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JMY is involved in anterograde vesicle trafficking from the *trans*-Golgi network

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ABSTRACT

Junction-mediating and regulatory protein (JMY) was originally identified as a transcriptional co-factor in the p53-response to DNA damage. Aside from this nuclear function, recent years have uncovered an additional function of JMY, namely in cytoskeleton remodelling and actin assembly. The C-terminus of JMY comprises a canonical VCA-module, the sequence signature of Arp2/3 complex activators. Furthermore, tandem repeats of 3 WH2 (V, or more recently also W) domains render JMY capable of Arp2/3 independent actin assembly. The motility promoting cytoplasmic function of JMY is abrogated upon DNA-damage and nuclear translocation of JMY.

To address the precise cellular function of JMY in cellular actin rearrangements, we have searched for potential new interaction partners by mass spectrometry. We identified several candidates and correlated their localization with the subcellular dynamics of JMY. JMY is localized to dynamic vesiculo-tubular structures throughout the cytoplasm, which are decorated with actin and Arp2/3 complex. Moreover, JMY partially colocalizes and interacts with VAP-A, which is involved in vesicle-based transport processes. Finally, overexpression of JMY results in Golgi dispersal by loss from the *trans*-site and affects VSV-G transport. These analyses, together with biochemical experiments, indicate that JMY drives vesicular trafficking in the *trans*-Golgi region and at ER-membrane contact sites (MCS), distinct from other Arp2/3 activators involved in vesicle transport processes such as the related WHAMM or WASH.

Introduction

Junction-mediating and regulatory protein (JMY) was originally identified as a transcription co-factor binding to p300-protein, which is involved in positively regulating the p53 response (Shikama et al., 1999). Moreover, JMY is targeted for degradation to disallow it from activating p53 by binding to the prominent p53 regulator Mdm2, until DNA damage signals its release (Coutts et al., 2007). In DNA damage conditions, JMY undergoes nuclear accumulation, which drives the p53 transcription response. In the cytoplasm, however, JMY was ascribed a role in cell motility by (i) directly driving actin nucleation at the cell front (Zuchero et al., 2009) and (ii) indirectly by negatively regulating cell–cell adhesion through suppression of cadherin expression (Coutts et al., 2009). Cellular processes that were assigned to JMY are as diverse as the suppression of neuritogenesis (Firat-Karalar et al., 2011) or cytokinesis in mouse oocytes, where it might act as upstream-regulator of Arp2/3-complex in asymmetric division (Liu et al., 2012). Nonetheless, the exact (sub-)cellular processes and the basic mechanisms underlying these findings are not well defined.

Sequence analysis groups JMY to both families, the *tandem monomer-binding nucleators* as well as the canonical class I activators of Arp2/3 complex (Coutts et al., 2010; Firat-Karalar and Welch, 2011; Rottner et al., 2010). Hence, JMY can contribute to actin assembly in Arp2/3-dependent as well as -independent ways: JMY comprises a C-terminal VCA module that comprises a g-actin

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binding Verprolin-homology (V- or WH2) domain, a central connectin (C) motif and a C-terminal acidic (A) motif for Arp2/3 binding. This canonical VCA-moduele has consequently been shown to be capable of activating the Arp2/3 complex (Coutts et al., 2009; Zuchero et al., 2009). In addition however, the C-terminus of JMY harbours two additional WH2 domains, which, in concert with the third WH2 domain, can initiate Arp2/3 independent nucleation, probably similar to Spire (Coutts et al., 2009; Zuchero et al., 2009). The major portion of JMY, which has a molecular weight of approx. 110 kDa, constitutes the central coiled coil domain, and is related to that found in the paralogous protein WHAMM (Campellone et al., 2008; Firat-Karalar and Welch, 2011). Hence, JMY and WHAMM form a subfamily within the group of class 1 nucleation promoting factors (NPFs) (Veltman and Insall, 2010). However, while WHAMM can also activate Arp2/3 complex, it lacks additional WH2 domains for Arp2/3-independent nucleation. Finally, WHAMM was found to drive vesicle traffic between the ER and the Golgi, a cellular compartment called ERGIC (ER-Golgi-Intermediate-Compartment), where it was required mainly for Arp2/3-dependent transport between these compartments (Campellone et al., 2008).

We here show that JMY is localized to tubular vesicles in the cytoplasm, where it drives actin dependent anterograde trafficking away from the *trans*-Golgi face. Overexpression of JMY affects integrity of the Golgi and impairs trafficking of the *stomatitis indiana virus* envelope glycoprotein VSV-G to the plasma membrane. Finally, when screening for interaction partners, we found that JMY interacts with the vesicle-associated membrane protein-associated protein A (VAP-A) at sites reminiscent of membrane contact sites (MCS) that are formed between the peripheral ER and other cellular membranes (Friedman and Voeltz, 2011).

