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# NOSTRIN functions as a homotrimeric adaptor protein facilitating internalization of eNOS

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## Summary

**Intracellular trafficking of endothelial nitric oxide synthase (eNOS) between different compartments is incompletely understood. Recently, we described a novel eNOS-interacting protein, NOSTRIN, which upon overexpression drives eNOS away from the plasma membrane towards intracellular compartments. Sequence similarity of NOSTRIN and pacsins/syndapins suggested a role for NOSTRIN in endocytosis. Accordingly, we show here that NOSTRIN interacts with the large GTPase dynamin and the actin nucleation promoting factor N-WASP by means of its SH3 domain, which also represents the docking site for eNOS. Via a coiled-coil region in the C-terminal portion of the protein, NOSTRIN oligomerizes, mainly forming trimers, which would allow simultaneous interaction with multiple binding partners of the SH3 domain. Consistent with this notion, expression of dynamin-2-GFP in CHO cells stably expressing eNOS (CHO-eNOS) results in recruitment of eNOS to dynamin-positive structures, only when NOSTRIN is present as well. Similarly, when N-WASP-GFP and NOSTRIN are co-expressed in CHO-eNOS cells, both proteins strongly co-localize with eNOS and are recruited to structures running along actin filaments. If, however, the actin cytoskeleton is depolymerized by cytochalasin D, NOSTRIN and eNOS are associated with extended structures in the cell periphery, possibly being unable to leave the plasma membrane. Together, these results indicate that NOSTRIN may facilitate endocytosis of eNOS by coordinating the function of dynamin and N-WASP.**

Key words: NOSTRIN, NO synthase, Pacsin/syndapin, Internalization, Dynamin, N-WASP

## Introduction

Endothelial nitric oxide synthase (eNOS) plays a key role in producing nitric oxide (NO) in endothelial and epithelial cells (Ortiz and Garvin, 2003; Sessa, 2004). Since NO is an extremely reactive and thus short-lived signaling molecule the exact subcellular distribution of the NO generating enzyme, eNOS, to distinct subcellular compartments is of crucial importance. Owing to irreversible myristoylation, the enzyme mainly localizes to membranes, and only a minute fraction is present in the cytosol (Govers and Rabelink, 2001). A major pool of eNOS resides at the Golgi complex where it is subjected to reversible palmitoylation targeting it to the plasma membrane (Shaul, 2002). Both, plasma membrane- and Golgi-bound eNOS are capable of activation (Fulton et al., 2002), but may be inhibited and activated through different mechanisms (Fulton et al., 2004; Govers et al., 2002). In addition to the established localization of eNOS to the Golgi and plasma membrane, eNOS is occasionally targeted to the cytoskeleton, representing an inactive pool of enzyme (Jiang et al., 2003; Sessa, 2004). A translocation of eNOS to vesicular structures throughout the cytoplasm was observed after bradykinin stimulation of endothelial cells (Thuringer et al., 2002); the functional relevance of this localization, however, is still elusive.

Given the fact that subcellular localization of eNOS affects enzymatic activity and thus NO availability, a tight control of eNOS targeting to different compartments appears to be essential. At present, it remains largely unknown how differential distribution of eNOS to various subcellular locales is achieved. Recently, we described two novel eNOS-interacting proteins termed NOSIP (eNOS interacting protein) and NOSTRIN (eNOS trafficking inducer), both of which influence localization of eNOS. For NOSIP, targeting of eNOS to the cytoskeleton appears to be one of the protein's major functions (Schleicher et al., 2005). Interaction of eNOS with NOSTRIN, by contrast, leads to translocation of eNOS from the plasma membrane to intracellular vesicular structures, most likely through an endocytic process (Zimmermann et al., 2002).

In the past few years, great progress has been made in discovering key players of endocytosis. A fast-growing group of proteins functioning in several steps of this process are multi-domain proteins that have one or more SH3 domains in common (e.g. intersectin, pacsin/syndapin or Grb2) (Schafer, 2002). These proteins are required to coordinate consecutive steps of endocytosis. Many of them first come into play through binding to dynamin. The dynamins (type I, II and III) are a unique family of large GTPases that spontaneously self-assemble into helical, supramolecular structures at the neck of newly formed endocytic membrane structures (Hinshaw, 2000; Praefcke and McMahon, 2004). This self-aggregation in turn stimulates GTPase activity of dynamin, ultimately leading to

vesicle fission (Sever, 2002). In addition to this function as a mechanochemical enzyme, a role for dynamin is emerging by interacting via its proline-rich domain with SH3-containing effectors of endocytosis (Schafer, 2004).

Accumulating evidence indicates that endocytic effector proteins provide a link between dynamin-mediated vesicle fission and subsequent steps of endocytosis that, among other things, require rearrangement of the cortical actin cytoskeleton (Orth and McNiven, 2003; Schafer, 2002). Contact of SH3 proteins with actin filaments can be direct, as for Abp1 or cortactin. Mostly, however, it is indirect through recruiting neural Wiskott-Aldrich syndrome protein (N-WASP), which stimulates the actin-nucleating activity of the Arp2/3 complex. Notably, N-WASP is involved in the formation of actin tails that propel pathogens as well as endogenous vesicles through the cytoplasm (Fehrenbacher et al., 2003). There is surmounting evidence for a role of N-WASP-mediated actin polymerization during vesicle internalization, possibly by driving vesicles away from the plasma membrane (Kessels and Qualmann, 2002; Merrifield, 2004).

Similarly to dynamin, interaction of N-WASP with endocytic effector proteins such as pacsins/syndapins or intersectin occurs via their SH3 domains binding to a proline-rich region within the N-WASP sequence (Hussain et al., 2001; Modregger et al., 2000; Qualmann et al., 1999). For N-WASP, interaction with SH3-containing proteins is one of several possible modes of activation. N-WASP is intrinsically inactive owing to an auto-inhibited conformation in which the N-terminal regulatory domain blocks the C-terminal Arp2/3 activating domain (Kim et al., 2000). Auto-inhibition can be released by many activators, the best characterized example being cooperative binding of the small GTPase Cdc42 and phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) (Caron, 2002). Alternatively, either Cdc42 or $PIP_2$ may synergize with SH3-containing proteins, such as Grb2 or the recently discovered Toca-1, to stimulate interaction of N-WASP with Arp2/3 (Carlier et al., 2000; Ho et al., 2004).

Pacsins/syndapins and Toca-1 are SH3 proteins that belong to the pombe cdc15 homology (PCH) family of proteins; by interacting with dynamin and N-WASP, pacsins/syndapins were shown to function in vesicle fission (Kessels and Qualmann, 2004). Overexpression of these PCH proteins leads to inhibition of receptor-mediated endocytosis but, to date, a direct interaction with the internalized target proteins has not been shown (Modregger et al., 2000; Qualmann and Kelly, 2000). Here, we report that NOSTRIN, a novel member of the PCH family, interacts with dynamin and N-WASP via its SH3 domain. Since NOSTRIN oligomerizes, mainly forming trimers, the protein can simultaneously interact with multiple binding partners. Consistent with this notion, we propose that NOSTRIN mediates internalization of eNOS through cooperation with dynamin and N-WASP.

