Disruption of the Sec24d Gene Results in Early Embryonic Lethality in the Mouse

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Abstract

Transport of newly synthesized proteins from the endoplasmic reticulum (ER) to the Golgi is mediated by the coat protein complex COPII. The inner coat of COPII is assembled from heterodimers of SEC23 and SEC24. Though mice with mutations in one of the four Sec24 paralogs, Sec24b, exhibit a neural tube closure defect, deficiency in humans or mice has not yet been described for any of the other Sec24 paralogs. We now report characterization of mice with targeted disruption of Sec24d. Early embryonic lethality is observed in mice completely deficient in SEC24D, while a hypomorphic Sec24d allele permits survival to mid-embryogenesis. Mice haploinsufficient for Sec24d exhibit no phenotypic abnormality. A BAC transgene containing Sec24d rescues the embryonic lethality observed in Sec24d-null mice. These results demonstrate an absolute requirement for SEC24D expression in early mammalian development that is not compensated by the other three Sec24 paralogs. The early embryonic lethality resulting from loss of SEC24D in mice contrasts with the previously reported mild skeletal phenotype of SEC24D deficiency in zebrafish and restricted neural tube phenotype of SEC24B deficiency in mice. Taken together, these observations suggest that the multiple Sec24 paralogs have developed distinct functions over the course of vertebrate evolution.

Introduction

Approximately one-third of all vertebrate proteins traverse the intracellular secretory pathway prior to being secreted into the extracellular space or transported to any of a number of intracellular compartments, including the Golgi, endosome, lysosome, or plasma membrane [1,2,3]. Following co-translational translocation into the endoplasmic reticulum (ER) lumen, newly synthesized proteins are folded and undergo initial post-translational modification, followed by exit from the ER at ribosome-free regions called ER exit sites (ERES) [4] via COPII-coated vesicles [1,5,6]. In yeast, the COPII coat is composed of the small GTP-binding protein Sar1p, the heterodimeric Sec23p/Sec24p complex and the heterotetrameric Sec13p/Sec31p complex [7]. Sar1p generates membrane curvature and initiates vesicle formation by inserting an N-terminal amphipathic helix into the ER membrane [8]. The active membrane-bound Sar1p-GTP recruits Sec23p/Sec24p, and Sec24p drives the selective recruitment of cargo proteins into budding vesicles [9,10,11]. Polymerization of the outer Sec13p/Sec31p complex is the final step in vesicle budding [12].

While the components of the COPII coat are highly conserved, and the fundamental interactions appear to be similar from yeast to mammals, most components exhibit multiple paralogs in higher eukaryotes. Studies in yeast suggest that Sec24p is the major cargo binding component of the COPII coat, with three cargo-binding sites in the N-terminal region interacting with either cytoplasmic domains of the cargo itself or cargo adaptors [9]. Deletion of yeast Sec24p is lethal, whereas deletion of either of two non-essential Sec24p paralogs, Lst1p and Iss1p, results in specific cargo-transport defects [13,14,15]. In vertebrates, four Sec24 paralogs (SEC24A-D) have been identified. These Sec24 paralogs fall into two subfamilies, a SEC24A/B subgroup and a SEC24C/D subgroup, based on protein sequence similarity, with the A/B subgroup closer to yeast Sec24p [16]. All four paralogs contain highly conserved C-terminal domains and a more variable N-terminal region, while the SEC24A/B and SEC24C/D subgroups appear to differ in their affinity for a subset of known cargo-sorting signals [17,18].

Mice with mutations in Sec24b exhibit neural tube closure defects as a result of decreased VANGL2 trafficking out of the ER [19], though no human disorders resulting from deficiencies of SEC24B or any of the other Sec24 paralogs have been reported. Mutations in the SEC24 binding partner SEC23, which has two paralogs (Sec23a and Sec23b), have been characterized both in humans and in fish. Missense mutations in human SEC23A lead to cranio-lenticulo-sutural dysplasia (CLSD), characterized by the persistence of wide-open fontanelles into childhood and the development of Y-shaped cataracts [20]. Mutations in human
SEC23B cause congenital dyserythropoietic anemia type II (CDAII), characterized by a specific defect in erythrocyte development [21] while Sec23b deficient mice have a markedly different phenotype, exhibiting pancreatic disruption and disintegration [22]. Disruption of either Sec23a or Sec23b in zebrafish both result in defects in extracellular matrix (ECM) protein secretion, producing a phenotype reminiscent of CLSD in humans [20,23]. Zebrafish lacking SEC24D exhibit similar craniofacial dysmorphology, presumably due to defects in the trafficking of extracellular matrix (ECM) proteins including type II collagen and matrilin [24] and medaka fish with a nonsense mutation in sec24d have also have skeletal defects [25]. We now report the characterization of murine SEC24D deficiency. Mice null for SEC24D exhibit very early embryonic lethality, suggesting an essential role for SEC24D in the transport of critical protein cargos from the ER.

