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Short Communication

The Sec domain protein Scfd1 facilitates trafficking of ECM components during chondrogenesis

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ABSTRACT

Chondrogenesis in the developing skeleton requires transformation of chondrocytes from a simple mesenchymal condensation to cells with a highly enriched extracellular matrix (ECM). This transition is in part accomplished by alterations in the chondrocyte protein transport machinery to cope with both the increased amount and large size of ECM components. In a zebrafish mutagenesis screen to identify genes essential for cartilage development, we uncovered a mutant that disrupts the gene encoding Sec1 family domain containing 1 (*scfd1*). Homozygous *scfd1* mutant embryos exhibit a profound craniofacial abnormality caused by a failure of chondrogenesis. Loss of *scfd1* was found to hinder ER to Golgi transport of ECM proteins and is accompanied with activation of the unfolded protein response in chondrocytes. We further demonstrate a conserved role for Scfd1 in differentiation of mammalian chondrocytes, in which loss of either SCFD1 or STX18, a SLY1 interacting t-SNARE, severely impair transport of type II collagen. These results show that the existence of a specific export pathway, mediated by a complex containing SCFD1 and STX18 that plays an essential role in secretion of large ECM proteins during chondrogenesis.

1. Introduction

Cartilage formation and skeletal morphogenesis depend on timely and abundant deposition of extracellular matrix (ECM) proteins (DeLise et al., 2000; Zuscik et al., 2008). The major ECM component of cartilage is type II collagen, which constitutes up to 60% of the total cartilage protein content (Mow et al., 1992; Sophia Fox et al., 2009). The synthesis and secretion of collagens and other ECM proteins are precisely and dynamically regulated as chondrogenesis proceeds. Failure to produce adequate mature collagen fibers leads to developmental defects and diseases such as osteogenesis imperfecta (Bateman et al., 2009; Rauch and Glorieux, 2004). Transport of ECM proteins to the extracellular space is dependent on the secretory machinery. Trafficking of ECM proteins is initiated as they leave their site of synthesis in the endoplasmic reticulum (ER) and are transported to the Golgi apparatus. Fibrillar collagen forms as a rigid rod-like triple helix with a length over 300 nm (Lamande and Bateman, 1999), presumably too large to fit into canonical COPII-coated transport vesicles, which are typically 60–80 nm in diameter. This strongly suggests that a distinct vesicular transport mechanism exists through which cartilage-specific collagens are actively packaged, likely requiring specific cargo receptors and/or chaperones.

SCFD1 (also known as SLY1) is a member of the Sec1/Munc18 family of proteins that cooperate with SNARE complexes in membrane fusion events (Carr and Rizo, 2010). The yeast ortholog of SCFD1 has been implicated in forward and retrograde trafficking to the ER (Li et al., 2005) and quality control of membrane fusion (Lobingier et al., 2014). In mammalian cells, several functions have been shown for SCFD1. SCFD1, in conjunction with Syntaxin 5, has been reported to be involved in ER to Golgi transport *via* assembly of pre-Golgi intermediates through interactions with Syntaxin 17 and 18 (Dascher and Balch, 1996; Rowe et al., 1998; Steegmaier et al., 2000; Yamaguchi et al., 2002). SCFD1 has also been suggested to play a role in intra-Golgi and retrograde transport *via* association with the COG4 complex (Laufman et al., 2009). Recently, Nogueira et al. reported that SCFD1 binds to TANGO1 and specifically regulates Procollagen VII export (Nogueira et al., 2014). While informative, these proposed roles of SLY1 in protein transport were primarily intuited based on the use of artificial cargoes such as a temperature-sensitive Glycoprotein mutant of the Vesicular Stomatitis Virus. The endogenous cargoes of SLY1-associated vesicles and the molecular mechanism underlying the transport of these vesicles is therefore a matter of debate. In previous

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work, a temperature sensitive zebrafish *scfd1* mutant was used to demonstrate a requirement for SCFD1 in regenerative outgrowth of the fin (Nechiporuk et al., 2003). However, due to the hypomorphic nature of this mutant allele, the *in vivo* function of SCFD1 was not fully addressed, particularly during embryogenesis.

In this current study, we present data on the identification and characterization of a zebrafish *scfd1* null mutant recovered from a gene trapping screen. We show that *scfd1* is essential for extracellular matrix secretion in cartilage. *Scfd1* mutants exhibit a failure in chondroblast differentiation and induce a strong unfolded protein response (UPR) in chondrocytes. We further demonstrate that SCFD1 and STX18, a SCFD1 interacting t-SNARE, are necessary for Procollagen II transport in a mammalian chondrocyte differentiation model. These data provide mechanistic insights into the central role of SCFD1 within the chondroblast to facilitate the formation of large ER transport vesicles involved in the exportation of cartilage collagens and, perhaps, collagen-associated molecules of the ECM. Our results further suggest that mutations affecting SCFD1 and other proteins mediating collagen export are potential causes for congenital craniofacial abnormalities and skeletal diseases of unknown genetic origin.

