Traffic jams in fish bones
ER-to-Golgi protein transport during zebrafish development

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Extracellular matrix (ECM) proteins, cell adhesion molecules, cytokines, morphogens and membrane receptors are synthesized in the ER and transported through the Golgi complex to the cell surface and the extracellular space. The first leg in this journey from the ER to Golgi is facilitated by the coat protein II (COPII) vesicular carriers. Genetic defects in genes encoding various COPII components cause a broad spectrum of human diseases, from anemia to skeletal deformities. Here, we summarize our findings in zebrafish and discuss how mutations in COPII elements may cause specific cellular and developmental defects.

COPII vesicle formation is initiated when the small, cytoplasmic GTPase Sar1 undergoes a conformational change upon GTP binding, exposing an amphipathic α-helix that allows Sar1 to associate with the ER membrane.1-3 Sar1 then recruits the Sec23/Sec24 heterodimer to the ER surface, forming a “pre-budding complex.” Sec23 acts as a GTPase-activating protein for Sar1, whereas Sec24 plays a role in protein cargo selection.4,5 These three proteins form the inner coat and are thought to impose the initial ER membrane deformation. Next, the COPII outer coat complex assembles by Sec13 and Sec31 heterotetramers, which form a cage that encompasses the pre-budding vesicle (Fig. 1A).6,7

COPII components are highly conserved throughout the plant and animal kingdoms. The yeast S. cerevisiae has one Sec23 gene and three Sec24 paralogs (Sec24, Lst1 and Iss), while vertebrate genomes contain four Sec24 (A–D) and two Sec23 paralogs (A and B).8,9 Although the yeast Sec23 and Sec24 are essential for survival, private variants in genes of COPII components in humans cause a broad spectrum of diseases with clinical manifestations as diverse as skeletal defects,10 anemia,11 or lipid malabsorption.12 The precise molecular and cellular mechanisms that lead to such outcomes are poorly understood, underscoring the importance of animal models to study these organ- and tissue-specific deficits.11,13

Sec23 and Sec24 Paralogs are Necessary for Skeletal Development

Unbiased forward genetic screens in zebrafish have been particularly useful to uncover the functions of genes that encode various COPII components. For example, the zebrafish bulldog/sec24d was recovered in a phenotype-driven screen for craniofacial dysmorphology mutations.14,15 The bulldog mutants are characterized by a short, flattened jaw, kinked pectoral fins, and short body length, but overall embryo patterning appears normal.16 This phenotype suggests that Sec24D activity is critical for the development of skeletal elements in the jaw, fin and notochord. The specificity of the phenotype caused by the loss of a universal component in a pervasive cellular pathway is striking but not unique. The zebrafish crusher/sec23a mutant13 manifests complex skeletal dysmorphology that closely resembles the bulldog phenotype. At the cellular level, bulldog and crusher chondrocytes fail to

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export type II collagen and other ECM proteins out of the ER (Fig. 2).

When considering how impairing ER-to-Golgi transport can lead to a tissue-specific phenotype, an important question to address is whether the bulldog and crusher mutants represent hypomorphic or null phenotypes affecting only certain proteins in a subset of cell types. The four alleles of bulldog lead to progressively longer truncations of the Sec24D protein, yet they all result in an identical phenotype, strongly suggesting that all of the bulldog alleles are genetic nulls (Fig. 1B). Similarly, the only identified Sec23A mutant in zebrafish, crusher, also results in a premature stop codon, leading to nonsense-mediated decay of the transcript (Fig. 1B).

Maternal contributions of proteins or transcripts could theoretically account for the largely normal early development of bulldog and crusher mutants, and both Sec23A and Sec24D are maternally deposited. However, activation of zygotic transcription in zebra-fish occurs concurrently with degradation of maternal mRNAs (during the midblastula transition at 3 h post-fertilization) making it unlikely that maternal proteins take part in COPII assembly beyond the initial stages of development. Moreover, if maternal contribution was a major factor, then antisense morpholino knockdown targeted to both maternal and zygotic mRNAs would result in a significantly more severe phenotype than either of the mutants present, but this is not the case. Thus, the most probable scenario is that bulldog and crusher represent genetic nulls. If these are null alleles, then this suggests that the phenotypes of crusher and bulldog reflect the specific functions of Sec23A and Sec24D in skeletal development.