Materials and Methods

Ethics Statement

All animal care and use complied with the Principles of Laboratory and Animal Care established by the National Society for Medical Research. The University of Michigan’s University Committee on Use and Care of Animals (UCUCA) approved all animal protocols in this study under protocol number 08571.

Generation of SEC24D Deficient Mice

ES cell clones RRT226 and RRR785 were obtained from the International Gene Trap Consortium (IGTC, Bay Genomics, San Francisco, CA), and will be referred to as Sec24dgt and Sec24dgt2, respectively. Both ES cell clones were cultured as described [26] and expanded for microinjection and preparation of total RNA and genomic DNA. ES cell-mouse chimeras were prepared by blastocyst microinjection as described [27] and bred with C57BL/6j mice to obtain germ-line transmission. ES cell-derived F1 agouti offspring were genotyped using primers Neo A and Neo B presence (Neo+ or absence (Neo-) of the gene trap allele. Sequences for all primers used in this study are listed in Table 1. Mouse carrying the gene trap allele were maintained by backcrossing to C57BL/6j.

Mapping of the Gene Trap Vector Insertion Sites

The gene trap vector insertion sites in intron 8 of Sec24dgt and intron 20 of Sec24dgt2 were determined by PCR amplification and DNA sequencing. A series of forward primers evenly spaced throughout the intronic sequence (I8F1-21 and I20F1-7, Table 1) were combined with a reverse primer (Vector 19 or Vector 20, Table 1) specific to the 5’ end of the gene trap vector sequence. Amplicons corresponding to a specific product spanning the insertion site were confirmed by DNA sequencing. Insertion site sequences for both gene trap alleles were deposited into GenBank.

Genotyping Mice by PCR and Southern Blot

Mice from Sec24dgt were genotyped using a three-primer competitive PCR assay consisting of a common forward primer, (In8F3) located upstream of the insertion site in intron 8, and two reverse primers, located downstream of the insertion site in intron 8, (In8R4) or within the gene trap vector (V19) (Figure 1A). This reaction produces products of different sizes from the wild-type (762 bp) and gene trap (666 bp) alleles, which are resolved by agarose gel electrophoresis (Figure 1B). Genotypes for four representative Sec24dgt2 mice were also confirmed by Southern blot analysis using a 371 bp probe amplified from C57BL/6j genomic DNA with the primers ApaI A and ApaI B. The probe was hybridized to ApaI-digested genomic DNA, as previously described [28]. Mice from Sec24dgt2 were also genotyped using a three-primer competitive PCR, with a common forward primer (In20F1) located upstream of the insertion site in intron 20, and two reverse primers, located downstream of the insertion site (In20R1) in intron 20 or within the gene trap vector (V20) (Figure 2A). This reaction produces a 715 bp product from the wild-type allele and a 530 bp product from the gene trap allele, which are resolved by agarose gel electrophoresis (Figure 2B).

Timed Mating

Timed matings were performed by intercrossing Sec24dgt heterozygous mice. Embryos were harvested at multiple time points, including day E10.5–11.5 for genotyping and histological analysis and E13.5 for the preparation of mouse embryonic fibroblasts. Genotyping was performed on genomic DNA isolated from embryonic yolk sacs. For blastocyst collection, female Sec24dgt/+ or Sec24dgt+/gt mice were superovulated by intraperitoneal injection of 0.5 IU pregnant mares’ serum gonadotropin (PMSG) on day 1 and 0.5 IU human chorionic gonadotropin (HCG on day 3. Females were then mated with Sec24dgt/+ or Sec24dgt+/gt males, and copulation plugs were noted on day 4, counted as day E0.5 of embryonic development. Blastocysts were harvested on day 7 (E3.5) as previously described [29], and crude lysates were genotyped by three-primer PCR. Morula (5-cell embryos) were collected from juvenile superovulated Sec24dgt/+ female mice mated with Sec24dgt/+ male mice and placed in culture as described [30].

Reverse-transcription PCR

Total RNA was isolated from a panel of frozen tissues from wild-type mice, Sec24dgt/+ mice and wild-type E10.5 embryos 10.5 embryos using the RNasy Mini Kit (Qiagen), as per manufacturer’s instructions, including the optional DNAseI digestion step. cDNA synthesis and PCR were carried out in one reaction using the SuperScript® III One-Step RT-PCR System with Platinum® Taq (Invitrogen). Primers were designed such that amplicons for each gene were approximately the same size. Primer sequences are listed in Table 1.

Quantitative Analysis by Southern Blot

To determine the limit of sensitivity for our PCR assay, serial dilutions of total RNA from wild-type into total RNA from Sec24dgt/+ embryos were used as template for RT-PCR. PCR products 190 bp in length were amplified from the resulting cDNA using Sec24dExon20-21F and Sec24dExon20-21R (Table 1) and analyzed by Southern blotting using a 144 bp 32P-labelled DNA probe generated from wild-type cDNA using primers 24dEx20-21ProbeF and 24dEx20-21ProbeR (Table 1).

