The *feelgood* mutation in zebrafish dysregulates COPII-dependent secretion of select extracellular matrix proteins in skeletal morphogenesis

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**SUMMARY**

Craniofacial and skeletal dysmorphologies account for the majority of birth defects. A number of the disease phenotypes have been attributed to abnormal synthesis, maintenance and composition of extracellular matrix (ECM), yet the molecular and cellular mechanisms causing these ECM defects remain poorly understood. The zebrafish *feelgood* mutant manifests a severely malformed head skeleton and shortened body length due to defects in the maturation stage of chondrocyte development. In vivo analyses reveal a backlog of type II and type IV collagens in rough endoplasmic reticulum (ER) similar to those found in coat protein II complex (COPII)-deficient cells. The *feelgood* mutation hinders collagen deposition in the ECM, but trafficking of small cargos and other large ECM proteins such as laminin to the extracellular space is unaffected. We demonstrate that the zebrafish *feelgood* mutation causes a single amino acid substitution within the DNA-binding domain of transcription factor Creb3l2. We show that Creb3l2 selectively regulates the expression of genes encoding distinct COPII proteins (*sec23a*, *sec23b* and *sec24d*) but find no evidence for its regulation of *sec24c* expression. Moreover, we did not detect activation of ER stress response genes despite intracellular accumulation of collagen and prominent skeletal defects. Promoter trans-activation assays show that the Creb3l2 *feelgood* variant is a hypomorphic allele that retains approximately 50% of its transcriptional activity. Transgenic rescue experiments of the *feelgood* phenotype restore craniofacial development, illustrating that a precise level of Creb3l2 transcriptional activity is essential for skeletogenesis. Our results indicate that Creb3l2 modulates the availability of COPII machinery in a tissue- and cargo-specific manner. These findings could lead to a better understanding of the etiology of human craniofacial and skeletal birth defects as well as adult-onset diseases that are linked to dysregulated ECM deposition, such as arthritis, fibrosis or osteoporosis.

**INTRODUCTION**

Extracellular matrix (ECM) serves as a structural scaffold and a reservoir for biologically active molecules (Hynes, 2009). Cartilage formation and skeletal morphogenesis depend on timely and abundant deposition of ECM proteins (DeLise et al., 2000). Failure to produce adequate mature ECM or form proper collagen fibers can lead to many developmental defects and diseases, such as osteogenesis imperfecta – which is typically characterized by fragile bones, scoliosis, short stature, hearing loss and teeth defects (Rauch and Glorieux, 2004). In adults, failure to maintain the ECM of the bone can lead to degenerative diseases such as osteoporosis, a debilitating condition characterized by a loss in bone density. Similarly, interstitial fibrosis leading to organ failure after injury, or pathological conditions in aging patients such as arthritis, have been associated with dysregulated protein secretion (Trojanowska et al., 1998; Heinegård and Saxne, 2011; Goldring and Goldring, 2010; Goldring and Goldring, 2007; Löppönen et al., 2004).

ECM proteins depend on the secretory machinery for transport to the extracellular space. The initial step of protein trafficking occurs when proteins leave the site of synthesis in the endoplasmic reticulum (ER) and are transported to the Golgi. This step is primarily conducted by coat protein II complex (COPII) vesicular carriers (Barlowe et al., 1994; Dancourt and Barlowe, 2010; Miller and Barlowe, 2010). The COPII complex is recruited to the ER membrane by the Sar1 GTPase and consists of an inner coat of Sec23-Sec24 heterodimers and an outer coat of Sec13-Sec31 proteins. Vertebrate genomes carry two highly similar paralogs of Sec23 (Sec23A and Sec23B) and four paralogs of Sec24 (Sec24A, Sec24B, Sec24C and Sec24D). Sec23 paralogs act as GTPase-activating proteins for Sar1, whereas Sec24 proteins play a role in cargo sorting (Wendeler et al., 2007).

Mutations in genes encoding COPII components have been implicated in a variety of human disorders, with defects in closely related paralogs causing widely different phenotypes (Bojadiev et al., 2006; Schwarz et al., 2009; Aridor and Hannan, 2002; Routledge et al., 2010). For example, mutations in SEC23B lead to congenital dyserythropoietic anaemia type II (CDAII), a disease characterized by ineffective erythropoiesis, bi- and multinucleated erythroblasts, and hypoglycosylation of red blood cell membrane proteins (Bianchi et al., 2009; Schwarz et al., 2009). By contrast, a point mutation in SEC23A leads to cranio-lenticulo-sutural dysplasia (CLSD), the distinctive marks of which include craniofacial skeleton malformations and short stature (Bojadiev et al., 2006). Zebrafish...
crusher (sec23a) mutant and sec23b morphant embryos present phenotypes that are similar to the corresponding human diseases, establishing zebrafish as a model system to study the molecular and cellular bases of COPII deficiencies (Lang et al., 2006; Schwarz et al., 2009).

Further establishing the paralog-specific defects seen with loss of COPII components, another zebrafish mutant, bulldog (sec24d), was recently described (Sarmah et al., 2010). Loss of Sec24D activity in zebrafish also results in craniofacial defects, whereas Sec24C morphants undergo normal development of head skeletal structures. Loss of bulldog (sec24d) does not prevent neural crest migration, formation of pharyngeal condensations, or proliferation of chondrocytes, but hinders normal maturation of highly secretory chondrocytes. Strikingly, the combined loss of Sec24C and Sec24D results in neural crest migration and condensation deficits, indicating that Sec24D activity is essential for chondrocyte maturation, but Sec24C or Sec24D compensate for each other in early stages of cartilage development (Melville and Knapik, 2011).

Considering the high levels of similarity between COPII paralogs and the basic cellular function that they perform, these findings suggest that COPII-dependent anterograde protein transport is a highly regulated process both during development and under physiological conditions in adulthood, and therefore is likely to be a factor in many more diseases than those already characterized. However, the mechanisms of this regulation are still in the early stages of being understood.

