4: Transcripts for representative ESCRT-encoding genes are enriched in cranial neural crest cells (CNCCs) at E10.5. A) Schematic representation of transverse sections

across the trigeminal stream (Tg) of migratory CNCCs at E10.5 used for RNAScope *in situ* hybridization and β-Galactosidase staining shown in **B-D&F**. OpC: optic cup. **B-D)** RNAScope shows enrichment of representative ESCRT-encoding genes (red) in migratory CNCC of the trigeminal stream (Tg), as observed by colocalization with the CNCC marker SOX10 (green): **B)** *Hgs* (ESCRT-0), **C)** *Chmp1a* (ESCRT-III), **D)** *Chmp4b* (ESCRT-III). NE: neuroepithelium. Scale bar=100 µm. **E)** β-Galactosidase staining of $Vps25^{LacZ/+}$ embryos carrying a copy of a reporter allele, harvested at the indicated developmental stages. Higher *Vps25* reporter signal is observed in the first branchial arch (BA1, black arrowhead) and frontonasal process (FNP, magenta arrowhead). Scale bar=200 µm for E8.5 and E9.5; Scale bar=500 µm for E10.5**. F)** Transverse section of β-Galactosidase stained E10.5 $Vps25^{LacZ/+}$ embryo reveals *LacZ* activity in trigeminal CNCC tissue (Tg, dashed line) and surface cephalic epithelium (black arrows). Scale bar=100 µm.

Figure 5: Representative ESCRT proteins are enriched in cranial neural crest cells (CNCCs) and surface cephalic epithelium (SCE) at E9.5. A) Schematic representation of transverse sections across the trigeminal stream (Tg) of migratory CNCCs at E9.5. CNCC migratory streams are depicted in green. Hy: Hyoid stream; BA1: first branchial arch; BA2: second branchial arch; OV: otic vesicle; OpV: optic vesicle. **B-J)** Immunofluorescence of ESCRT proteins (red) representative of ESCRT-0 (**B**), ESCRT-I (**C**), ESCRT-II (**D**), ESCRT-III (**E-H**), VPS4 complex (**I**), and the ESCRT-associated proteins protein ALIX (**J**). ESCRT proteins are not only enriched in migrating CNCCs (labeled with SOX10, green) but also in the SCE (white arrowheads). DNA is stained with DAPI (blue). NE: neuroepithelium; Tg: trigeminal stream. Scale bar=100 µm.

Figure 6: Representative ESCRT proteins are enriched in cranial neural crest cells (CNCCs) and surface cephalic epithelium (SCE) at E10.5. A) Schematic representation of transverse sections across the trigeminal stream (Tg) of migratory CNCCs at E10.5. OpC: optic cup. **B-L)** Immunofluorescence of ESCRT proteins (red) representative of ESCRT-0 (**B**), ESCRT-I (**C**), ESCRT-II (**D, E**), ESCRT-III (**F-J**), VPS4 complex (**K**), and the ESCRT-associated proteins protein ALIX (**L**). ESCRT proteins are not only enriched in migrating CNCCs (labeled with SOX10, green) but also in the SCE (white arrowheads). DNA is stained with DAPI (blue). NE: neuroepithelium; Tg: trigeminal stream. Scale bar=100 µm.

Figure 7: ESCRT gene transcripts and encoded proteins are present in both epithelium and mesenchyme of the mouse embryonic midface as well as in developing heart and limb buds. A) Bulk RNA-seq from E11.5 midface epithelium and CNCC-derived mesenchyme shows that ESCRT-encoding genes are expressed in both tissues. Multiple unpaired t-test. p value ≤ 0.05 = *. Error bars=SD. **B, C)** qRT-PCR from E10.5 **(B)** and E11.5 **(C)** embryonic frontonasal process (FNP), branchial arch 1 (BA1), forelimb and heart reveals that representative ESCRT-encoding genes are expressed at higher levels in the developing heart, while exhibiting similar expression levels in FNP, BA1 and forelimb. Error bars=SD. **D)** Western blot analysis shows that representative ESCRT-encoded protein products are present in the developing face, as well as heart and limbs. More than one isoform is present for some ESCRT proteins.

Figure 8: Hypomorphic mutant mouse embryos for the ESCRT-II-encoding gene *Vps25* exhibit severe craniofacial defects. A) WISH of *Vps25* in E10.5 *Vps25* wild-type ($Vps25^{+/+}$) and *Vps25* homozygous hypomorphic mouse mutants ($Vps25^{ENU/ENU}$) shows lower levels of *Vps25* transcript in mutant embryos, particularly in frontonasal process (FNP, black arrowhead) and branchial arch 1 (BA1, magenta arrowhead). **B)** Gross morphology of E15.5 $Vps25^{ENU/ENU}$ embryos reveals edema (blue asterisk), hypoplastic lower jaw (magenta arrowhead), stunted snout (white arrowhead), and polydactyly (white star) in comparison to control littermates. **C)** Alcian blue staining of E14.5 embryos shows normal overall chondrogenesis but confirms decreased size of Meckel's cartilage (magenta arrowhead) of mutant embryos *versus* wild-type. Scale bar=500 µm. **D)** Superimposition of 3D renderings from µCT scans of wild-type (blue) and mutant $Vps25^{ENU/ENU}$ (yellow) heads at E15.5 highlights the absence of lower jaw structures (empty blue arrowhead). **E)** Lateral view (upper panel), sagittal sections (middle panel), and coronal sections (lower panel) of µCT scans from E15.5 wild-type and mutant embryos reveal multiple craniofacial morphological abnormalities in $Vps25^{ENU/ENU}$ mutants, including low-set dysmorphic ear pinna (black star), hypoplastic lower jaw (magenta arrowhead), and cleft of the secondary palate (black arrows). Ey: eye; T: tongue. **F)** H&E staining of coronal histological sections through the secondary palate confirms fully penetrant clefting in E15.5 $Vps25^{ENU/ENU}$ embryos. Np: nasopharynx; PS: palatal shelves; MC: Meckel's cartilage. Scale bar=500 µm.