Further support for this notion comes from the clinical analysis of the Cranio-lenticulo-sutural dysplasia (CLSD), a rare genetic disease with a recessive mode of inheritance, that is caused by a point mutation in the SEC23A gene. CLSD patients have skeletal dysmorphologies and cellular defects that are very similar to the crusher zebrafish variant, also suggesting that at least some of the tissue-specific roles of COPII elements have been conserved during evolution. To date, no human syndromes have been identified that disrupt Sec24D; thus, the zebrafish mutations are the primary tools to determine the function of Sec24D in vertebrates.

Although traditionally the COPII-dependent anterograde protein transport was considered an essential pathway with universal functions in all cells, these recent clinical data and animal studies challenge this view. How, then, is the
bulldog embryos have similar numbers of chondrocytes as wild-types, further suggesting that the early stages of chondrogenesis that involve NCC migration, condensation, differentiation and proliferation proceed normally. However, sox9a downregulation, which typifies maturing chondrocytes, does not take place in bulldog mutants, and chondrocytes fail to become hypertrophic. Collectively, these findings indicate that whereas Sec24D-dependent protein transport is dispensable for the early steps of chondrogenesis, it is required for chondrocyte maturation and hypertrophy. Therefore, we postulate that Sec24D function is required at specific time points of cartilage development.

Figure 2. Immunostaining of WGA and Col2a1 in the Meckel’s cartilage of 4 dpf bul and cru embryos. bul and cru chondrocytes accumulate intracellular type-II collagen deposits not seen in wild-type. Methods as in Sarmah et al.¹⁶

Let’s consider the possibility that distinct cargo adaptors, e.g., Sec24D, function at defined developmental stages. The chondrocytes of craniofacial cartilage elements are derived from cranial neural crest cells (NCC) that migrate to form mesenchymal condensations at the pharyngeal arches. These processes require diverse cell-cell and cell-matrix interactions. NCC then differentiate into chondroblasts, proliferate and begin secreting high levels of type-II collagen and other ECM proteins. The end-stage chondrocytes, embedded in dense ECM, subsequently become hypertrophic and eventually die by chondroptosis.¹⁸

In bulldog/sec24d mutants, NCC migration is not disrupted.¹⁶ Proteins involved in the initial steps of mesenchymal condensations of NCC, cell-cell interactions and cell-matrix interactions (i.e., N-cadherin and its partner β-catenin, Fibronectin and Integrin β1) are properly localized to the plasma membrane. Additionally, bulldog embryos have similar numbers of chondrocytes as wild-types, further suggesting that the early stages of chondrogenesis that involve NCC migration, condensation, differentiation and proliferation proceed normally. However, sox9a downregulation, which typifies maturing chondrocytes, does not take place in bulldog mutants, and chondrocytes fail to become hypertrophic. Collectively, these findings indicate that whereas Sec24D-dependent protein transport is dispensable for the early steps of chondrogenesis, it is required for chondrocyte maturation and hypertrophy. Therefore, we postulate that Sec24D function is required at specific time points of cartilage development.

An alternative explanation for the late skeletal-specific defects could relate
to the heavy secretory load in maturing chondrocytes that could overwhelm the available pool of COPII components. It is conceivable that at early developmental stages there is sufficient supply of coat proteins to meet the secretory demand, but as development progresses and the load of transported proteins increases, phenotypes emerge in highly secretory cells such as chondrocytes. We find this possibility unlikely, because collagen secretion is already disrupted at earlier stages in the notochord where the secretory load is significantly lower than in chondrocytes. Furthermore, components of the collagen receptor complex, such as Integrin β1, are transported to the plasma membrane at the stage when ER is already backlogged with ECM proteins. Therefore, it seems improbable that the trafficking defects in bulldog chondrocytes are due solely to cargo overload, although cargo load might contribute to the overall phenotype.