Forward genetic screens in model organisms, such as the one that isolated the crusher and bulldog mutants (Neuhauss et al., 1996), provide an unbiased approach to discover physiologically relevant mutations that affect skeletal development. Here, we have characterized the feelgood (felm662) mutant, which belongs to the same phenotypic series as crusher and bulldog. We found that the feelgood mutation disrupts head skeleton and notochord development through loss of secretory capacity. The feelgood defect specifically disrupts collagen trafficking, thus decoupling its transport from that of other secretory and ECM proteins. We show that the feelgood phenotype is caused by a missense mutation in the DNA-binding domain of the transcription factor cAMP responsive element binding protein 3-like 2 (Creb3l2). We provide the first evidence of paralog-specific regulation of COPII components by showing that loss of Creb3l2 activity decreases the expression of sec23a, sec23b and sec24d, but not of sec24c. Our results suggest that Creb3l2 acts primarily as a transcriptional regulator of specific COPII components, establishing a mechanism that enables cell-type, cargo- and tissue-specific functions of COPII vesicles during development and providing a possible mechanism for the diverse disease manifestations caused by loss of COPII. Our results also suggest that skeletal development is highly sensitive to the level of Creb3l2 activity in vivo and advance Creb3l2 as a candidate cause for skeletal diseases of unknown genetic origin.

RESULTS
The zebrafish feelgood mutation causes craniofacial defects
The primary features of the feelgood phenotype include a reduced lower jaw, shortened body length, and compact head, trunk and tail as measured from the posterior edge of the ear capsule (Fig. 1A,B, white arrow) to the tip of the tail (Fig. 1A-D). Alcian blue staining (Fig. 1E-F’) reveals that all cartilage elements of the head skeleton are present in feelgood mutants, but they are shortened and malformed, including abnormal curvature of the Meckel’s and ceratohyal cartilages, and failure of the Meckel’s cartilage to taper towards the anterior end (Fig. 1F’).

Histological analysis by toluidine blue staining (Fig. 1G,H) shows reduced ECM surrounding the chondrocytes and tightly packed nuclei in feelgood mutants (Fig. 1H, arrow). Furthermore, the overall pattern of chondroblast intercalations and stacking is disrupted. These features are shared with the head skeleton defects observed in the crusher and bulldog mutants (Lang et al., 2006; Sarmah et al., 2010; Melville and Knapik, 2011).

Type II collagen trafficking is disrupted in feelgood mutant chondrocytes
To investigate whether abnormal cartilage shape in feelgood mutants is associated with trafficking deficits of ECM proteins,
as is the case with *crusher* and *bulldog*, we examined the cellular distribution of Collagen2α1 (Col2α1) in chondrocytes at 80 hpf, the earliest stage at which *feelgood* mutants can be clearly identified, and in 5-dpf embryos. In addition, we compared collagen trafficking to bulk transport of secreted glycoproteins by staining N-acetylglucosamine or sialic acid residues with wheat germ agglutinin (WGA).

In 80-hpf wild-type embryos, both Col2α1 and WGA labeling was primarily localized to the extracellular space (Fig. 2A–A′), with small clusters of intracellular staining probably representing the Golgi apparatus (Allen et al., 1989). In *feelgood* mutants, WGA staining also appeared in the extracellular space and the Golgi apparatus (Fig. 2B), suggesting that *feelgood* mutants traffic the bulk of WGA-binding glycoproteins at close to normal levels. By contrast, immunofluorescence staining in *feelgood* mutants revealed deposition of Col2α1 to the extracellular space but also accumulation in cytosolic vesicle-like structures, which were larger and denser than the corresponding intracellular compartments in wild types (Fig. 2A′,B′). By 5 dpf, *feelgood* and wild-type WGA staining patterns were similar (Fig. 2C,D), whereas the intracellular type II collagen accumulation in *feelgood* chondrocytes had increased (Fig. 2C′,D′).

To identify the intracellular localization of protein buildup, we used transmission electron microscopy (TEM). TEM images showed that chondrocytes were stacked in a regular fashion in wild-type embryos (Fig. 2E,I), whereas, in *feelgood*, chondrocytes had a round morphology and were irregularly spaced, suggesting stacking defects (Fig. 2G,K). At this stage, rough ER, identified as ribosome dotted membranes, was severely, but not uniformly, distended in *feelgood* mutants (Fig. 2F,H). By 5 dpf, chondrocytes in wild-types had become hypertrophic, lacking dense rough ER (Fig. 2J). Conversely, chondrocytes in *feelgood* seem to be arrested at earlier stages of morphogenesis.
maturation, containing large vacuoles of distended ER membranes filled with electron dense material (Fig. 2.K.L).

Taken together, these data indicate that the jaw deformity in feelgood mutants is probably a consequence of abnormal collagen secretion and continuous intracellular protein buildup, leading to a progressively more severe phenotype. They also suggest that the maturation of feelgood chondrocytes towards a hypertrophic state is delayed or stalled, subsequent to insufficient matrix deposition, which is known to cause deficits in matrix-mediated intracellular signaling (Hickok et al., 1998).

**Cartilage matrix is progressively lost in feelgood mutants**

Cartilage matrix is continuously turned over during development and tissue homeostasis. Maintenance of cartilage matrix requires synchronization of a number of cellular functions, including coordinated protein secretion, cell-matrix signaling and cartilage degradation. To query how the intracellular defect in collagen transport affects ECM formation, we compared the ultrastructure of cartilage matrix in TEM images of wild-type and feelgood embryos (Fig. 2.M-T). In this analysis, we examined tissue sections from the earliest stage at which we can morphologically distinguish the feelgood phenotype (80 hpf), followed by analyses on days 4, 5 and 6 of development. We found that feelgood mutant matrix at 80 hpf contains collagen fibrils (Fig. 2.Q), suggesting that collagen is successfully synthesized and secreted by chondrocytes at the initial stages of chondrogenesis. However, as collagen fibrils grow progressively denser in wild-type cartilage (Fig. 2.M-P), ECM matrix in feelgood becomes gradually more sparse and devoid of collagen bundles with an almost complete absence of organized fibrils by 6 dpf (Fig. 2.Q-T).