## Materials and Methods

### Reagents and antibodies

Disuccinimidyl suberate (DSS), methotrexate and antibodies to Myc, GST, GFP and $(His)_6$ tags were from Sigma (Taufkirchen, Germany); cytochalasin D was purchased from Alexis (Gruenberg, Germany). Murine monoclonal antibody to human NOSTRIN was generated using GST-tagged $NOSTRIN_{242-506}$ purified from *E. coli* (Nanotools, Teningen, Germany). Antiserum to N-WASP was from Hiroaki Miki (University of Tokyo, Japan) (Miki et al., 1996). Rabbit and mouse antibodies to human eNOS as well as monoclonal antibodies to rat dynamin-1 and -2 were from BD Transduction Laboratories (Heidelberg, Germany). Phalloidin-Alexa 546 was purchased from Molecular Probes (Eugene, USA) and Pefabloc from Roche (Penzberg, Germany).

### cDNA constructs

Vectors pcDNA-dynamin-1 (human) and pEGFP-N-WASP (rat) were generous gifts of Ivan Dikic (University of Frankfurt, Germany), and pEGFP-dynamin-2 (rat) was kindly provided by Mark A. McNiven (Rochester, MN). For expression in cell lines, full-size human NOSTRIN cDNA was cloned into pcDNA3.1-myc/$(His)_6$ (Invitrogen, Karlsruhe, Germany). cDNAs encoding amino acids (aa) 250-434 or 433-506 of NOSTRIN were cloned into pSG8-VSV, which is derived from pSG5 (Stratagene, La Jolla, CA) modified for in-frame expression of a vesicular stomatitis virus (VSV) tag. NOSTRIN-ΔSH3 (aa 1-440) was expressed in cell lines from a pCMV-5B construct (Stratagene). To produce purified $(His)_6$- or GST-tagged NOSTRIN, full-size NOSTRIN cDNA was cloned into pET22b and pGEX-4T-1 (Amersham Biosciences, Freiburg, Germany) vector, respectively. To express GST-tagged NOSTRIN-SH3 and NOSTRIN-ΔSH3, the cDNAs encoding aa residues 1-440 and 433-506, respectively, were ligated into pGEX2T vector. For Semliki forest virus (SFV) infection of cells, pSFV-NOSTRIN, pSFV-NOSTRIN-GFP or pSFV-GFP were used (Zimmermann et al., 2002).

### Cell culture and transfection

COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transient transfections were performed with DEAE-dextran. In brief, a 10 cm dish containing $6\times10^5$ cells was washed with phosphate-buffered saline (PBS), and expression plasmids were applied in 5.7 ml serum-free medium mixed with 300 μl of DEAE-dextran (1 mg/ml) and 12 μl of chloroquine (50 mg/ml). After incubation for 2.5 hours, cells were treated with 10% dimethlysulfoxide (DMSO) in PBS for 2 minutes and cultured in DMEM with 10% FCS for 24-30 hours prior to use. Chinese hamster ovary (CHO) cells stably expressing eNOS (CHO-eNOS) were cultured in DMEM supplemented with 10% FCS and 200 nM methotrexate (Dedio et al., 2001). CHO-eNOS cells were transiently transfected using PolyFect (Qiagen, Hilden, Germany) according to the manufacturer's instructions. SFV constructs were prepared and used as described (Zimmermann et al., 2002).

### Yeast two-hybrid analyses

Yeast mating assays were performed using the Matchmaker yeast two-hybrid (Y2H) system (Clontech, Palo Alto, CA). To narrow down the region involved in oligomerization of NOSTRIN, the following constructs were produced in pEG and pJG vectors: NOSTRIN, $NOSTRIN_{1-288}$, $NOSTRIN_{242-506}$, $NOSTRIN_{250-434}$, $NOSTRIN_{323-470}$ and $NOSTRIN_{433-506}$. Yeast strains EGY48/pSH18-34 and RFY206 were transformed with pEG and pJG plasmids, respectively. Yeast mating assays were carried out according to the manufacturer's instructions. Empty vectors served as negative controls. Interaction was tested using an X-galactosidase (X-Gal) assay on 4× deficient plates (–His, –Ura, –Trp, –Leu).

### Chemical crosslinking

COS cells transfected with Myc/$(His)_6$-tagged NOSTRIN or VSV-tagged $NOSTRIN_{250-434}$ and $NOSTRIN_{433-506}$ were harvested 36 hours post-transfection. Cells were washed twice with PBS and resuspended in buffer containing 20 mM HEPES, pH 7.4, 10 mM $MnCl_2$ and 1

mM dithiothreitol (DTT). Cells were lysed by repeated freezing/thawing in liquid nitrogen and sonification. For crosslinking, DSS was added (final concentration 0.3 mM DSS in DMSO) and incubated with the lysate for 30 minutes on ice. DMSO alone was used for control. The reaction was stopped by adding Tris-HCl, pH 7.4 (final concentration 70 mM), and the samples were analyzed by SDS-PAGE and western blotting using anti-Myc or anti-VSV.

### Pull-down assays

Full-length $(His)_6$-tagged NOSTRIN (pET22b vector) was purified from *E. coli* BL21 using a Ni-NTA matrix (Qiagen) according to the manufacturer's instructions. GST and GST-NOSTRIN were purified on glutathione (GSH)-Sepharose (Amersham). $(His)_6$-NOSTRIN immobilized on Ni-NTA matrix was incubated with purified GST and GST-NOSTRIN in Triton buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 μM Pefabloc) for 2 hours at 4°C and washed three times with PBS. Bound proteins were eluted with sample buffer (63 mM Tris-HCl, pH 6.8, 2.5% SDS, 5% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue) and analyzed by SDS-PAGE and western blotting using anti-GST or anti-$(His)_6$. For GST pull-down assay, full-length NOSTRIN, NOSTRINΔSH3 and NOSTRIN-SH3 were expressed in *E. coli* BL21. The resultant GST fusion proteins were purified on GSH-Sepharose. Untransfected COS and CHO-eNOS cells, or COS cells transiently transfected with pcDNA3-dynamin-1, were lysed in Triton buffer 24 hours after transfection. GST fusion proteins immobilized on GSH-Sepharose were incubated with cell lysates for 2 hours at 4°C and washed three times with Triton buffer. Precipitated proteins were eluted with sample buffer and analyzed by western blotting using anti-dynamin-1 or anti-N-WASP.