Another possibility is that the nature of transport cargo could demand specialized adaptors for ER exit. For example, large fibrillar collagens (type I or type II) fail to exit the ER in bulldog mutants. It is conceivable that Sec24D is essential specifically for the transport of collagen. This could help explain the characteristics of the bulldog phenotype, because cells in all of the affected tissues, (the jaw, fin and notochord) secrete collagens. Fibrillar collagens are transported somewhat differently than other cargos, because of their large size and inflexible shape.19-21 This possibility is bolstered by the fact that crusher/sec23a also fails to secrete type II collagen, whereas a variety of other proteins are sent to the extracellular space. However, our results also show that failure to transport might not exclusively depend on the size of the trafficked protein. For example, Fibronectin is properly secreted from Sec24D-deficient cells, whereas Matrilin, which is comparable in size to Fibronectin, is not secreted.22,23

Finally, another possibility is that Sec23A and Sec24D have specialized functions in cargo sorting. Although the four Sec24 paralogs are highly divergent from each other, each paralog is conserved among vertebrate species. This finding implies that their functions are also evolutionarily conserved, lending support to the hypothesis that Sec24A, Sec24B, Sec24C and Sec24D each have distinct cargo-binding affinities.31 Alternatively, Sec24D may recruit an accessory protein that is required for type II collagen secretion, similar to Tanger1,25 an adaptor required for type VII collagen secretion. This possibility is further strengthened by the fact that fibrillar collagens type I, II and VII are stalled in the ER under various COPII-deficient conditions.10,13,16,19,25-26 This would argue for Sec24D being required for sorting as opposed to handling large proteins.

## Redundant and Specialized Functions of Cargo Adaptors

Another possible explanation for the tissue and stage-specific defects of bulldog/sec24d could come from redundancy among Sec24 proteins, especially between Sec24D and its closest paralog, Sec24C. Indeed, in HeLa cells, SEC24C and SEC24D recognize similar cargo binding motifs.24 In zebrafish, Sec24C knockdown by antisense morpholino oligonucleotides does not result in any obvious craniofacial defects, even though sec24c morphant embryos are shorter, similarly to the bulldog/sec24d phenotype.16 Nevertheless, although craniofacial primordia migrate in sec24c or sec24d single morphants, NCC migration is disrupted in sec24c/sec24d double morphants, chondrocytes fail to form and NCC-derived craniofacial skeletal elements are completely absent. Thus, it appears that either of the two Sec24 paralogs is sufficient for adequate protein transport during neural crest cell migration and the initial stages of chondrogenesis. In contrast, Sec24D is uniquely essential for chondrocyte maturation.

Of note, the eyes of sec24c morphant embryos are reduced in size, a defect that is not seen in bulldog. Hence, Sec24D cannot compensate for lack of Sec24C during eye development, arguing that there are distinct requirements for the two paralogs in various tissues. Taken together, these results suggest that within the context of a whole organism, Sec24C and Sec24D have both redundant and essential functions.

While the paralog-specific phenotypes seen in Sec24 depletions could be explained by differences in cargo affinity due to primary sequence divergence, the differences in the phenotypes caused by the loss of Sec23A or Sec23B in both humans and zebrafish are less clear. Human mutations in SEC23B result in congenital dyserythropoietic anemia type II (CDAII), an anemia caused by abnormal erythroblast development. In CDAII, erythroblasts have multiple nuclei and membrane abnormalities, possibly caused by impaired glycosylation of membrane proteins. CDAII stands in stark contrast to CLSD, which is caused by a mutation in SEC23A. CLSD has a very similar manifestation to crusher/bulldog mutants. CLSD patients are characterized by skeletal defects, including craniofacial abnormalities and late closing fontanelles, short stature, neuronal deficits and suture cataracts. At the cellular level, CLSD causes enlarged ER in fibroblasts. In contrast, CDAII patients have no apparent skeletal defects, while CLSD patients do not have a reported anemia; therefore these diseases manifest differently both at the clinical and cellular levels.

SEC23A and SEC23B share >95% similarity other than a string of 18 amino acids. The location of the divergent sequence may provide some clues about the specialized functions of SEC23A and SEC23B and the disease manifestations of the corresponding mutations. The 18 amino acids form a flexible loop near the Sec24D binding site, so it is likely that they allow the two SEC23 paralogs to partner preferentially with different SEC24 proteins (Fig. 1C). If this is true, then the diverse SEC23A and SEC23B phenotypes might reflect the cargo sorting affinities of their respective SEC24 partners.