Thus, cartilage collagen is being initially secreted by feelgood chondrocytes, albeit at lower levels than in wild types. However, the typical increase in matrix secretion during cartilage differentiation at the 4 dpf stage is absent, suggesting that feelgood is required for sustained, high-volume traffic of ECM proteins.

**Notochord sheath formation, but not secretion of glycosaminoglycans, is disrupted in feelgood mutants**

feelgood mutant embryos are shorter than wild types, pointing to disrupted notochord development. In zebrafish embryos, the notochord functions as a hydrostatic skeleton, the mechanical properties of which depend on an external fibrous sheath – which consists primarily of collagen and laminin matrix – and numerous internal vacuoles in notochord sheath cells – which enclose secreted glycosaminoglycans (GAGs) with high affinity for water (Scott and Stemple, 2005; Adams et al., 1990). Both of these biomechanical components exert the appropriate notochord stiffness that is essential for embryo lengthening and maintenance of straight posture.

To determine the secretory status of GAG proteins in the notochord sheath, we used WGA staining. The results show that, at 28 hpf, WGA-stained proteins are trafficked through the notochord sheath cells of feelgood embryos to the extracellular space (Fig. 3.A,B,E,F). The major components of the notochord sheath are type II and type IV collagen fibrils that are interlinked with laminins, nidogen and fibulins: the typical components of basement membranes (Timpl and Brown, 1996). We analyzed the secretory status of the type II and type IV collagens by immunofluorescence and found that both are abnormally localized within large vesicle-like structures similar to those seen in the craniofacial cartilage (Fig. 3.C-E,I-L).

To determine whether secretory defects extend to other ECM proteins, we analyzed the localization of laminin in the notochord and somitic boundaries, and we detected no deficits in deposition in feelgood embryos compared with wild types (Fig. 3.M,N, and data not shown). Histological analysis of transverse sections stained with toluidine blue in 80-hpf embryos further corroborated the findings at earlier stages and revealed a less robust notochord sheath in feelgood compared with wild types (Fig. 3.O-R). These data suggest that the transport of GAGs to internal vacuoles, as well as the secretion of WGA-binding glycoproteins and laminin, is not disrupted in feelgood mutants. By contrast, feelgood specifically affects the secretion of type II and type IV collagens, resulting in an overall weaker and smaller fibrous sheath surrounding the notochord, which is consistent with the shorter body length of feelgood embryos.

To further assess the secretory capability of the feelgood embryos, we analyzed total protein extracts from 4-dpf embryos on western blots probed with antibodies against type II collagen. We found an increase in unprocessed procollagen, consistent with a trafficking defect (Fig. 3.S).

Furthermore, immunoblot analysis of protein extracts using N-cadherin antibody found that feelgood N-cadherin was resistant to endoglycosidase H (Endo H; which cleaves mannos-rich structures) but sensitive to PNGase F (which cleaves high-mannose as well as complex-type N-glycans). This indicates that N-cadherin is normally processed in feelgood mutants and progresses along the intracellular secretory pathway, consistent with its plasma membrane localization in feelgood chondroblasts (Fig. 3.T, and data not shown).

**The feelgood mutation affects melanosome maturation**

To test whether the feelgood phenotype affects other cell types that are characterized by high levels of protein trafficking, including those that do not secrete collagen, we analyzed pigment cell maturation. This process involves intensive protein transport during the biogenesis of melanosomes, a set of morphologically and functionally unique organelles that accumulate melanin and translocate within melanocytes in response to pigment modulating stimuli (Marks and Seabra, 2001). Our results show that, although pigment appears normal, the ability of feelgood melanocytes to respond to stimuli is disrupted (Fig. 4.A-D). Specifically, when stress was induced by a low concentration of the melanotoxic 4-hydroxyanisole (Riley et al., 1975), melanosomes in wild-type pigment cells responded by aggregating into small dense structures as previously described (Logan et al., 2006). However, in feelgood embryos, melanosomes failed to aggregate when stimulated with 4-hydroxyanisole and melanosome concentrating hormone (Fig. 4.C,D, and data not shown).

To gain insight on pigment aggregation phenotype, we compared the ultrastructural characteristics of melanosomes between wild-type and feelgood fish (Fig. 4.E-G). TEM images revealed that, in wild types, melanosomes mature to flattened, oval shape structures, whereas, in feelgood mutants, they appear uniform in size and circular in shape (Fig. 4.F,G). This phenotype is similar to the melanosome maturation defects observed in the murine and
zebrafish silver mutants (Theos et al., 2006; Schonthaler et al., 2005). The silver phenotype is caused by failure to transport Pmel17, a structural protein that is required to maintain the oval shape of melanosomes, across the ER membrane (Theos et al., 2006).

Taken together, these results show that feelgood disrupts normal melanosome maturation in melanocytes. Thus, it appears that feelgood has critical functions in distinct cell types and not only in collagen-producing cells.

The feelgood mutation disrupts the creb3l2 locus

The similarities between the feelgood phenotype and the phenotypes of the crusher and bulldog mutants, which abolish the function of two key COPII components, suggested that the feelgood locus encodes a protein participating in COPII-mediated transport. To identify the chromosomal location of the feelgood mutation, we utilized a positional cloning strategy. We genotyped fish from an F2 intercross and used the zebrafish genetic linkage map (Knapik