Generation of BAC Transgenic Mice

Two bacterial artificial chromosome (BAC) clones containing the entire Sec24d gene, RP23-355K12 (RP23) and RP24-271N12 (RP24) were obtained from the BACpac Resources Center at Children’s Hospital Oakland Research Institute (CHORI, http://bacpac.chori.org/). BAC DNA was purified using a NucleoBond BAC 100 kit (Machery-Nagel), per manufacturer’s instructions. C57BL/6j x SJL F1 female mice were generated by the University of Michigan Transgenic Animal Model Core and crossed to Sec24d heterozygous mice. Zygotes from this cross were injected with BAC DNA and transgenic founders for RP23 and RP24 were
detected by PCR using pBACe3.6F1 and pBACe3.6R1 (Table 1), primers specific for the vector backbone.

Transgenic founders carrying the BAC transgene (Tg+) were generated using Sec24d+/gt females as the egg donors, resulting in both Sec24d+/+ and Sec24d+/gt founders for RP23 or RP24. Sec24d+/gt founders were immediately crossed with mice heterozygous for the Sec24d gene trap allele (Sec24d+/gt) to generate potential Sec24dgt/gt Tg+ rescues. To generate Sec24d+/gt Tg+ for lines with wild-type founders, an additional cross between Sec24d+/+ Tg+ and mice heterozygous for the Sec24d gene trap allele (Sec24d+/gt) was required. The resulting Sec24d+/+ Tg+ mice were crossed with Sec24d+/gt mice to generate potential Sec24d+/+ Tg+ mice. All progeny were subjected to genotyping for Sec24d as well as the presence of the BAC transgene. However, these assays cannot distinguish the endogenous wild-type allele from the copy of Sec24d present on the BAC-transgene. Thus, Sec24d+/+ Tg+ mice were distinguished from Sec24d+/gt Tg+ mice by genotyping for microsatellites differing between the Sec24d''+'' and ''gt'' alleles (see below). All Sec24d+/gt mice used for this study had been backcrossed to C57BL/6J (N8).

### Microsatellite Genotyping

A microsatellite genotyping assay was designed to distinguish the wild-type allele from the gene trap allele originally targeted on the 129S1/SvImJ background. To ensure that the correct genotype assignments were given, four independent microsatellites near Sec24d but outside both BAC transgenes were chosen, two on either side of Sec24d (Figure 3). These microsatellites, three tetra-nucleotide repeats and one tri-nucleotide repeat, were selected for use in the microsatellite genotyping assay using the Tandem Repeat Database [31] because they differed in allele size among the relevant mouse strains to distinguish the endogenous Sec24d