### Co-immunoprecipitation

CHO-eNOS cells infected with SFV-NOSTRIN-GFP or SFV-GFP (control) for 7 hours were lysed in Triton buffer containing 60 mM *N*-octylglycoside. After pre-clearing with Pansorbin™ (Calbiochem), the lysates were incubated at 4°C overnight with rabbit anti-GFP (Clontech) followed by incubation with Protein-A-Sepharose (Amersham) for 2 hours. Precipitates were washed three times with Triton buffer, eluted with sample buffer and analyzed by western blotting using mouse antibodies to GFP, eNOS and dynamin-2, and rabbit anti N-WASP.

### Gel filtration

A Superdex 200 HR column connected to ÄKTApurifier HPLC system (Amersham) was pre-equilibrated with PBS and calibrated with molecular mass standards (Amersham). Purified recombinant GST-$NOSTRIN_{242\text{-}506}$ in PBS was loaded onto the column and eluted at a flow rate of 0.5 ml/minutes. Elution of protein was monitored by measuring absorbance at 280 nm. The peak fractions (fraction volume 300 μl) were collected and analyzed by western blotting using anti-GST.

### Confocal immunofluorescence microscopy

CHO-eNOS cells on coverslips were transiently transfected with pcDNA3.1-NOSTRIN or pCMV-NOSTRIN-ΔSH3 plus pEGFP-dynamin-2 or pEGFP-N-WASP for 24-28 hours. For treatment with cytochalasin D, cells on coverslips were infected with SFV-NOSTRIN for 6 hours, followed by a 2 hour incubation with 2 μM cytochalasin D. Cells were fixed with ice-cold methanol for 7 minutes, blocked with BPT (1% BSA, 0.1% Tween20 in PBS), incubated with primary antibodies (dilution 1:100 in BPT if not stated otherwise), and probed with Cy3-, Cy5- and/or FITC-coupled secondary antibodies (Jackson Immunoresearch, West Grove, PA). Phalloidin-Alexa 546 was added during incubation with primary antibodies. The coverslips were embedded in Gelmount mounting media (Biomeda, Foster City, CA) and analyzed with an LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany).

### Computational analysis

Analysis of protein domain structures was performed by comparing results of SMART (Simple Modular Architecture Research Tool; EMBL, Heidelberg, Germany) and ScanProsite software (Swiss Institute of Bioinformatics). Alignment searches were done with ClustalW, Prosite, Smart, Pfam and BLAST, and used for phylogenetic tree construction applying Phylodraw0.8 software (Graphics Application Lab., Pusan National University, South Korea).

## Results

### NOSTRIN belongs to the PCH family of proteins

Recently, we described a novel eNOS-interacting protein, NOSTRIN, which influences both localization and activity of the enzyme. Sequence analysis revealed that NOSTRIN is related most closely to proteins of the PCH family, which comprises proteins such as human pacsin and its rat ortholog syndapin, and human CD2BP1 and its mouse ortholog PSTPIP (Fig. 1A,B). Typically PCH proteins are involved in endocytic processes and/or rearrangement of the (actin) cytoskeleton (Kessels and Qualmann, 2004; Lippincott and Li, 2000). Members of the PCH family of proteins are defined by a characteristic domain composition comprising an N-terminal Fes/CIP4 homology (FCH) domain, a C-terminal SH3 domain and one or two coiled-coil domains (Fig. 1A). NOSTRIN, FBP17, CIP4 and Toca-1 additionally contain an HR1 (protein kinase C-related kinase homology region1) motif in their central region that was originally described as a Rho GTPase-interacting module. The HR1 motifs of CIP4 and Toca-1 interact with Cdc42, and that of FBP17 interacts with Rnd2 (Fujita et al., 2002; Ho et al., 2004; Tian et al., 2000). For NOSTRIN, the function of the HR1 motif remains to be elucidated. The overall sequence identity within the PCH family is rather low, ranging between 15 and 20%; however, single domains display a higher degree of homology. For example the SH3 domains of NOSTRIN and pacsins display an identity of about 37%. Like most proteins of the PCH family, NOSTRIN is well conserved throughout eukaryotes. Rat and mouse orthologs share 81-82% sequence identity with the human protein. NOSTRIN orthologs have also been identified in *D. melanogaster* (33% identity) and *C. elegans* (20%) demonstrating that NOSTRIN is evolutionarily well conserved.

### NOSTRIN forms homooligomers

Several members of the PCH family of proteins have been shown to homooligomerize, presumably facilitating simultaneous interaction with multiple binding partners (Kessels and Qualmann, 2004). We hypothesized that NOSTRIN may also form oligomers, thereby functioning as a multivalent adaptor for recruiting other factors to its complex with eNOS. To test this notion, we applied a pull-down assay where immobilized $(His)_6$-tagged NOSTRIN was incubated with purified GST-NOSTRIN or GST as control. GST-NOSTRIN but not GST co-precipitated with $(His)_6$-tagged NOSTRIN, which suggests a direct NOSTRIN-NOSTRIN

interaction (Fig. 2A). To confirm this observation and to analyze how the various subdomains of NOSTRIN contribute to oligomerization, yeast two-hybrid analysis was employed. Full-size NOSTRIN and several deletion constructs of NOSTRIN (Fig. 2B) were cloned into bait (pEG) and prey (pJG) vectors, respectively, and probed in a yeast mating assay. In addition to full-size NOSTRIN, the C-terminal fragments $NOSTRIN_{242-506}$, $NOSTRIN_{250-434}$ and $NOSTRIN_{323-470}$ were found to self-interact strongly as indicated by intense blue staining in an X-Gal assay (Fig. 2C). Judging by growth and blue staining, binding of full-length NOSTRIN to itself and its fragments appears to be weaker than among the truncated forms themselves; this unexpected observation correlates with a lower expression level of full-length NOSTRIN, as shown by western blotting of total yeast lysates (data not shown). In contrast to the C-terminal fragments of NOSTRIN, $NOSTRIN_{1-288}$ did not interact with itself and displayed only a weak interaction with full-size NOSTRIN and the C-terminal fragments $NOSTRIN_{242-506}$ and $NOSTRIN_{250-434}$. Likewise, the very C-terminal SH3-domain ($NOSTRIN_{433-506}$) failed to induce blue staining beyond background. These results suggest that the region spanning positions 250-434 of NOSTRIN plays a key role in NOSTRIN oligomerization. Of note, this region contains a coiled-coil domain, i.e. a motif often involved in oligomer formation (Burkhard et al., 2001).