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**Fig. 3. Collagen trafficking is preferentially disrupted in feelgood mutants, leading to notochord defects.** (A-F) Immunofluorescence of WGA, Col2α1 and merged images of 15-μm sagittal sections of the notochord of 28-hpf embryos. Arrow indicates vesicle-like collagen staining outside the notochord sheath. (G,H) DIC images of the corresponding sections in A-F. (I-N) Immunofluorescence of type-IV and type-II collagen (I-L) and laminin (M,N) in 15-μm sagittal sections of the notochord at 28 hpf. Scale bar: 5 μm. (O-R) Toluidine blue staining of transverse sections through the notochord at the level of the posterior parachordal plate (O,P) and posterior medulla oblongata (Q,R) in 80-hpf embryos. Schematic diagrams mark the plane of the corresponding sections. Arrows indicate the less robust notochord sheath in feelgood compared with wild types. (S) Immunoblot analysis of type II collagen processing. Molecular forms are indicated as: processed, the fully processed form; pN, pN-collagen II; pNpC, unprocessed procollagen II. (T) EndoH sensitivity assay for N-cadherin. Embryo lysates were either untreated (−) or treated with either EndoH (H) or PNGase F (F) before immunoblotting for N-cadherin.
et al., 1998; Bradley et al., 2007) to establish that the *feelgood* mutation is located in a 5.2 cM region on the proximal arm of chromosome 4 (Fig. 5A). We then built a physical map of the critical region between these markers using the Zv7 genomic assembly to obtain contigs, and bridged the gaps between contigs using BAC information from the Zebrafish Genome Fingerprinting Project and sequences from the Ensembl trace repository. We confirmed the accuracy of our physical map by developing simple sequence length polymorphism (SSLP) markers from BAC or contig sequences and counting recombination events in a 2510 meioses F2 map cross. Finally, we restricted the critical interval to a 58 kb region flanked proximally by a marker within an intron of the *creb3l2* gene, and distally by a marker in intron 20 of the *diacylglycerol kinase, iota* (*dgki*) gene. No other known genes are present in the critical region (Fig. 5A).

Sequencing of the coding region of the two candidate genes revealed a single C>G transversion at base pair 1128 of the *creb3l2* gene that results in an N301K missense mutation (Fig. 5B,C). The *Creb3l2* protein contains a basic leucine zipper domain consisting of a basic motif that mediates sequence-specific DNA binding, a leucine zipper motif that facilitates protein dimerization (Vinson et al., 1989; Hope and Struhl, 1987) and a single-pass transmembrane domain preceding a Site-1 protease (S1P) recognition site (Fig. 5B). The *feelgood* mutation is located within the DNA-binding basic motif of *Creb3l2*, in a segment that is conserved from *Caenorhabditis elegans* to human (Fig. 5D). *Creb3l2* belongs to a family of five paralogs that are highly conserved among vertebrate species, including zebrafish. Phylogenetic analysis showed that zebrafish *creb3l2* is the most similar zebrafish paralog to human *CREB3L2* (supplementary material Fig. S1).

To determine whether loss of *Creb3l2* function is responsible for the *feelgood* phenotype, we designed a Tol2-based rescue construct (Kwan et al., 2007) containing wild-type *bactin2*:*creb3l2-mCherry* that was injected into *feelgood* mutants to create chimeric transgenic fish overexpressing wild-type and mutant proteins (Fig. 5E). Approximately 1/4 of the injected *feelgood* mutant embryos showed a partial rescue of the *feelgood* phenotype as indicated by the longer jaw, which protrudes past the eyes, and the presence of cartilage elements whose shape closely resembles that of wild types (Fig. 5F,H). A similar construct that contained the putative *feelgood* (N301K) mutation in *creb3l2* failed to rescue, indicating that the N301K substitution disturbs *creb3l2* function and accounts for the *feelgood* mutant phenotype (Fig. 5G,I,J).

creb3l2 is expressed in the developing pharyngeal arches

To determine whether the spatiotemporal expression pattern of *creb3l2* during development matches the structures that are most affected in *feelgood* mutants, we analyzed RNA samples by reverse transcriptase (RT)-PCR and embryos by whole mount in situ hybridization at sequential developmental time points. RNA analysis revealed that *creb3l2* mRNA is maternally deposited and that the amount of transcript decreases after the onset of zygotic transcription at the mid-blastoalla transition. As development proceeds, *creb3l2* is steadily expressed throughout morphogenesis, with the highest level at 4 dpf (supplementary material Fig. S2A). Probing embryos by in situ hybridization with digoxigenin-labeled *creb3l2* riboprobes showed that *creb3l2* is ubiquitously expressed during early development (supplementary material Fig. S2B). By 36 hpf, *creb3l2* RNA became primarily localized to the developing jaw, pectoral fins and the otic capsule (supplementary material Fig. S2C), and expression is maintained throughout jaw development (supplementary material Fig. S2D-E’). These results show that *Creb3l2* is highly expressed in tissues that show developmental deficits in *feelgood* embryos, consistent with a direct role for *Creb3l2* in craniofacial morphogenesis.

creb3l2 knockdown phenocopies the feelgood defects

The *feelgood* mutation most probably disrupts the DNA-binding ability of Creb3l2, resulting in partial or complete loss of function. To determine whether *feelgood* is a null or a hypomorphic allele, we knocked down Creb3l2 protein using two morpholinos (MO-1 and MO-2; Fig. 6A). Although both produced similar results, MO-2, which straddles the exon 4 splice acceptor site of the *creb3l2* gene locus in order to disrupt transcript processing, was more effective. MO-2 is predicted to cause a frame shift that generates a stop codon at amino acid 209 and leads to a truncated peptide lacking all characterized functional domains (Fig. 6A). Injection of MO-2 resulted in depletion of the spliced *creb3l2* transcript as evaluated by RT-PCR (Fig. 6A). The observed MO-2 phenotype is similar to that of *feelgood*, although the morphant defects are more
severe than the ones in feelgood mutants (Fig. 6B-D). Decreasing the amount of morpholino resulted in a milder phenotype more similar to feelgood (Fig. 6C,C’, quantified in Fig. 6F). To determine the specificity of the MO-2 phenotype, we co-injected morphants with mRNA of correctly spliced creb3l2 and the corresponding mutant creb3l2, and observed a suppression of the MO-2 phenotype (I). The chromatograms of sequences of these fish was confirmed by sequencing.

The feelgood mutation reduces the transcriptional activity of Creb3l2

The feelgood variant results in a change of a single amino acid residue (N301K) within the DNA binding domain of Creb3l2. To assess whether this change affects transcriptional activity, we cloned zebrafish cDNAs encoding wild-type Creb3l2, the N-terminal 374 aa of Creb3l2 (which is similar to the S1P cleaved form of Creb3l2), and the corresponding feelgood variants into expression vectors (Fig. 7A). We also cloned the 895 bp fragment of the zebrafish sec23a promoter, which is located immediately upstream from the translation start site, in front of the Luciferase reporter gene. We chose this fragment because it contains three putative CREB-binding elements: 5’-TCACGTTT-3’ at position –3 to –10; 5’-TCAGCTT3’ at –347 to –354 and 5’-AGACGTGG-3’ at –888 to –895 bp from the transcriptional start site.