### Table 1. Primer sequences used in this study (denoted 5' to 3').

| Mapping and Genotyping Primers | Primers for BAC Transgene Rescue |
|-------------------------------|----------------------------------|
| In8F1 CCTGCAAGCTACACAAAGC     | pBACe3.6F1 GCTCACGATCCCTAAGGAGC |
| In8F2 CTGGCAAGCTACACAAAGC     | pBACe3.6R1 TTCCGTCTCGTGATGAAA |
| In8F3 CATGCGACGTGAGAACAGA     | MS-1F TGGTAGGACGACAGCTGTGTA |
| In8F4 ACTGCGAGCAATGGGAAGCG    | MS-1R GGTCTAACACGAGAATTTG |
| In8F5 CCCGGTACCTCAGACAAATA   | MS-2F GCACTGGAAAACACCTGTCTC |
| In8F6 GCCGAGCTGTCGATGAGCA    | MS-2R CACATTCGAACTGATGTTT |
| In8F7 ATGGATGCTGAGCTGAAAAT   | MS-3F TGTGGCATTGTCTCGCAG |
| In8F8 CACAGGAGCAACAGTGAAAG  | MS-3R ACGGGGTAGGACAGGAG |
| In8F9 CTGGATCTCAGCAGACTCAA   | MS-4F TGAGTCTCTTGTGAGGAC |
| In8F10 CATGCGGAGACATGAGGAGC | MS-4R GATGGGAGGAGCATTGTG |
| In8F11 GGATGCTTTAAAATGGCAGC  | Southern Blot Probe Primers |
| In8F12 CATGGGAGAAGCAGGCCTAC | Apal A AATCCCTGTTGTAGGTTG |
| In8F13 CACTGGAGATGGAAACTGT   | Apal B CAAAGATCTCCCTCCTG |
| In8F14 GAGGTGCAAGGAACTTGTA  | 24dx20-21ProbeF ACAGTTTGTGAAC |
| In8F15 CTGGCCCTTCTTACCTTCTG  | 24dx20-21ProbeR ACGTCTGAGGACAG |
| In8F16 AAGAAGCAGGACGCACTCAA | RT-PCR Primers |
| In8F17 TTTTCCTCGGCTACCAGAC | GAPDH-F TGTGATGGTGTAGGTTG |
| In8F18 CACCCCCCTCTCTCTCCTC | GAPDH-R ACCAGGTGAGGACAGGAG |
| In8F19 AGGACAGGAAAGCCTAAGTG| Sec24dEx20-21F TGAAGGTGCTGAGTTG |
| In8F20 GCCCGAGCTCTAGCTGCTG | Sec24dEx20-21R ACATGTCGAGGACAG |
| In8F21 GCCAGAGGAAGGAAGAGA | Vector 19 GGGCTCAGAAGACTGCTG |
| In8F22 GCCCTGAGTTGCACTATAAA | Vector 20 GACCTGAGTTGCACTG |
| In8F23 CAGATTTGCTCAGAGAAGA | NeoA CTGGGACAGATGTAGTCAG |
| In8F24 CATCCTGAGTTGCACTATA | NeoB TCTTCCTCAGATCTCCTGAGC |
| In8F25 TGGTAGGACGACAGCTGTGTA |
| In8F26 CACACTAGGCTAGTACAGGCT | NeoC TCTTCTCAGATCTCCTGAGC |
| In8F27 GCCATGGAAAGCAGGCCTAC | NeoD TCTTCTCAGATCTCCTGAGC |
| In8F28 GCCCATGCTCTAGCTGCTG | NeoE TCTTCTCAGATCTCCTGAGC |
| In8F29 GCCCTGAGTTGCACTATAAA | NeoF TCTTCTCAGATCTCCTGAGC |
| In8F30 GCCCTGAGTTGCACTATAA | NeoG TCTTCTCAGATCTCCTGAGC |
| In8F31 GCCCTGAGTTGCACTATAA | NeoH TCTTCTCAGATCTCCTGAGC |
| In8F32 GCCCTGAGTTGCACTATAA | NeoI TCTTCTCAGATCTCCTGAGC |
| In8F33 GCCCTGAGTTGCACTATAA | NeoJ TCTTCTCAGATCTCCTGAGC |
| In8F34 GCCCTGAGTTGCACTATAA | NeoK TCTTCTCAGATCTCCTGAGC |
| In8F35 GCCCTGAGTTGCACTATAA | NeoL TCTTCTCAGATCTCCTGAGC |
| In8F36 GCCCTGAGTTGCACTATAA | NeoM TCTTCTCAGATCTCCTGAGC |
| In8F37 GCCCTGAGTTGCACTATAA | NeoN TCTTCTCAGATCTCCTGAGC |
| In8F38 GCCCTGAGTTGCACTATAA | NeoO TCTTCTCAGATCTCCTGAGC |
| In8F39 GCCCTGAGTTGCACTATAA | NeoP TCTTCTCAGATCTCCTGAGC |
| In8F40 GCCCTGAGTTGCACTATAA | NeoQ TCTTCTCAGATCTCCTGAGC |
| In8F41 GCCCTGAGTTGCACTATAA | NeoR TCTTCTCAGATCTCCTGAGC |
| In8F42 GCCCTGAGTTGCACTATAA | NeoS TCTTCTCAGATCTCCTGAGC |
| In8F43 GCCCTGAGTTGCACTATAA | NeoT TCTTCTCAGATCTCCTGAGC |
| In8F44 GCCCTGAGTTGCACTATAA | NeoU TCTTCTCAGATCTCCTGAGC |
| In8F45 GCCCTGAGTTGCACTATAA | NeoV TCTTCTCAGATCTCCTGAGC |
| In8F46 GCCCTGAGTTGCACTATAA | NeoW TCTTCTCAGATCTCCTGAGC |
| In8F47 GCCCTGAGTTGCACTATAA | NeoX TCTTCTCAGATCTCCTGAGC |
| In8F48 GCCCTGAGTTGCACTATAA | NeoY TCTTCTCAGATCTCCTGAGC |
| In8F49 GCCCTGAGTTGCACTATAA | NeoZ TCTTCTCAGATCTCCTGAGC |

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from the copy of the Sec24d gene in the BAC-transgene. Each microsatellite was evaluated for every potentially transgenic Sec24d<sup>gt/gt</sup> mouse by PCR on genomic DNA using GoTaq<sup>H</sup> Hot Start Green Master Mix (Promega). A forward primer located upstream and a reverse primer located downstream of the microsatellite repeat were used for amplification (see primer sequences, Table 1). Primers were designed using Primer3 such that the amplicon size was approximately 200 bp in length, based on the C57BL/6J reference sequence. PCR was performed as per manufacturer’s instructions, using 29 cycles and scaled up to a 30 μl reaction volume. Amplification annealing temperatures were optimized for each primer set. PCR products were separated by PAGE using 20% polyacrylamide gels and ethidium bromide staining. The gene trap allele is expected to be 129/SvImJ within the congenic interval, in contrast to wild-type alleles, which should be either C57BL/6J, DBA/2J, or SJL/J, based on the breeding strategy. (SJL/J was introduced with some of the original transgenic founders, and DBA/2J with some early matings, though all subsequent backcrosses were to C57BL/6J). Genomic DNA isolated from pure C57BL/6J, DBA/2J, 129S1/SvImJ, and SJL/J mouse strains was also used as templates to determine the amplicon size corresponding to each strain for each microsatellite marker. The genotypes of mice identified as Sec24d<sup>gt/gt Tg</sup><sup>+</sup> were confirmed by progeny testing through crosses with Sec24d<sup>+</sup>/gt mice (<sup>N7 on C57BL6/J</sup>). Data shown in the tables excludes 6 mice in which a recombination event occurred between the upstream and downstream sets of markers. This number is consistent with the predicted recombination frequency of ~1:50 within this 4.2 Mb interval. We cannot exclude the possibility that we missed a double recombination event, though the chance of that occurring within our sample size is unlikely (predicted frequency for double recombinants, ~1:2500).