There are also species-specific defects associated with loss of Sec23 proteins. For example, although CDAII patients, who have mutations in SEC23B, do not have skeletal defects, sec23b knockdown in zebrafish results in complete loss of the ventral head skeleton, whereas the dorsal skeleton is less affected. This phenotype is more severe than that of crusher/sec23a, where both the ventral and dorsal skeleton failings are milder and more similar to the dorsal defects of sec23b mutants. The differences in phenotypes may be due to divergent transcriptional regulation and, thus, availability of...
various COPII components. For example, Sec23B is expressed at five-fold higher levels than SEC23A in human erythroblasts. Similarly, we observed sec24c expression upregulation in bulldog mutants, suggesting that transcriptional regulatory loops are in place to offset the loss of individual COPII components. It is possible that such genetic circuits are wired differently among species, resulting in distinct compensatory mechanisms. However, it is also plausible that the differences highlight a null phenotype in the zebrafish and a weak hypomorphic allele of the CDAA1 patients.

Loss of both Sec23A and Sec23B in crus/hr/sec23b morphants leads to a more severe phenotype than either mutation alone. Double mutant-morphant embryos are very short and have little head skeleton remaining, yet they are able to undergo embryonic patterning. Considering that there are only two known paralogs of Sec23 and Sec23 activity should be that there are only two known paralogs remaining, yet they are able to undergo a severe phenotype than either mutation alone. Double mutant-morphant embryos have essential and redundant roles both in development and disease. In this knowledge could eventually help us to treat human diseases caused by defective or excessive secretory activity.

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References

1. Barlowe C, Orci L, Yeung T, Hoosobuchi M, Hamamoto S, Salama N, et al. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 1994; 77:895-907; PMID: 8004676; DOI:10.1016/0092-8674(94)90138-4.

2. Matsuo K, Orci L, Amherdt M, Bednarek SY, Hamamoto S, Scheckman R, et al. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. Cell 1998; 93:263-75; PMID: 9568718; DOI:10.1016/0092-8674(96)80157-9.

3. Huang M, Weisman JT, Béraud-Dufour S, Luan P, Wang C, Chen W, et al. Crystal structure of Sar1-GDP at 1.7 Å resolution and the role of the NH2 terminus in ER export. J Cell Biol 2001; 155:97-48; PMID:11759406; DOI:10.1083/jcb.200106039.

4. Miller E, Antony B, Hamamoto S, Scheckman R. Cargo selection into COPII vesicles is driven by the Sec24p subunit. EMBO J 2002; 21:6103-15; PMID:12426382; DOI: 10.1093/emboj/cdf605.

5. Mancias JD, Goldberg J. Structural basis of cargo membrane protein discrimination by the human COPII coat machinery. EMBO 2008; 27:2918-28; PMID: 18483296; DOI:10.1038/emboj.2008.208.

6. Stagg SM, Gurkan C, Fowler DM, LaPointe P, Foss TR, Potter CS, et al. Structure of the Sec31/31 COPII coat cage. Nature 2006; 439:234-8; PMID: 16407955; DOI:10.1038/nature04339.

7. O’Donnell J, Maddox K, Stagg S. The structure of a COPII tubule. J Struct Biol 2010; In press; PMID: 20828620; DOI:10.1016/j.jsb.2010.09.002.

8. Paccaud JP, Reith W, Carpentier JL, Ravazzola M, Amherdt M, Scheckman R, et al. Cloning and functional characterization of mammalian homologues of the COPII component Sec23. Mol Biol Cell 1996; 7:1535-46; PMID:8893607.

9. Tang BL, Kausalya J, Low DY, Lock ML, Hong W. A family of mammalian proteins homologous to yeast Sec23p. Biochem Biophys Res Commun 1999; 258:679-84; PMID:10329445; DOI:10.1006/bbrc.1999.0574.

10. Boyadjiev SA, Fromme JC, Ben J, Chong SS, Nauta MM, Eramian D, et al. Comparative protein structure modeling using Modeller. Curr Protoc Bioinformatics 2006; 4:184276; PMID:184276; DOI:10.1002/0471259503.ps00294-6.

11. Eswar N, Webb B, Marti-Renom MA, Madhusudan MS, Eramian D, Shen M, et al. Comparative protein structure modeling using Modeller. Curr Protoc Bioinformatics 2006; 5:184276; PMID:184276; DOI:10.1002/0471259503.ps00294-6.