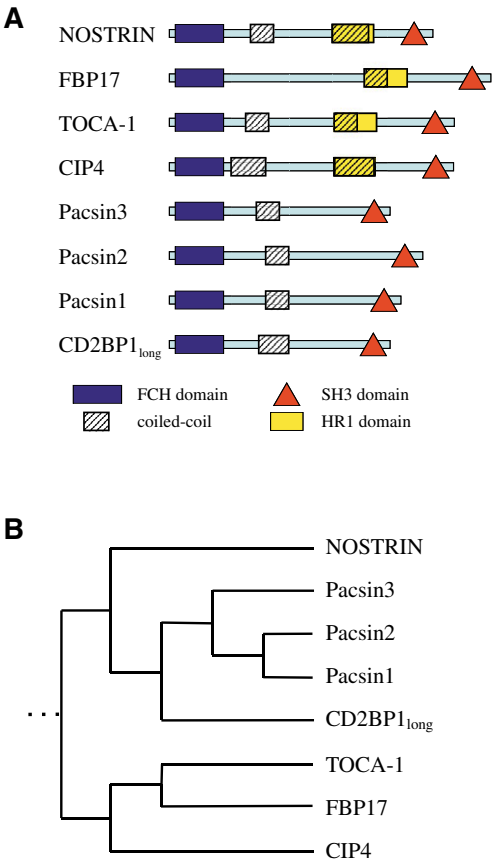


**Fig. 1.** NOSTRIN belongs to the PCH family of proteins. (A) Domain structure of selected members of the human PCH protein family. The proteins share a characteristic composition comprising an N-terminal FCH domain, a C-terminal SH3 domain and a coiled-coil domain located C-terminally of the FCH domain. Some members including NOSTRIN, FBP17, CIP4 and Toca-1 also contain an HR1 motif, which harbors a coiled-coil domain and has been described as a Rho GTPase binding unit. (B) Phylogenetic tree of PCH family proteins. The phylogenetic tree was constructed by alignment of sequence data of denoted proteins (ClustalW), applying the Phylodraw0.8 software. According to this, pacsins represent the closest relatives of NOSTRIN.

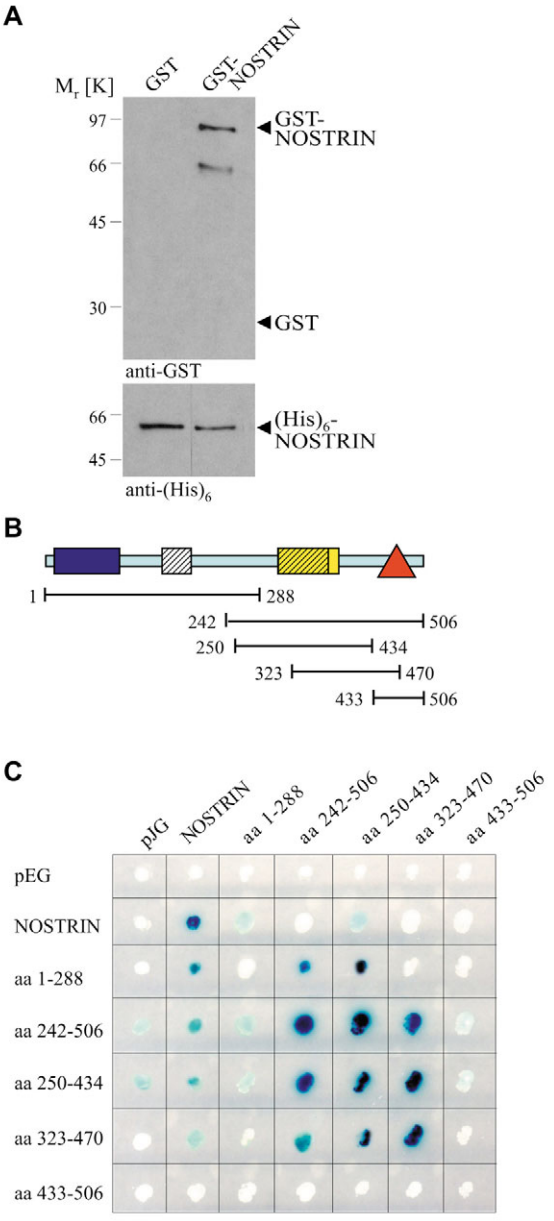


**Fig. 2.** NOSTRIN is able to self-associate. (A) Immobilized $(His)_6$-tagged NOSTRIN was incubated with purified GST-NOSTRIN, or GST as control. Matrix-bound proteins were eluted and analyzed by immunoblotting using anti-GST and anti-$(His)_6$. (B) NOSTRIN constructs used in the yeast two-hybrid assay. (C) A yeast mating assay was performed using the Matchmaker system (Clontech). Interaction of proteins was judged by growth and blue staining on 4× deficient plates (–His, –Ura, –Trp, –Leu) containing X-Gal.

### NOSTRIN forms trimers both in vitro and in vivo

To determine the stoichiometry of NOSTRIN oligomers, GST-NOSTRIN purified from *E. coli* was subjected to gel filtration. In this assay, GST-NOSTRIN$_{242\text{-}506}$ lacking the N-terminal FCH and coiled-coil domain was analyzed since full-size GST-NOSTRIN tended to aggregate into insoluble clusters. Following purification of GST-NOSTRIN$_{242\text{-}506}$ (57 kDa), the protein was applied to size exclusion chromatography; fractions were collected and analyzed by western blotting. GST-NOSTRIN$_{242\text{-}506}$ eluted predominantly as a complex of approximately 170 kDa, matching the size of the trimer (Fig. 3A). A minor fraction of GST-NOSTRIN$_{242\text{-}506}$ was present as a dimer of 115 kDa, whereas the NOSTRIN monomer of 57 kDa was almost undetectable in the eluate.

To explore NOSTRIN oligomerization in cells, we incubated lysates of COS cells overexpressing full-size NOSTRIN (58 kDa) with DSS, which covalently links interacting proteins. Western blotting revealed that, in the DSS-treated sample, a complex of approximately 180 kDa was stabilized by crosslinking which, judged by its apparent size, might well represent a NOSTRIN trimer (Fig. 3B). Under these conditions, a minor fraction of NOSTRIN was present as a complex of 120 kDa, which corresponds to the size of the dimer, while a major portion was present in the monomeric form. These data are in line with the results from gel filtration, pointing to equilibrium between NOSTRIN monomer, dimer and trimer. In this crosslinking experiment, additional bands were seen at approximately 140 kDa and 200 kDa, which may represent complexes of NOSTRIN with other interacting proteins.

In the yeast two-hybrid assay, a truncated form of NOSTRIN comprising positions 250-434 (cf. Fig. 2B) was sufficient for self-association. Consequently, we repeated the crosslinking experiment with this 24 kDa fragment and found that it prevalently occurred as a trimer and dimer (approximately 70 kDa and 50 kDa, respectively), as does full-length NOSTRIN (Fig. 3C, left panel). Consistent with the results of the yeast mating assay, the C-terminal fragment NOSTRIN$_{433\text{-}506}$ failed to oligomerize, being present exclusively as a 12 kDa monomer (Fig. 3C, right). Together, these findings demonstrate that NOSTRIN self-associates preferentially to a homotrimeric complex, and that NOSTRIN$_{250\text{-}434}$ holds the minimum trimerization motif.