**Statistical Analysis**

To determine statistical deviation from the expected Mendelian ratios of genotypes from a given cross, the p-value reported is the chi-squared value of observed ratio of genotypes compared to the
expected ratio. Complete blood counts parameters were evaluated for significance using Student’s T-test comparing levels from wild-type mice to levels from Sec24d−/− mice. An initial analysis showed no significant difference between males and females for each genotype, therefore data from males and females were pooled. The wild-type group consisted of 1 male and 2 females, and the Sec24d−/− group consisted of 2 males and 2 females. Alpha levels were adjusted for multiple observations according to the Bonferroni correction.

Results

SEC24D is Required for Early Embryonic Development in the Mouse

Genomic PCR and sequencing identified the Sec24dnull gene trap insertion site at position 3378 of intron 8, numbering from the start of the intron (GenBank accession number KC763189) (Figures 1A,B, 4A–C). This insertion is consistent with the mRNA insertion site at position 3378 of intron 8, numbering from the start (average of 58% and 37.5%, respectively) and within founder lines penetrance was observed, both between the RP23 and RP24 paralogs at E10.5, E14, and E18.5, consistent with data from the EMAGE gene expression database (http://www.emouseatlas.org/emage/) [33]. RT-PCR analysis of Sec24d expression in adult mouse tissues demonstrated broad expression in a wide range of tissues, similar to previous reports of human expression patterns [16] from the RNA Atlas [34]. The latter identifies SEC24D expression in all measured human tissues (including colon, heart, hypothalamus, kidney, liver, lung, ovary, skeletal muscle, spleen testes, and adipose tissue). Taken together, these data indicate that Sec24d is expressed early and broadly across tissues.

Sec24d BAC Transgenes Rescue the Embryonic Lethal Sec24d−/− Phenotype

Two independent BAC transgenes, both spanning the full Sec24d gene (Figure 3), exhibited rescue of the Sec24d−/− embryonic lethal phenotype (Table 4). Sec24d+/− Tg mice appeared healthy, and exhibited normal fertility and lifespan with no apparent abnormalities on gross autopsy. The Sec24d+/− Tg mice were adjusted for multiple observations (Table 5). Electron microscopy of pancreas and liver tissues from Sec24d+/− mice, as well as mouse embryonic fibroblasts derived from Sec24d+/− mice, showed no abnormalities in the cellular organization or ER structure compared to tissues or fibroblasts derived from littermate Sec24d+/+ controls (Figure 5A–D).

Sec24d is Ubiquitously Expressed

Analysis of Sec24a-d mRNA expression by real-time RT-PCR detected expression of all four Sec24 paralogs at E10.5, E14, and E18.5, consistent with data from the EMAGE gene expression database [33]. RT-PCR analysis of Sec24d expression in adult mouse tissues demonstrated broad expression in a wide range of tissues, similar to previous reports of human expression patterns [16] from the RNA Atlas [34]. The latter identifies SEC24D expression in all measured human tissues (including colon, heart, hypothalamus, kidney, liver, lung, ovary, skeletal muscle, spleen testes, and adipose tissue). Taken together, these data indicate that Sec24d is expressed early and broadly across tissues.

No Phenotypic Abnormalities in Sec24d−/+Tg Mice

Sec24d+/− mice are viable and fertile and exhibit no gross or microscopic abnormalities on standard autopsy examination. Complete blood count analysis identified no significant differences between Sec24d+/− and Sec24d+/+ littermates for most parameters measured after correction for multiple observations (Table 3).

A Hypomorphic Sec24d+/− Allele Supports Development to Mid-embryogenesis

A second Sec24d null mouse line (Sec24d−/−) was generated from ES cells derived from an independent gene trap insertion and
**Figure 4. Sec24d
**

**(A) Sequence of the Sec24d
**

Gene trap insertion site in intron 8 of the Sec24d

Gene. The arrowhead indicates

The locations of genotyping primer sequences

are underlined.