Transient transfection experiments in human fibroblasts showed that the wild-type Creb3l2 and the processed, cytosolic Creb3l2 activate the reporter gene by 5.5- and 7.5-fold, respectively (Fig. 7B). By contrast, both the full-length and cytosolic feelgood Creb3l2 variants were considerably less active, inducing the promoter by 2.5- and 4.0-fold, respectively. The transcriptional activity assays suggest that the N301K feelgood variant diminishes the transcriptional activity of Creb3l2 by approximately 50%. Thus, the molecular data are consistent with the genetic and MO-mediated mutational analyses described above (Figs 5, 6), which show that feelgood is a hypomorphic allele. The fact that both the full-length and the processed Creb3l2 mutants display a similar loss of activity compared with the corresponding wild-type proteins strongly suggests that the primary molecular defect in feelgood leads to loss of transcriptional activity, or nuclear transport rates, rather than...
incorrect processing by S1P or inefficient transport of Creb3l2 from ER to Golgi.

The feelgood mutation leads to decreased expression levels of select cargo adaptor proteins

Collectively, our results indicate that feelgood (creb3l2) mutants have a similar, although milder, craniofacial phenotype to crusher (sec23a) and bulldog (sec24d) mutants (Lang et al., 2006; Sarmah et al., 2010). This raises the possibility that the feelgood deficit leads to lower expression levels of sec23a and sec24d. Sec23A was previously shown to be a direct target of Creb3l2 (Saito et al., 2009). We also found that Creb3l2 itself has a conserved cre site in its promoter, and therefore might be self-regulated.

In order to determine whether the putative targets of Creb3l2 – creb3l2, sec23a and sec24d – are misregulated in feelgood mutants, we analyzed total RNA samples from 80-hpf embryos. We also included sec24c and sec23b for comparison. Quantitative real-time PCR (qPCR) results showed that the expression levels of creb3l2, sec23a and sec24d are decreased in feelgood mutants relative to wild types (Fig. 7C). By contrast, sec24c levels are upregulated in feelgood mutants as compared with wild types. This outcome could be either due to sec24c upregulation through Creb3l2-independent, compensatory mechanisms, or a Creb3l2-mediated, indirect suppression of sec24c.

To determine whether expression levels of creb3l2 targets are altered by disruption of ER-to-Golgi protein trafficking independently of Creb3l2, we analyzed RNA samples from crusher and bulldog mutants. The results show that the expression levels of creb3l2, sec23a and sec24d are increased in these mutants relative to wild types, except for sec23a and sec24d in their respective mutants, which might be due to nonsense-mediated decay (Fig. 7C), whereas sec24c is increased in all three mutants.

Taken together, these results demonstrate that Creb3l2 plays an important role in regulating multiple components of COPII carriers, as well as regulating its own expression. Interestingly, there is no evidence that the expression of sec24c is regulated by Creb3l2, consistent with the lack of craniofacial dysmorphology phenotype in sec24c morphants (Sarmah et al., 2010).

The feelgood mutation does not cause ER stress response

To test whether the unfolded protein response (UPR) is disrupted in feelgood mutants, we analyzed the expression of bip and sil1 (Fig. 7D), both of which are induced in crusher and bulldog. Neither was upregulated in a statistically significant manner in 80-hpf feelgood mutants compared with wild types, suggesting a lack of ER stress response. Finally, expression differences in the Creb3l2 processing enzyme S1P were negligible in the three mutants. The lack of UPR probably reflects the less severe nature of the feelgood phenotype, consistent with the hypothesis that the primary feelgood defect is in collagen trafficking.

DISCUSSION

Here we show that the zebrafish feelgood mutation manifests skeletal phenotypes resembling those observed in crusher (sec23a)
...and bulldog (sec24d), i.e. accumulation of type II and type IV collagens within enlarged rough ER in chondrocytes and notochord sheath cells. At the molecular level, the feelgood mutation causes an amino acid substitution within the DNA-binding domain of the transcription factor Creb3l2.

feelgood mutants present deficits in the late developmental stages of the craniofacial skeleton formation. The early stages of head skeleton development, including neural crest migration, mesenchymal condensation and specification into type II collagen secreting chondrocytes, proceed normally, indicating that these steps do not depend on fully functional Creb3l2. However, in the later developmental steps, characterized by robust collagen secretion by chondrocytes, ECM fails to densify and mature, leading to skeletal elements that are specified, but structurally malformed. This phenotype of normal skeletal patterning and misshapen individual elements is similar to human birth defects including CLSD (Boyadjiev et al., 2006; Fromme et al., 2007), and, as such, feelgood might provide a powerful tool for understanding the etiology of this class of congenital dysmorphologies. Moreover, the initial presence of collagen fibrils in the extracellular space, but lack of ECM maintenance seen in feelgood mutants, is reminiscent of adult-onset diseases characterized by microarchitectural skeletal deterioration such as observed in osteoporosis or arthritis (Goldring and Goldring, 2010; Heinegård and Saxne, 2011).

Our results uncovered that Creb3l2 function is not confined to skeletal tissues, but is also required in other cell types. Specifically, feelgood mutant embryos displayed defects in the maturation of melanosomes; these defects are similar to those previously described in the zebrafish and mouse mutation silver (Schonthaler et al., 2005; Theos et al., 2006). The silver locus encodes Pmel17, a type I membrane-inserted glycoprotein that is an integral part of the melanosomal matrix. Pmel17 is trafficked from the ER to endosomes and finally to melanosomes (Theos et al., 2005; Harper et al., 2008). In feelgood, as revealed by TEM, the trafficking of melanosome matrix proteins that are responsible for change in melanosome shape is de-coupled from pigment synthesis and secretion, indicating that Creb3l2-mediated regulation of cargo secretion extends to different cell types.