### NOSTRIN binds to dynamin and N-WASP via its SH3 domain

Having established that NOSTRIN oligomerizes, we next asked what the consequences of this self-association might be. We have previously shown that NOSTRIN interacts with eNOS via its SH3 domain (Zimmermann et al., 2002). Thus, formation of NOSTRIN oligomers would allow for the recruitment of other interaction partners to the eNOS-NOSTRIN complex via the SH3 domain. Given the fact that the PCH family proteins represent the closest relatives to NOSTRIN, we asked whether NOSTRIN is able to interact with dynamin or N-WASP, one or both of which have been shown to interact with PCH proteins (Ho et al., 2004; Kessels and Qualmann, 2004; Tian et al., 2000). To follow this idea, we used GST-NOSTRIN and lysates from CHO-eNOS cells that stably express eNOS. Under these conditions, GST-NOSTRIN pulled down eNOS, dynamin as well as N-WASP (Fig. 4A). Moreover, the same proteins were found in precipitates from lysates of CHO-eNOS cells infected with

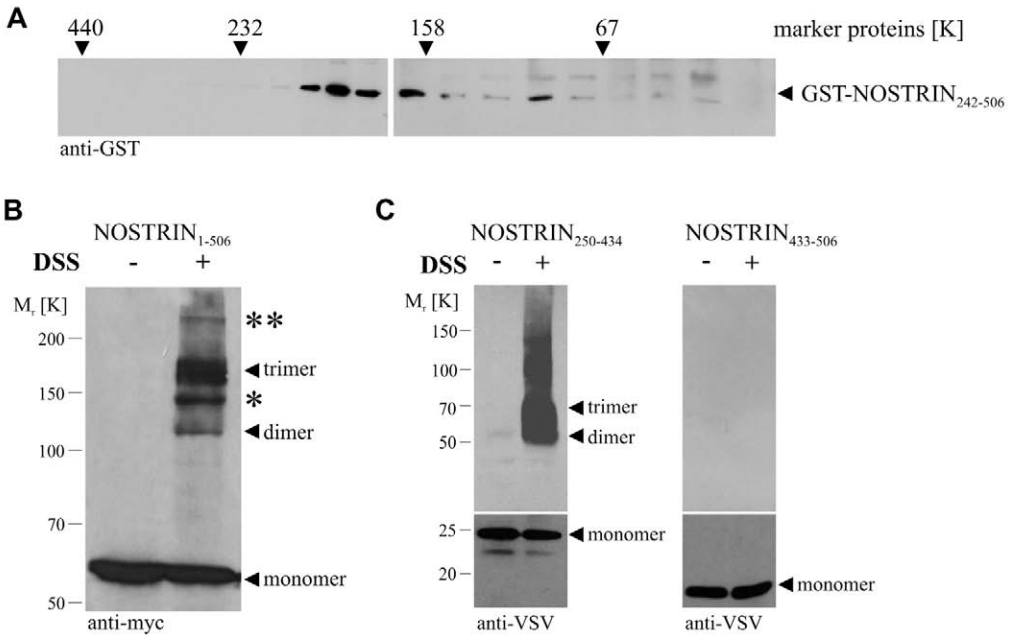


**Fig. 3.** NOSTRIN trimerizes in vitro and in vivo. (A) Gel filtration of purified recombinant GST-NOSTRIN$_{242\text{-}506}$. Fractions were collected and analyzed by SDS-PAGE and western blotting using anti-GST. (B,C) Lysates of COS cells transiently transfected with Myc/(His)$_6$-tagged full-size NOSTRIN or VSV-tagged NOSTRIN$_{250\text{-}434}$ and NOSTRIN$_{433\text{-}506}$, respectively, were treated with 0.3 mM DSS or mock-treated for control. Samples were analyzed by western blotting using anti-Myc or anti-VSV, respectively. The asterisks mark non-identified complexes of NOSTRIN.

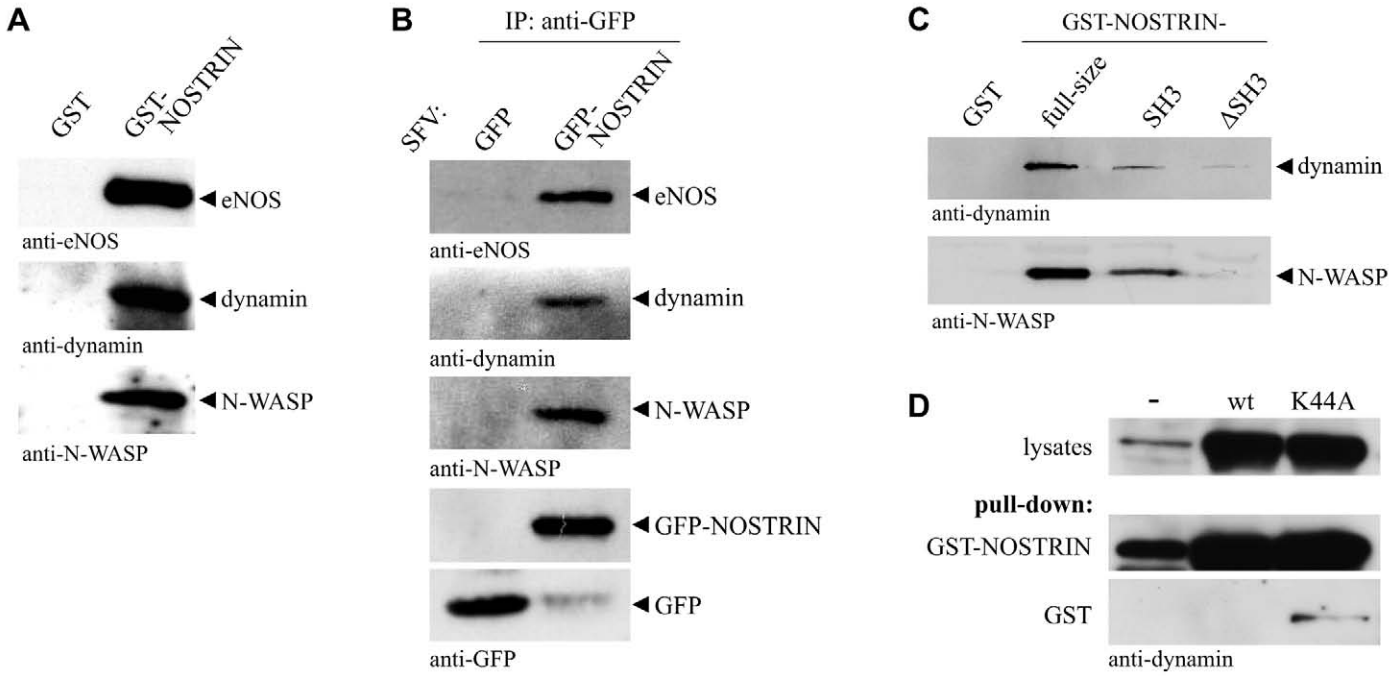


**Fig. 4.** NOSTRIN, by means of its SH3 domain, interacts with multiple proteins. (A) GST-NOSTRIN was used in a pull-down assay using lysates of CHO-eNOS cells. GST alone served as control. (B) Co-immunoprecipitation from CHO-eNOS cells infected with SFV-NOSTRIN-GFP or SFV-GFP (control), using anti-GFP. (C) Lysates of COS cells transfected with dynamin cDNA for 24 hours (upper panel) or untransfected (lower) were subjected to precipitation with GST-NOSTRIN, GST-NOSTRIN-SH3, GST-NOSTRIN-ΔSH3 or GST alone. (D) Immobilized GST-NOSTRIN and GST were incubated with lysates of COS cells expressing wild-type dynamin (wt) or the dominant-negative mutant dynamin-K44A (K44A). Empty vector (–) served as control.