**(B) 5' end of the vector sequence inserted into intron 8. Sequence aligning to intron 8 is in bold, while the lowercase sequence represents a 77 bp insertion that is not present in the mouse genome. The position of primer V19 within the 5' vector sequence is indicated.

**(C) 3' end of the vector sequence inserted into Sec24d intron 8.



Table 2. Results of Sec24d

Intercrosses, and backcrosses to Sec24d

mice.

| Sec24d genotype: | +/- | +/-/gt | gt/gt | p-value |
|------------------|-----|--------|-------|---------|
| **Sec24d
**

Intercrosses: 25% | 50% | 25% | <7.1 x 10

17 |
| **N2 to N17** Progeny at weaning (n = 634) | 49.5% (314) | 50.5% (320) | >0.81 |
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Table 3. Complete blood count analysis of Sec24d+/+ and Sec24d+/− mice.

| Genotype            | WBC (× 10^3 cells/μl) | RBC (× 10^6 cells/μl) | HGB (g/dL) | HCT (%) | MCH (pg) | MCHC (g/dL) | RDW (%) | PLT (× 10^3 cells/μl) | MPV (fL) |
|---------------------|-----------------------|-----------------------|------------|----------|----------|-------------|---------|----------------------|----------|
| Sec24d+/+           | 5.50 ± 0.74           | 9.57 ± 0.22           | 15.67 ± 0.88 | 45.0 ± 1.0   | 46.93 ± 0.35 | 34.37 ± 1.68 | 12.07 ± 0.09 | 950.0 ± 120.1 | 6.13 ± 0.52 |
| Sec24d+/−           | 7.25 ± 0.63           | 9.93 ± 0.13           | 16.0 ± 0.0  | 46.25 ± 0.75 | 47.35 ± 0.23 | 34.08 ± 0.13 | 11.75 ± 0.09 | 1035.0 ± 46.3 | 6.50 ± 0.17 |

Whole blood was drawn by retro-orbital puncture and analyzed on an Advia120 whole blood analyzer. All values are ± or – standard error of the mean. *Based on the Bonferroni correction for multiple observations, the level of significance corresponding to p<0.05 for a single observation would be adjusted to p<3.8 × 10^-3.

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Table 4. Rescue of Sec24d+/− mice by BAC transgenes.

| Genotype:               | Expected Ratio with Rescue | Genotype:               | Expected Ratio with Rescue |
|------------------------|-----------------------------|------------------------|-----------------------------|
| Sec24d+/− Tg+          | 14.3% (1/7)                 | Sec24d+/− Tg+          | 85.7% (6/7)                 |
| Total for RP23 BAC     | 8.3% (9)                    | Total for RP24 BAC     | 91.7% (100)                 |
| (n = 109)              |                             | Total for RP23 BAC     |                             |
|                       |                             | Total for RP24 BAC     |                             |
|                       |                             | Total (n = 352)        | 6.25% (22)                  |

Genotypes indicated are of 2 week old progeny resulting from a cross between Sec24d+/− Tg+ mice and Sec24d+/− mice.

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Figure 5. Transmission electron micrograph of Sec24d+/− and Sec24d+/− cells. (A) pancreatic islet cells, (B) pancreatic acinar cells, (C) hepatocytes, and (D) mouse embryonic fibroblasts. Islet cells are identified by the presence of specialized secretory granules and acinar cells by the presence of zymogen granules. Samples were viewed at 10,500–13,500× direct magnification. A scale bar at the top right of each image corresponds to 1 μm. Abbreviations: N = nucleus, ER = endoplasmic reticulum (black arrowheads), SG = secretory granules, ZG = zymogen granules, MT = mitochondria.

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mapped to position 639 of intron 20 in Sec24dgt2 (GenBank accession number KC763190) (Figures 2, 6). The fusion transcript encoded by Sec24dgt2 contains SEC24D exons 1 through 20 (encoding the first 872 amino acids out of 1032) fused to the β-geo selection cassette. Sec24dgt2 mice, like the Sec24dggt2 mice, are healthy and exhibit no apparent abnormalities upon standard autopsy examination. Intercrosses also yielded no Sec24dgt2/gt2 pups at weaning (Table 6), confirming the embryonic lethality of SEC24D deficiency. In contrast to the Sec24dggt2 allele, analysis at both the blastocyst-stage and E10.5–11.5 identified Sec24dgt2/gt2 embryos in the expected Mendelian ratio, though no Sec24dgt2/gt2 embryos were observed beyond E13.5. The Sec24dgt2/gt2 E10.5 embryos appeared grossly and histologically indistinguishable from their wild-type and heterozygous littermates, with visible heartbeats just prior to dissection. RT-PCR of total RNA prepared from Sec24dgt2/gt2 embryos at E10.5 detected a low level of residual normal splicing around the gene trap, though quantitative analysis by PCR and Southern blotting suggests that the level of this residual full-length transcript in Sec24dgt2/gt2 mice is less than 0.1% of the wild-type allele.