Creb3l2 is a protein of 519 amino acids, coded by 12 exons spanning a genomic region of over 120 kb. The overall genomic structure as well as the amino acid sequence of the DNA-binding domain have been highly conserved from zebrafish to human (Storlazzi et al., 2003). Creb3l2 is part of a class I membrane-bound basic leucine zipper proteins that must be cleaved by S1P and Site-2 protease (S2P) in order to be activated (Fig. 8) (Seidah et al., 1999; Kondo et al., 2007). Because Creb3l2 is synthesized in the ER, whereas the proteases that cleave it are localized in the Golgi, Creb3l2 proteolysis might be regulated by the availability of the COPII machinery as with the similarly cleaved sterol regulatory element binding proteins (SREBPs) (Espenshade et al., 2002). Thus, by virtue of being an ER-resident protein, Creb3l2 is in an ideal location to optimize both the availability and composition of the ER-to-Golgi trafficking machinery by selective regulation of secretory pathway components.

Previously, Creb3l2 was shown to directly bind the promoter of Sec23a in mouse (Saito et al., 2009). Our analysis using the zebrafish sec23a promoter and constructs encoding wild-type and mutant Creb3l2 show that Creb3l2 N301K has lost approximately 50% of its transcriptional activity. This result further corroborated the mild skeletal defects observed in feelgood mutants. Thus, we concluded that the feelgood mutation represents a hypomorphic allele that is much less severe than the zebrafish creb3l2 morphants and knockout mouse, both of which have very severe skeletal deficits.

The analysis of Creb3l2 KO mice has suggested that the Creb3l2-mediated Sec23a pathway regulates the ER stress response...
machinery during chondrogenesis (Saito et al., 2009). We detected similar increases in the ER stress response in crusher (sec23a) and bulldog (sec24d) mutants, but not in feelgood. It has been difficult to discern which aspects of the phenotype are due to overactivation of the ER stress response and which are due to the inability of cells to secrete proteins to build normal ECM. However, the hypomorphic nature of the feelgood allele allowed us to parse the ER stress response phenotype from the requirement of Creb3l2 for COPII-dependent ER-to-Golgi transport. We postulate that the primary function of Creb3l2 is not direct regulation of the ER stress response. This conclusion is consistent with our preliminary results using additional ER stress response markers, such as CHOP and Xbp1 (Tabas and Ron, 2011) (data not shown). These experiments did not reveal significant upregulation of ER stress response in feelgood mutants or in the much more severe creb3l2 morphants.

We have shown that Creb3l2 does not only regulate the expression of sec23a, but also that of other COPII components, namely sec23b and sec24d. Although we observed diminished expression of sec24d in feelgood, we did not find any evidence that Creb3l2 regulates the expression of the closest paralog of sec24d, sec24c. Therefore, it is likely that the feelgood mutation mostly affects the Creb3l2-Sec24D secretory axis without influencing Sec24C-dependent cargos. The loss-of-function phenotypes of creb3l2, sec24d and sec24c mutants and/or morphants support this model (Sarmah et al., 2010; Melville and Knapik, 2011). For example, the four bulldog zebrafish alleles, which abolish Sec24D function, share many features with feelgood, i.e. they display intracellular backlogs of collagen molecules and sparse ECM but relatively normal trafficking of other ECM molecules such as fibronectin (Sarmah et al., 2010). By contrast, chondrocyte maturation and skeletal development proceed normally in sec24c morphants.

The differential transcriptional regulation of COPII components could also explain the tissue-specific deficits observed in some human syndromes. For example, mutations in SEC23B result in the human disease CDAII, which manifests in anemia but not skeletal defects, whereas mutations in SEC23A result in the human disease CLSD, which is characterized by skeletal defects but not anemia (Boyadjiev et al., 2006; Bianchi et al., 2009). The differences in phenotypes might be due to divergent transcriptional regulation, resulting in differential availability of various COPII components in different cell types. This hypothesis is supported by the finding that SEC23B is expressed at fivefold higher levels than SEC23A in human erythroblasts (Schwarz et al., 2009). Because both Sec23 paralogs are sex-limited targets, it is likely that additional unknown regulators modulate the availability of Sec23A, Sec23B, Sec24C and other COPII-associated proteins. Future discovery research in this area is likely to help crack the secretory code.

The high ECM secretory load has been postulated as a cause of the craniofacial dysmorphology in the COPII mutants (Lang et al., 2006; Fromme et al., 2007). Our results suggest that Creb3l2 is also required for the secretion of certain protein classes independently of secretory volume. For example, Collagen II and IV secretion to the extracellular space is affected in both chondrocytes and notochord sheath cells, yet the latter produce far fewer collagen fibrils than the former. Moreover, although fibrillar collagens accumulate in intracellular compartments, other abundantly...
deposited ECM proteins such as laminins, as well as vacuolar GAG proteins, are trafficked to the extracellular space.

There are several explanations of how this phenotype might occur. It is plausible that large fibrillar molecules such as collagen compete less efficiently with other protein classes that are synthesized in the ER and destined for transport to Golgi and subsequent destinations. This might occur because, whereas cargo-free self-assembled COPII vesicles are 40-80 nm in diameter (Barlowe et al., 1994; Miller and Barlowe, 2010; Dancourt and Barlowe, 2010), fibrillar procollagen bundles form rigid 300 nm rods (Fraser et al., 1979; Stephens and Pepperkok, 2002; Canty and Kadler, 2005). Hence, collagen transport requires specialized, oversized COPII structures and an ample supply of coat proteins (Stagg et al., 2008; O’Donnell et al., 2010). As a result, in cells with high levels of protein trafficking, the availability of COPII carriers might become a limiting factor, and other proteins might displace collagen molecules. Alternatively, there might be insufficient levels of COPII components to build the more complex tubular structures required for large cargos, leading to intracellular backlogs of collagen within distended ER compartments (Fig. 8, right).