SFV-NOSTRIN-GFP, using anti-GFP for immunoprecipitation (Fig. 4B). These findings point to the possibility that (multimeric) NOSTRIN, eNOS, dynamin and N-WASP form a larger protein complex within CHO-eNOS cells.

To determine whether the SH3 domain of NOSTRIN indeed represents the binding domain for dynamin and N-WASP and to show that binding of these two proteins to NOSTRIN is independent of eNOS, truncated versions of NOSTRIN were used in a pull-down assay using COS cells that do not express eNOS. Both dynamin and N-WASP were precipitated by full-size NOSTRIN and its SH3 domain but not by NOSTRIN-ΔSH3, indicating that the SH3 domain is necessary and sufficient for the interaction of NOSTRIN with dynamin and N-WASP (Fig. 4C). We also did a pull-down assay with lysates of cells overexpressing wild-type dynamin or its K44A mutant known to be defective in GTP binding. GST-NOSTRIN precipitated dominant-negative mutant K44A as well as wild-type dynamin (Fig. 4D), indicating that GTP binding is not a prerequisite for the interaction. The weak band observed in untransfected cells represents endogenous dynamin that was also present in the lysates. Finally, we also observed interaction of dynamin with NOSTRIN via the SH3 domain in a yeast two-hybrid assay (data not shown), confirming direct binding of the two proteins.

## NOSTRIN assembles eNOS and dynamin into common structures

Next, we set out to analyze the functional relationship of NOSTRIN, dynamin and eNOS in living cells. For this purpose, CHO-eNOS cells were used, which display a typical distribution of eNOS where the enzyme is mainly at the plasma membrane and at the Golgi complex (Fig. 5G). We analyzed these cells at 24 hours post-transfection to keep expression levels of dynamin moderate. Under these conditions, NOSTRIN localized to various subcellular structures – the plasma membrane, vesicular structures throughout the cytoplasm and to a network of elongated structures – with rather one or the other appearance prevailing in different cells (cf. Fig. 5B, Fig. 6B). With this moderate expression of NOSTRIN, eNOS was translocated to intracellular structures and was to a lesser extent found at the plasma membrane, although translocation of eNOS was not complete (Fig. 5A-D; Fig. 6A-D, co-localization, pink). In accordance with previous reports (Cao et al., 1998), dynamin-2-GFP localized to punctuate spots at the cell periphery (Fig. 5E). These dynamin-positive structures were mainly devoid of eNOS staining in the absence of NOSTRIN (Fig. 5E-H; co-localization, light blue). However, when dynamin-2-GFP and NOSTRIN were co-expressed in CHO-eNOS cells, NOSTRIN, dynamin and eNOS co-localized extensively in distinct structures mainly at the cell periphery (Fig. 5I-L; triple co-localization, white). As expected from the pull-down assays, NOSTRIN-ΔSH3 was not able to induce this co-localization (Fig. 5M-P). Expression of the ΔSH3-protein did not change localization of either eNOS or dynamin-2-GFP (Fig. 5P). These observations suggest that NOSTRIN, in a process requiring multimerization, is able to bring together dynamin and eNOS to common structures.