**Table 5. Progeny testing of Sec24dgt2/gt Tg+ mice.**

| Genotype: | Sec24d+/+, Sec24d+/−, Sec24d−/− | Tg+ | other |
|----------|---------------------------------|------|-------|
| **Expected Ratio if parent was Sec24d+/+, BAC-Tg−** | 28.6% (2/7) | 71.4% (5/7) | p-value |
| Total for RP23 BAC (n = 47) | 0% | 100% (47) | <1.5 × 10−05 |
| RP23-677 (n = 53) | 0% | 100% (47) | <1.5 × 10−05 |
| Total for RP24 BAC (n = 83) | 0% | 100% (83) | <8.4 × 10−06 |
| RP24-139 (n = 53) | 0% | 100% (53) | <4.2 × 10−06 |
| RP24-157 (n = 30) | 0% | 100% (30) | <5.4 × 10−04 |
| **Total (n = 130)** | 0% | 100% (130) | <5.6 × 10−13 |

Tested mice were crossed with Sec24dgt2/gt Tg− mice, and progeny were genotyped. The presence of any Sec24d+/− mice would indicate that the test parent was heterozygous for the gene trap allele. P-values are calculated for the observed genotypes compared to the expected if the tested transgenic mouse was Sec24d+tg rather than Sec24dgt2. doi:10.1371/journal.pone.0061114.t005

**Discussion**

Our data demonstrate that SEC24D is absolutely required for early embryonic development in the mouse, with complete deficiency resulting in uniform loss prior to the blastocyst stage. Low levels of SEC24D expression (<0.1% of wild-type) are sufficient to support development through mid-embryogenesis, though incompatible with survival to term, whereas SEC24D haploinsufficiency results in no apparent phenotypic abnormalities. The lack of a heterozygous phenotype, together with transgenic rescue of the homozygous null phenotype, excludes a contribution from a dominant negative effect of the truncated SEC24D fusion protein to the embryonic lethality. The transgenic rescue also excludes a passenger gene effect at a nearby locus related to the gene targeting [35]. The transgenic rescue also demonstrates that the critical cis-regulatory sequences required for SEC24D expression are contained within the ~140 Kb genomic segment shared by the 2 BACs used in these experiments.

The reduction in null embryos as early as the 8-cell stage (Table 2) suggests that residual maternal SEC24D is insufficient to maintain normal cellular function beyond the first few cell divisions. These data suggest a role for an essential secretory cargo in the early embryo that is specifically dependent on SEC24D for transport from the ER. Alternatively, SEC24D could be the major or only Sec24 paralog expressed at this early developmental stage. In either case, the low level of normal Sec24d mRNA (<0.1%) resulting from residual splicing around the hypomorphic Sec24dg gene trap allele appears to be sufficient to support development past this stage, though the corresponding level of wild-type SEC24D protein was not directly measured. Also, a higher level of residual splicing in the early embryo, or partial function of the SEC24D β-gal fusion protein cannot be excluded.

The early essential role for SEC24D contrasts with the isolated neural tube developmental defect resulting from SEC24B deficiency in the mouse [19]. These results are surprising, in light of the observation of a higher extent of sequence identity for SEC24A and B to the essential yeast Sec24p protein than the SEC24C and D vertebrate paralogs, which are closer to the nonessential yeast genes Lst1p and Iss1p. The profound dependence of the early mammalian embryo on SEC24D was also unexpected, given the much milder phenotype observed in SEC24D deficient zebrafish. The latter animals exhibit craniofacial dysmorphology, thought to result from a specific defect in collagen secretion from chondrocytes [24], but otherwise develop normally. Variances in the level of maternal mRNA deposition between mice and zebrafish is not a likely explanation for these differences given the lengthy embryonic survival of the zebrafish compared to the mouse [24]. Rather, these observations suggest that the specific functions of the vertebrate SEC24s, mediated either through unique cargo selectivity or tissue-specific expression programs, may have shifted over evolutionary time. Consistent with this notion, the phenotypes of SEC23B deficiency differ markedly between humans, mice and zebrafish [21,22,23,36].

The initial F1 and N2 intercrosses of Sec24d+/− heterozygous mice revealed a puzzling excess of heterozygotes compared to wild-type offspring, significantly exceeding the expected 2:1 ratio (Table 2, p<0.0094). This apparent selective advantage to the Sec24d+/− heterozygote was no longer evident after further backcrossing into C57BL/6j, with genotyping of 634 backcross animals no longer showing an imbalance between the Sec24d+/− and Sec24d−/− genotypes. Intercrosses of the second gene trap allele (Sec24dgeo) also failed to confirm an excess of heterozygous offspring (Table 6). Taken together, these data suggest the presence of an incidental “passenger” gene mutation at a locus tightly linked to the initial Sec24d allele [35], which was eventually lost as a result of backcrossing to C57BL/6j.