Craniofacial and skeletal dysmorphologies account for the majority of birth defects and most of them are of unknown origin. The fact that random mutagenesis screens in zebrafish have yielded multiple mutants that disrupt the transport machinery suggests that at least some of the birth defects of unknown nature are due to abnormal protein transport (Neuhauss et al., 1996). Besides developmental defects, a host of adult-onset human diseases, including arthritis or interstitial fibrosis after organ injury, have been linked to dysregulated collagen production (Goldring and Marcu, 2009; Goldring and Goldring, 2007; Heinigéard and Saxne, 2011; Trojanowska et al., 1998). The decoupling of collagen transport from that of other cargos as well as the hypomorphic nature of the feelgood allele has important implications in the understanding of human diseases linked to collagen defects. Because the genetic basis for these diseases is often highly complex, tools such as feelgood are important for identifying potential candidate genes and regulatory networks. Better understanding of the roles of the regulatory and structural components of the intracellular transport machinery could lead to better diagnostic tools and novel treatments of human diseases.

METHODS

Fish maintenance and breeding
Fish were raised and kept under standard laboratory conditions at 28.5°C as previously described (Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006). feelgood (allele designated m662, isolated in the MGH genetic screen (Neuhauss et al., 1996; Drievery et al., 1996]) was kept in AB genetic background for phenotypic analysis. Embryos were staged and fixed at specific hours (hpf) or days (dpf) post-fertilization as described by Kimmel et al. (Kimmel et al., 1995). For some experiments embryos were incubated in 0.2 mM 1-phenyl-2-thiourea (Sigma) to block pigmentation.

Genetic mapping and cloning
The feelgood locus was mapped in an F2 intercross using bulked segregate analysis. DNA samples were PCR-genotyped with SSLP markers evenly spaced across the zebrafish genome. The mapped feelgood mutation was confirmed by sequencing genomic DNA flanking the mutation site from three homozygous wild-type F2 animals, five heterozygous F2 animals, six homozygous mutant F2 animals and six animals each from three different genetic backgrounds of wild-type fish (AB, IN and TL).

Cartilage staining
Embryos (80 hpf or 5 dpf) were fixed in 4% phosphate buffered PFA overnight. After two washes in PBS (1×) for 5 minutes, embryos were bleached with 10% H2O2 and 30 µl of 1 M KOH for 1 hour. Following two washes in PBT for 5 minutes, embryos were incubated in 0.1% Alcian blue solution overnight at room temperature (RT) on a shaker. After one wash in acidic ethanol (70% ethanol, 5% HCl) followed by an overnight destaining in fresh acidic ethanol, embryos were dehydrated in 85% and 100% ethanol for 15 minutes each and transferred to 80% glycerol.

Histology
Histological sections were prepared in JB-4 plastic resin medium (Polysciences). Phosphate buffered PFA (4%)-fixed 5-dpf embryos were washed with PBS and subsequently dehydrated with 25%, 50%, 70% and 95% ethanol (each step for 5 minutes). Dehydrated embryos were equilibrated with JB-4 infiltration solution for 10 minutes at RT. The embryos were then placed in plastic molds and mounted in JB-4 embedding solution. Blocks were sectioned in 5 µm thickness using a Leica RM2265 microtome. Sections were collected on adhesive coated slides (Superfrost plus, Fisher), dried on a heating plate and stained with metachromatic dye Toluidine blue (Sigma), and mounted using cytoseal-XYL as a mounting medium as previously described (Granero-Moltó et al., 2008).

Immunofluorescence and WGA staining
Type II collagen and WGA staining was performed as previously described (Sarmah et al., 2010). Whole-mount embryos were fixed in 4% PFA at 4°C overnight and incubated with 1:200 diluted primary antibody against collagen type II (Polysciences) and WGA–Alexa-Fluor-488 conjugate (1:200) followed by 1:300 Alexa Fluor 555 fluorescently conjugated secondary antibody (Molecular Probes). The WGA lectin binds to N-acetylgalosamine and N-acetylneuraminic acid residues of membrane and matrix glycoproteins. For staining on sections, embryos were fixed in 4% or 2% PFA, embedded in 1.5% agarose in 5% agarose, and stored in 30% sucrose solution at 4°C overnight. Agarose blocks were mounted with O.C.T. (Sakura Finetechinal). 15-µm sections were cut using a Leica CM 3050 cryostat at −20°C and transferred onto Superfrost slides (Fisher). Sections were washed in PBS, blocked in 2 mg/ml BSA, 2% goat serum, 2% DMSO in PBS and incubated with collagen type II antibody (1:250 dilution), collagen type IV antibody (1:200 dilution, Lab Vision), Laminin Ab-1 (1:100; LabVision) or WGA (1:250) at 4°C overnight. Alexa-Fluor-555 or Alexa-Fluor-488 conjugate was applied as secondary antibody (1:500). TO-PRO-3 (Molecular Probes) was used for nuclear counterstaining. Confocal images were taken with a Zeiss LSM510 inverted confocal microscope (Vanderbilt Cell Imaging Shared Resource).

Electron microscopy
After being anesthetized with tricaine (Sigma), zebrafish embryos were placed into fresh 2% gluteraldehyde and incubated overnight.
at 4°C. Fish were washed in PBS, transferred to 1% osmium tetroxide and washed in diH2O. Fish were stained en bloc in 1% aqueous uranyl acetate for 1 hour and washed in diH2O. The samples were then taken through a series of dehydration steps starting with 30% and followed by 50%, 70%, 95% and absolute ethanol. Propylene oxide was used as a transitional solvent to replace the dehydration solution. Samples were transferred to a 1:1 araldite:propylene oxide mixture then placed in pure araldite in a vacuum oven. Pure resin specimens were then transferred into embedding molds containing fresh resin and finally placed into a 16°C oven overnight. Ultra-thin serial sections (50-60 nm) from the vacuum oven. Pure resin specimens were then transferred into a 1:1 propylene oxide mixture then placed in pure araldite in a dehydration solution. Samples were transferred to a 1:1 propylene oxide:araldite solution and washed in diH2O. Fish were stained en bloc in 1% uranyl acetate and 0.5% lead citrate and imaged. 7 dpf embryos were placed in egg water containing 0.5 μM melanin concentrating hormone (Sigma) and imaged. 6-dpf embryos were placed in egg water containing 0.5 μM melanin concentrating hormone (Sigma) and imaged. 6-dpf embryos were placed in egg water containing 0.5 μM melanin concentrating hormone (Sigma) and imaged. 6-dpf embryos were placed in egg water containing 0.5 μM melanin concentrating hormone (Sigma) and imaged. 6-dpf embryos were placed in egg water containing 0.5 μM melanin concentrating hormone (Sigma) and imaged.