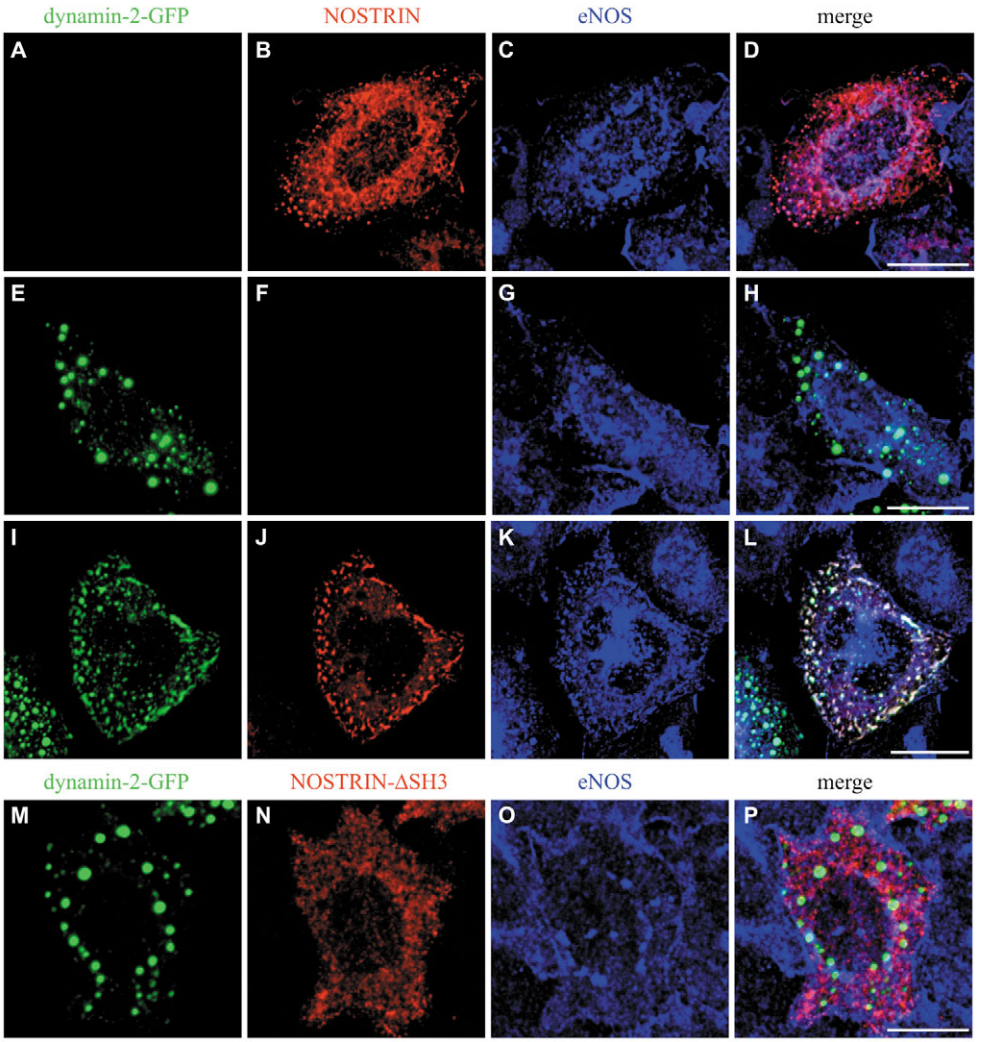


**Fig. 5.** Dynamin and eNOS are assembled into common structures by NOSTRIN in a process requiring its SH3 domain. CHO-eNOS cells stably expressing eNOS were transiently transfected with cDNA encoding NOSTRIN (A-D), dynamin-2-GFP (E-H), NOSTRIN and dynamin-2-GFP (I-L), or NOSTRIN-ΔSH3 and dynamin-2-GFP (M-P). Following fixation, cells were stained with anti-NOSTRIN (B,J,N) and anti-eNOS (C,G,K,O). (E,I,M) Fluorescence of GFP-fused dynamin-2. (D,H,L,P) Merged images of each row. (A-D) NOSTRIN and eNOS co-localize at vesicular and elongated structures throughout the cytoplasm. (E-H) In the absence of NOSTRIN, eNOS and dynamin-2-GFP hardly overlap. (I-L) Upon co-expression of dynamin-2-GFP and NOSTRIN, dynamin and eNOS are brought together at common structures. (M-P) NOSTRIN-ΔSH3 does not affect localization of either eNOS or dynamin-2-GFP. Bars, 10 μm.

### NOSTRIN, N-WASP and eNOS co-localize at actin filaments

To study the functional relevance of the interaction between NOSTRIN and N-WASP with regard to eNOS, we co-expressed NOSTRIN and N-WASP-GFP in CHO-eNOS cells. Consistent with previous findings in other cell lines (Vetterkind et al., 2002), N-WASP-GFP was diffusely distributed throughout the cytoplasm and the nucleus (Fig. 6E). There was no apparent overlap of N-WASP-GFP with eNOS, which remained in its characteristic location at the plasma membrane and at the Golgi complex (Fig. 6E-H). Co-expression of NOSTRIN with N-WASP-GFP significantly changed this picture in that NOSTRIN and N-WASP were associated with a network of elongated structures to which eNOS co-localized as well (Fig. 6I-L). These observations suggest that NOSTRIN, which partially localized to filamentous structures by itself (Fig. 6B), may recruit N-WASP from its diffuse cytoplasmic localization to these structures. We also noted that N-WASP expression may increase the degree of branching of these structures (compare Fig. 6B,J), which most likely run along actin filaments (see below). A co-precipitation assay confirmed that NOSTRIN is indeed required to achieve interaction of eNOS and N-WASP-GFP (data not shown).

To date, the best characterized function of N-WASP is to recruit the Arp2/3 complex, thereby initiating actin filament polymerization (Stradal et al., 2004). Bearing this in mind, we tested whether the elongated structures to which NOSTRIN recruits N-WASP and eNOS indeed overlap with actin. Staining of CHO-eNOS cells transfected with N-WASP and NOSTRIN confirmed this notion showing a profound co-localization of N-WASP, NOSTRIN and phalloidin-labeled actin filaments (Fig. 6M-P). Taken together, these observations indicate that co-expression of N-WASP-GFP, NOSTRIN and eNOS in CHO cells leads to co-localization of this protein triad at structures running along actin filaments.

### NOSTRIN-mediated translocation of eNOS requires an intact actin cytoskeleton

Co-localization of NOSTRIN and eNOS with N-WASP and phalloidin suggested that rearrangement of the actin cytoskeleton may be necessary for NOSTRIN-mediated processes. To test this hypothesis, we used cytochalasin D to depolymerize the actin cytoskeleton (Cooper, 1987) and analyzed the consequences of this treatment. Again, CHO-