The early embryonic lethality observed in SEC24D-deficient mice is consistent with the absence of a previously identified human SEC24D phenotype, though human deficiencies have also not yet been identified for any of the other Sec24 paralogs.
A  **Sec24d intron 20 sequence around the vector insertion site**

\[
\begin{align*}
\text{GCATGCTCATGTGCTCAGCGTGGCGAGTGGAAGGTAGGCGTCTTCACTGGCTTGGTTCAGTGTCGTTT} \\
\text{GCTCTGCTATTACACCTCAGGGTGAAGAGGCGCTTCCATTAGAGCTTAACACAGTGTGCT} \\
\text{CAGAAGACTTCATCGAATTCCATACATTAGAAAGCACCTACTGATTCTCATATCGGCTGGGGGACATCTGTGTTG} \\
\text{CTGTGTTGCTCTTTCTTATAATTGTTGTGACTAGGTACACCAGTGACGTGAGCCTAACAGATGAACCTG} \\
\text{TACTTGAGGCTACATGCAATGCAAGTGCTCTAGGTCTGCTGCCATGTGACACACTCTTCTCTGTGACG} \\
\text{TTATGTGCTCGATTTATATATATCAGAATGACCAGCGCCCAACATATAATTTCGACAGGGGCGGTCTG} \\
\text{TGATAATGCTACATAAA} \\
\end{align*}
\]

\[\text{In20F1} \]

B  **5’ end of pGT0Lxf vector sequence inserted into Sec24d intron 20**

\[
\begin{align*}
\text{TAAGGTCAGCCCCCCCCCCCCATATATAGGAGGGCAGGTCCCTCTCGAGGAGGTGGTCCCAAGGTCTG} \\
\text{GGTAGAAAGTTAGGAGGAGCGACCAGGCACCAGAGCTGACGGGGAGATTGCAAGAGTTGCTG} \\
\end{align*}
\]

\[\text{vector 20 primer} \]

C  **3’ end of pGT0Lxf vector sequence inserted into Sec24d intron 20**

\[
\begin{align*}
\text{GCAGAATGTGTTGAGAGGCGATCGGTGGCGGCTTCTCTGGCATTACCGAAGCTGTGCGAAGGAGGATGTCG} \\
\text{AGCGGATATTAGTTGGGTAAACCAGCGAGGTGGTGAATACGACGGGCGTGGCCAGCGCTCG} \\
\end{align*}
\]

\[\text{CTAG} \]

Figure 6. **Sec24d-gt2 allele sequence.** (A) Sequence of the Sec24d-gt2 gene trap insertion site in intron 20 of the Sec24d gene. The arrowhead indicates the site of the gene trap vector pGT0LxF insertion. Intron 20 sequences flanking the insertion site are in bold. The locations of genotyping primer sequences are underlined. (B) 5’ and (C) 3’ ends of the vector sequence inserted into intron 20. The position of primer V20 within the 5’ vector sequence is indicated, and flanking intron 20 sequences are in bold.

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Table 6. Results of Sec24d-gt2 intercrosses, and backcrosses to Sec24d+/+ mice.

| Sec24d genotype: | +/- | +/-gt2 | gt2/gt2 | p-value |
|------------------|-----|--------|---------|---------|
| Sec24d-/- x Sec24d-gt2 expected: | 25% | 50% | 25% | |
| Pregancy at weaning (n = 88) | 34.1% (30) | 65.9% (58) | 0 | <6.1 x 10^-08 |
| E13.5 to E18.5 (n = 29) | 37.9% (11) | 62.1% (18) | 0 | <1.9 x 10^-03 |
| E10.5 to E11.5 (n = 105) | 24.8% (26) | 56.2% (59) | 19% (20) | <1.6 x 10^-01 |
| Blastocyst (n = 99) | 26.3% (26) | 47.4% (47) | 26.3% (26) | <7.8 x 10^-01 |
| Sec24d-gt2 x Sec24d-gt2 expected: | 50% | 50% | – | |
| Backcrosses at N2 to N12 (n = 367) | 56% (207) | 44% (160) | – | >1.0 x 10^-02 |

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However, the specific phenotypes of human mutations at SEC23A and SEC23B suggest the possibility of unique disorders associated with more subtle SEC24D mutations. Only 2 SEC23A-deficient mice have been identified, each carrying unique missense mutations, likely associated with a partial change/loss of function. Similarly, though many different SEC23B mutations have been identified in patients with CDAII, no patients have yet been identified who are homozygous or compound heterozygous for 2 null mutations, suggesting that complete SEC23B deficiency might also result in early lethality. The diverse phenotypes of humans, mice, and zebrafish with mutations in genes encoding components of the COPII coat suggests a complex balance of function among the multiple paralogous genes in each family. The availability of genetic models for deficiency in COPII component genes should enable future studies of COPII function and cargo selection in vivo.

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Author Contributions
Conceived and designed the experiments: ACB EJA BZ DG. Performed the experiments: ACB EJA. Analyzed the data: ACB EJA BZ DG. Wrote the paper: ACB EJA DG.

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