**Western blotting**

Proteins were isolated by homogenizing 4-dpf embryos in RIPA buffer containing protease inhibitor (Sigma). Glycoproteins in the lysates were cleaved by Endo H (NEB) or PNGase F (NEB) according to manufacturer specifications. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Mini-Protean TGX gels (Bio-Rad). For immunoblotting, proteins were transferred to polyvinylidene fluoride (PVDF) membrane using an electrophoretic transfer apparatus (Bio-Rad). The membrane was blocked with 1% non-fat milk (Bio-Rad) and incubated with 1:100 diluted primary antibody against type II collagen (Polysciences), or N-Cadherin (Sigma) followed by 1:10,000 HRP-conjugated anti-rabbit secondary antibodies (Promega). Signal detection was performed using Pico West Chemiluminescent Substrate (Thermo Scientific).

**Generation of Tol2kit-based transgenic fish**

The *bactin*2*creb3l2*:*mCherry construct was created using the *Tol2kit* approach (Kwan et al., 2007). In brief, PCR was used to add attB1 and attB2 sites to the coding region of *creb3l2*, and the product was recombined into pDONR221 to create a middle entry clone. A construct containing the N301K mutation was created by recombining the middle entry clone and 20 bp of the *mCherry* coding sequence into the pCS2+ vector. The firefly luciferase reporter plasmid was generated by subcloning 895 bp of promoter sequence into the pCS2+ vector (Promega). After 30 hours, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s specifications.

**In situ hybridization**

The *creb3l2* probe was made by cloning 427 nucleotides from the 3’ UTR of *creb3l2* cDNA into pGEM-T Easy vector (Promega) with primers 5’-ATCTACTGCGCTGGGCGAT-3’ and 5’-GCCATGTTGAGCTTCAATG-3’. MO-1 was designed to target the *creb3l2* probe was made by cloning 427 nucleotides from the 3’ UTR of *creb3l2* cDNA into pGEM-T Easy vector (Promega) with primers 5’-ATCTACTGCGCTGGGCGAT-3’ and 5’-GCCATGTTGAGCTTCAATG-3’. MO-1 was designed to target the *creb3l2* probe was made by cloning 427 nucleotides from the 3’ UTR of *creb3l2* cDNA into pGEM-T Easy vector (Promega) with primers 5’-ATCTACTGCGCTGGGCGAT-3’ and 5’-GCCATGTTGAGCTTCAATG-3’.

**Luciferase assay**

Effector protein expression plasmids were generated by subcloning full-length and cytosolic-domain (1-374) *Creb3l2* and *Creb3l2* into pCS2+ vector. The firefly luciferase reporter plasmid was generated by subcloning 895 bp of promoter sequence upstream of zebrafish *sec23a* into the pGL3 luciferase reporter vector (Promega). Human foreskin fibroblasts (System Biosciences) were grown to 95% confluence and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications with reporter plasmid (0.4 μg), the corresponding effector plasmids (0.4 μg), and a reference plasmid pRL-SV40 (0.04 μg) carrying the *Renilla luciferase* gene under the control of the SV40 enhancer and promoter (Promega). After 30 hours, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Firefly luciferase activity was normalized to that of *Renilla* luciferase.

**Phylogenetic analysis**

Protein sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A phylogenetic tree was constructed using the neighbor joining method (Saitou and Nei, 1987).
**TRANSLATIONAL IMPACT**

**Clinical issue**
Normal cellular function depends on an elaborate network of intracellular organelles and molecular carriers that direct traffic of individual proteins to specific destinations. The journey of membrane-embedded or secreted proteins such as cell adhesion molecules, growth factors and extracellular matrix (ECM) begins in the endoplasmic reticulum (ER). Proteins synthesized in the ER are subsequently transported to the Golgi complex, where they become glycosylated before being sent to other organelles, the cell surface or the extracellular space. Transport from the ER to the Golgi is conducted by the coat protein II (COPII) vesicular carriers, which take part in various enzymatic activities, cargo recognition and structural assembly.

Over the last few years it has become evident that genetic defects in genes encoding COPII components cause developmental abnormalities in embryos, as well as a wide range of human diseases in adults, from anemia to skeletal deformities and lipid malabsorption. Besides genetic defects, interstitial fibrosis leading to organ failure after injury, or pathological conditions in aging patients such as arthrits, have been associated with dysregulated, often excessive, protein secretion.

**Results**
The authors show that the feelgood mutation in zebrafish is caused by a single amino acid substitution within the DNA-binding domain of transcription factor Creb3l2, which is known to be a positive regulator of secretory pathway activities, cargo recognition and structural assembly.

**Implications and future directions**
The findings presented here imply that skeletal morphogenesis is particularly sensitive to both the precise levels and molecular composition of the transport machinery. A large number of identified craniofacial mutations in animal models disrupt protein trafficking with high frequency, strongly suggesting that at least some of the birth defects of unknown origin are due to abnormal protein transport. Future analyses will focus on genetic screens and genomic approaches to search for human variants in key regulatory and structural proteins that at least some of the birth defects of unknown origin are due to abnormal protein transport. Future analyses will focus on human variants in key regulatory and structural proteins that are involved in the secretory pathway.

**Statistical analysis**
Data in bars represent average ± s.d. Statistical analyses were performed using unpaired two-tailed Student’s t-test and P-values <0.05 were considered as significant.

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**COMPETING INTERESTS**
The authors declare that they do not have any competing or financial interests.

**AUTHOR CONTRIBUTIONS**

**SUPPLEMENTAL MATERIAL**
Supplementary material for this article is available at [dmm.biologists.org](http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.007625/-/DC1)

**REFERENCES**


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