Materials and methods

Antibodies, expression constructs and reagents

GFP-JMY-full length construct was obtained by PCR from the murine cDNA-full length clone #IRAKp961B15195Q (Source BioScience). JMY deletion mutants and domains were cloned from the GFP-JMY-FL plasmid via PCR as indicated in Table S1 and ligated into pEGFP-C1 expression vector (Clontech). Murine VAP-A was cloned from the cDNA full length clone #IRAVp968F054D (Source BioScience). GFP-tagged full length WHAMM was as described and kindly provided by Matthew D. Welch (Campellone et al., 2008). VSV-G temperature sensitive mutant GFP-VSV-G ts045 was as described (Toomre et al., 1999). GFP-ER was subcloned from the original Clontech pEYFP-ER. Plasmids were maintained in *E. coli* DH5α (life technologies). Commercial primary antibodies were as follows: rabbit polyclonal anti-JMY (ab#1: ab69945; Abcam); goat polyclonal anti-JMY (#ab2: sc-10027; Santa Cruz); rabbit polyclonal anti-ERGIC53 (#E1031; Sigma–Aldrich); mouse monoclonal anti-GM130 (#610823; BD Transduction Laboratories); rabbit polyclonal anti-TGN46 (#PAB0110; Abnova); mouse anti-VSV-G ectodomain antibody was kindly provided by Jean Gruenberg (University of Geneva). Anti myc was from Abcam (#9E10) and anti GFP was from Synaptic Systems (#101G4). Alexa-Fluor-594-labelled phalloidin and antibodies as used in immunofluorescence stainings were obtained from Life Technologies. HRP-coupled antibodies used in Western-Blot experiments were from Dianova.

Cells, treatments and transfections

COS-7 cells (DSMZ; #ACC 60) were grown in DMEM, 4.5 g/L glucose (Life Technologies) with 10% FCS (Sigma–Aldrich), 2 mM glutamine and 50 U/ml Penicillin/Streptomycin (Life Technologies) at 37 °C and 5% CO_2. HeLa cells (ATCC; #CCL-2) were grown in MEM with Earle's salts (Life Technologies) with 10% FCS (Sigma–Aldrich), 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids and 50 U/ml Penicillin/Streptomycin (Life Technologies) at 37 °C and 5% CO_2. Transfections were carried out with X-tremeGene 9 (Roche), according to the manufacturer' protocols. For microscopy, cells were transfected in a 3 cm diameter dish and plated to 15 mm glass coverslips on the next day. For immunoprecipitations (IPs), cells were transfected in a 10 cm diameter dish and processed after 16–24 h.

Immunoprecipitation

For IPs, transfected cells were washed once with PBS, lysed in 1 ml ice-cold lysis buffer (8 mM Tris base, 12 mM HEPES, pH 7.5, 50 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 1% Triton X-100, and EDTA-free protease inhibitor cocktail Complete Mini (Roche)) for 20 min on ice. Clarification of the lysates was performed for 15 min at 12,000 × *g*. After removing a sample as loading control, supernatants were incubated for 2 h in a spinning wheel at 4 °C. G-Sepharose slurry was equilibrated two times in IP-Buffer (8 mM Tris base, 12 mM HEPES, pH 7.5, 50 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$). 30 μl of equilibrated G-Sepharose slurry was added to supernatants and incubated for additional 45 min at 4 °C. Bead material was separated by centrifugation for 2 min at 500 × *g* and a sample for Western-Blot analysis prepared. The resulting pellet was washed twice in IP-buffer without Triton-X-100, supplemented with SDS sample-buffer and stored at −20 °C. Western Blots were developed using HRP-labelled secondary antibodies, LumiLight (Roche), and images were acquired using a GeLiance imaging system (Perkin Elmer).

VSV-G transport assay

To determine the rate of GFP-VSV-G transport, 2×10^5 HeLa cells grown on glass cover slips were transfected either with pEGFP-VSVG3 alone or together with myc-tagged JMY using Turbofect® (Thermo Fisher Scientific, Germany) according to the manufacturer's instructions. After 6–14 h at 37 °C, cells were shifted to 39.5 °C for 24 h. VSV-G transport was induced by replacing culture media with media containing 10 mg/ml cycloheximide at 32 °C. After 1 h, cells were washed 3× with ice cold PBS and fixed in 8% PFA for 10 min on ice followed by an additional fixation step using 4% PFA at room temperature. Fixed cells were washed thrice with PBS and incubated with 50 mM NH_4Cl for 5 min. VSV-G at the plasma membrane was stained in non-permeabilized cells using an anti-VSV-G ectodomain specific antibody. Myc-JMY-expression was confirmed by permeabilizing and staining parallel samples with rabbit anti myc (A14, Santa Cruz Biotechnology), revealing a co-expression in more than 95% of the GFP-VSV-G-expressing cells (not shown). The secondary antibody was goat anti-mouse Oyster594 (Luminartis, Münster, Germany). Cells were analysed using a Leitz Diaplan microscope equipped with an Olympus XM10 camera. For image analysis, ImageJ (Schneider et al., 2012) was used. GFP-VSV-G positive cells were outlined manually, and the mean grey values of green ($I_{VSV\text{-}G}$) and red (I_{ecto}) channels were measured. Background levels were assessed from non-transfected cells in the same image and subtracted. Transport rates were calculated by dividing the corrected values of I_{ecto} by $I_{VSV\text{-}G}$.

Fluorescence microscopy

For immunofluorescence, cells grown on fibronectin were fixed with pre-warmed formaldehyde (4%) in PBS for 20 min, extracted with 0.1% Triton X-100 (1 min), and stained with the indicated antibodies and/or phalloidin. Effects of JMY and mutants were quantified by assessing the percentage of GFP-tagged JMY variant