Figure 9: The NOTCH1 receptor is downregulated in *Vps25* homozygous hypomorphic embryos. **A)** WISH of migratory CNCC markers *Sox10* (upper panel) and *Tfap2α* (lower panel) shows that migration of CNCCs is not affected by the *Vps25* hypomorphic mutation. Gene

transcripts for *Shh* and *Fgf8* remain unaffected in the developing head of *Vps25*$^{ENU/ENU}$ mutants from E9.5 to E10.5 (*Shh*; **B**) and from E9.5 to E11.5 (*Fgf8*; **C**). **D)** As shown by WISH, *Notch1* transcript is decreased and its distribution perturbed in E11.5 mutant nasal pits and branchial arch 1 (BA1, black arrowheads) compared to controls. Scale bar=200 μm for E9.5 and E10.5; Scale bar=500 μm for E11.5. MNP: medial nasal process; LNP: lateral nasal process; MxP: maxillary process of BA1; MdP: mandibular process of BA1. **E)** Western blot analyses confirm decreased levels of the active form of NOTCH1 (cleaved) in mutant BAs versus controls at E10.5 and E11.5, whereas levels of full-length NOTCH1 are increased at E10.5. **F)** Cleaved NOTCH levels are decreased in frontonasal process (FNP) of *Vps25*$^{ENU/ENU}$ embryos compared to control littermates at E10.5 and E11.5, whereas levels of full length NOTCH1 are increased at E10.5. **G)** Quantification of NOTCH receptor levels shown in **E. H)** Quantification of NOTCH receptor levels shown in **F.** FL: full length; Cl: cleaved. Two-way ANOVA with Tukey's post test. p value ≤ 0.05 = *; p value ≤ 0.01 = **; p value ≤ 0.001 = ***. Error bars=SD.

Figure 10: Aberrant ESCRT-II function results in downregulation of the NOTCH signaling pathway. A) At E10.5, epithelium of *Vps25*$^{ENU/ENU}$ nasal prominences exhibit lower levels of the active form of NOTCH1 (NOTCH ICD, green) compared to wild-type tissue as shown by immunofluorescence. EpCAM (magenta) is used as an epithelial marker. Scale bar=100 μm. **B)** Immunofluorescence using antibodies against the extracellular domain of NOTCH1 (ECD, green) shows that the protein is similarly present in wild-type and mutant tissue. The late endosomal marker LAMP1 (magenta) colocalizes with NOTCH1 ECD (green) in large LAMP1-positive intracellular structures (white arrowheads) in *Vps25*$^{ENU/ENU}$ nasal epithelium. Scale bar=100 μm. LNP: lateral nasal process; MNP: medial lateral process. **C)** qPCR analyses of NOTCH receptors, ligands, and target genes on FNP tissues isolated from E10.5 wild-type and *Vps25* mutant embryos demonstrate that the NOTCH signaling pathway is severely downregulated in mutant tissue. Multiple unpaired t-test. p value > 0.05 = ns; p value ≤ 0.05 = *; p value ≤ 0.01 = **; p value ≤ 0.001 = ***; p value ≤ 0.0001 = ****. Error bars=SD. **E)** Cartoon representation of suggested model for NOTCH1 signaling and intracellular trafficking in wild-type versus *Vps25*$^{ENU/ENU}$ cells. In the wild-type signal-receiving cell (left black rounded box), the NOTCH intracellular domain (red rectangle; ICD) is cleaved from the extracellular domain (blue rectangle; ECD) by γ-secretase when the NOTCH ligand (DLL/JAG; orange rectangle) from the signal-sending cell (star-shaped, left panel) interacts with the ECD on the plasma membrane. The cleaved ICD is then translocated into the nucleus, where it initiates transcription of NOTCH1 target genes (green arrow). Concurrently, full-length NOTCH1 receptors are

endocytosed in early endosomes and subsequently recycled back to the plasma membrane. In parallel, as the endosomes mature, full-length NOTCH1 receptors are internalized in an ESCRT-dependent manner into intraluminal vesicles of LAMP1-positive late endosomes for further degradation down the lysosomal pathway. Conversely, in *Vps25*$^{ENU/ENU}$ mutant cells (right panel), there are decreased levels of NOTCH1 ligand (DLL/JAG) and defective cleavage of the full-length NOTCH1 receptors by γ-secretase (black X). This results in the decreased presence of ICD molecules inside the cell nucleus and consequent lack of transcription of NOTCH1 target genes (red circle with backslash). Moreover, there is decreased recycling of full-length NOTCH1 receptors from early endosomes back to the plasma membrane. In parallel, due to abnormal functioning of the ESCRT machine (ESCRT*), full-length NOTCH1 receptors are abnormally trafficked and accumulate into engorged LAMP1-positive late endosomes due to failed lysosomal-mediated degradation. ESCRT* denotes ESCRT machinery with *Vps25* hypomorphic mutation.

Suppl. Video 1: 3D rendering of ESCRT-encoding gene expression patterns in E10.5 mouse embryo.

Suppl. Video 2: 3D rendering of ESCRT-encoding gene expression patterns in E11.5 mouse embryo.

Suppl. Video 3: 3D rendering of microCT from wild-type embryonic head at E15.5.

Suppl. Video 4: 3D rendering of microCT from *Vps25*$^{ENU/ENU}$ embryonic head at E15.5.