2. Materials and methods

2.1. Zebrafish lines

Zebrafish embryos were maintained and staged using standard techniques (Westerfield, 1993). *Tg(sox10:EGFP)^{ir937}* fish has been previously described (Piloto and Schilling, 2010). The RP-T gene trap vector was modified from RP2 and co-injected with Tol2 transposase mRNA as previously described (Clark et al., 2011). To identify the affected gene in the gene trap lines, 5' RACE was carried out as previously described (Clark et al., 2011). The *hsp70:scfd1*; *α-crystallin:EGFP* transgenic line was generated by using standard Tol2 transgenesis (Kawakami, 2005). Full-length zebrafish *scfd1* coding sequence was subcloned downstream of a *hsp70* promoter in a backbone vector carrying a α-*crystallin:EGFP* marker cassette (Burrows et al., 2015). Heat shock was performed for 30 min in pre-warmed 37 °C media.

2.2. Alcian blue staining, immunohistochemistry and WGA staining

Alcian blue staining (Walker and Kimmel, 2007), immunohistochemistry and WGA staining (Sarmah et al., 2010) were performed as previously described. Primary antibody specific to Collagen II (II-II6B3, obtained from DHSB, 1:200 dilution) was used. Fluorescent immunocytochemistry was performed using anti-mouse antibody conjugated with Cy3 (1:1000 dilution, Sigma) and WGA–Alexa-Fluor-488 conjugate (1:200 dilution, Life Technology).

2.3. Imaging

Imaging was performed using a Leica DFC320 camera on a Leica M205FA stereomicroscope. All confocal images were taken on a Zeiss LSM510 confocal microscope.

2.4. *RNA* in situ *hybridization*

Transcription of DIG-labeled antisense RNA probes was performed using standard methods. RNA *in situ* hybridization (ISH) was carried out as previously described (Thisse and Thisse, 2008).

2.5. Cell death assay

Cell death assays were performed as previously described (Caron et al., 2008; Verduzco and Amatruda, 2011) using a Cell Death Assay Detection Kit, POD (Roche).

2.6. Quantitative real-time PCR

Total RNA was prepared using TRIzol (Invitrogen, Life Technologies Corp.) from 50 combined control or *scfd1* mutant embryos, with mutants being identified morphologically. Control larvae were phenotypically wild type sibling embryos (mixture of nullizygous and hemizygous for the gene trap allele). Primers used for qPCR analysis are listed in Table S1.

2.7. Electron microscopy

After being anesthetized with tricaine (Sigma), zebrafish embryos were placed into fresh 4% paraformaldehyde and incubated overnight at 4 °C. Sample processing, sectioning and imaging were carried out as previous described (Melville et al., 2011).

2.8. Western blotting

Proteins were isolated by homogenizing 3 dpf embryos in RIPA buffer containing protease inhibitor (Sigma). Procedures of western blotting and antibodies were described in Supplementary Experimental Procedures.

2.9. Cell culture and Alcian blue staining of ATDC5 cells

Culture, insulin stimulation and Alcian blue staining of ATDC5 cells were carried out as previously described (Yang et al., 2009).

2.10. Establishment of knockdown stable cell lines

The mammalian pSUPER-puro vectors were used for expression of short hairpin (sh) RNAs corresponding to mouse cDNAs (target sequences listed in Table S2). ATDC5 cells were transfected with the Sly1, TANGO or Syntaxin 18 shRNA constructs, and 36 h later were transferred into selection medium containing 0.5 mg/ml puromycin. Depletion of gene expression in puromycin-resistant clones was verified by western blotting. A stable cell line harboring an empty pSUPER vector was established and used as a control.

2.11. Flow cytometric analysis of apoptosis

Membrane and nuclear events during apoptosis were analyzed by flow cytometry using FITC-Annexin V and propidium iodide (PI) staining and an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher).

3. Results and discussion

3.1. Loss of scfd1 leads to defects in craniofacial chondrogenesis

In a *Tol2* transposon-mediated gene trapping screen to identify novel genes essential for embryogenesis (Fig. 1A), we identified a line, RT-104B, showing chondrocyte specific GFP expression from 60 h post-fertilization (hpf) until 7 day post-fertilization (dpf) (data not shown and Fig. 1B–L). GFP expression also could be observed in gastrointestinal organs, brain, ovary and fin in larvae and adult fish (Fig. S1). This dynamic expression pattern suggested a role for the trapped gene in development and physiological function of multiple organs. Homozygous mutant embryos from incrosses of heterozygous RT-104B fish showed no obvious morphological defects until 48 hpf (Fig. 2A and B), and died by 7 dpf. By 72 hpf, RT-104B homozygous mutant embryos had a reduced lower jaw, with no cartilage formation evident by Alcian blue staining (Fig. 2C and D and data not shown). At 96 hpf, only posterior portions of trabeculae and residual parachordal plate were formed in mutant embryos, whereas the viscerocranium (lower jaw) was completely absent (Fig. 2G–I). In addition to defects in