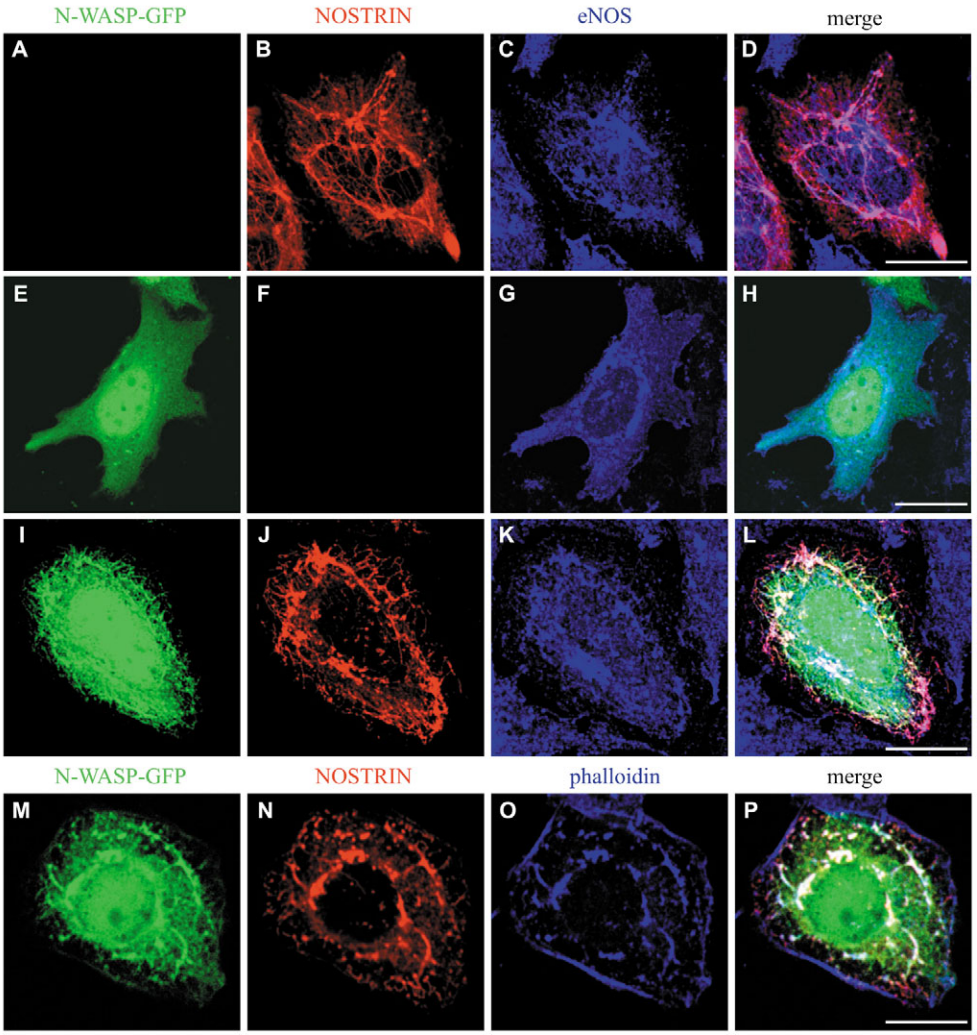


**Fig. 6.** NOSTRIN, eNOS and N-WASP co-localize at actin filaments. CHO-eNOS cells were transiently transfected with cDNA encoding NOSTRIN (A-D), N-WASP-GFP (E-H), or NOSTRIN and N-WASP-GFP (I-P). Following fixation, cells were stained with anti-NOSTRIN (B,J,N), anti-eNOS (C,G,K) and phalloidin (O). (E,I,M) Fluorescence of GFP-fused N-WASP. (D,H,L,P) Merged images of each row. (A-D) Upon moderate expression, NOSTRIN localizes to vesicular and filamentous structures, where it co-localizes with eNOS. (E-H) When N-WASP-GFP is expressed alone, it is mainly diffusely dispersed in the cytoplasm and does not co-localize with eNOS. (I-L) Co-expression with NOSTRIN brings N-WASP-GFP to eNOS-positive, mostly filamentous structures. (M-P) NOSTRIN and N-WASP-GFP co-localize at structures running along phalloidin-labeled actin filaments. Bars, 10 μm.

eNOS cells were used, this time infected with SFV-NOSTRIN to reach a high expression level of NOSTRIN (Zimmermann et al., 2002) resulting in a near-complete redistribution of eNOS to intracellular structures positive for NOSTRIN (Fig. 7A-C). Upon destabilization of the actin cytoskeleton with cytochalasin D, localization of NOSTRIN changed dramatically to large structures residing at the cell periphery (Fig. 7D-F). Along with NOSTRIN, eNOS also localized to these structures suggesting that disruption of the actin cytoskeleton arrests the complex of NOSTRIN and eNOS at the cell periphery (Fig. 7F, arrowheads). These findings indicate that the redistribution of eNOS to intracellular compartments, observed upon overexpression of NOSTRIN, most likely represents internalization of eNOS from the plasma membrane, rather than a halt of eNOS on its way to the cell surface.

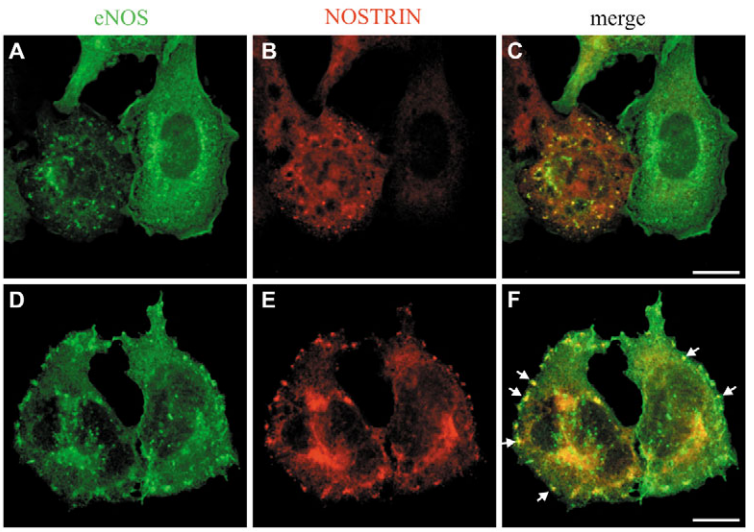


**Fig. 7.** NOSTRIN-dependent translocation of eNOS requires an intact actin cytoskeleton. CHO-eNOS cells infected with SFV encoding NOSTRIN for 8 hours were treated with cytochalasin D within the last 2 hours of infection (D-F) or mock-treated (A-C) as control. After fixation, cells were stained with anti-NOSTRIN (B,E) and anti-eNOS (A,D). NOSTRIN and eNOS co-localize in intracellular vesicle-like structures of untreated cells (A-C), whereas they accumulate in large patches in the periphery of cytochalasin-treated cells (arrows) (D-F). Bars, 10 μm.

## Discussion

The past few years saw great progress in unraveling mechanisms of eNOS regulation, which proved to be mainly governed by interacting proteins. These eNOS regulators either directly associate with the enzyme, as proposed for caveolin-1 and hsp90, or reversibly modify eNOS, as described for a number of kinases (Balligand, 2002; Gratton et al., 2004; Sessa, 2004). Growing evidence suggests that the capability to be activated and the resultant activity of eNOS strongly depend on the enzyme's whereabouts within the cell, yet the molecular mechanisms orchestrating the intracellular targeting processes of eNOS have remained largely unknown (Fulton et al., 2004; Shaul, 2002). Recently, we discovered a novel binding partner for eNOS, which we called NOSTRIN (eNOS trafficking inducer) due to the observation that, upon overexpression of NOSTRIN, eNOS translocates from the plasma membrane to intracellular vesicular structures (Zimmermann et al., 2002). In support of this notion the data we presented herein provide insight into the molecular details of NOSTRIN-mediated eNOS trafficking.

NOSTRIN classifies as a novel member of the PCH family of proteins. Similar to pacsins/syndapins (Kessels and Qualmann, 2004), NOSTRIN interacts with the large GTPase dynamin and the Arp2/3 activator N-WASP via its SH3 domain. Since there is a growing list of SH3-containing proteins binding to dynamin and N-WASP, we asked what the specific function of NOSTRIN might be in this context. The answer was provided by co-expression studies of NOSTRIN with dynamin or N-WASP in CHO-eNOS cells. Here, NOSTRIN was able to bring together dynamin and eNOS to common vesicular and tubular structures, suggesting that it is critically involved in the endocytosis of eNOS. In principal, SH3-containing proteins may have opposing effects on dynamin-mediated endocytosis. For instance, amphiphysin enhances dynamin activity (Takei et al., 1999), whereas endophilin perturbs dynamin-mediated vesiculation (Farsad et al., 2001). Given that high levels of NOSTRIN drive eNOS away from the plasma membrane, the functional outcome of the interaction between NOSTRIN and dynamin is likely to be enhanced internalization of eNOS.

Previous reports have described a direct interaction of dynamin and eNOS leading to an increased catalytic activity of eNOS in vitro (Cao et al., 2001; Cao et al., 2003). Subsequently, it was proposed that dynamin may also be involved in the internalization of eNOS from the plasma membrane through a mechanism that does not necessarily involve physical interaction between the two proteins (Chatterjee et al., 2003). Accordingly, the localization of dynamin with respect to eNOS varies in different cell types. For instance, in bovine aortic endothelial cells, eNOS and dynamin co-localize at the Golgi apparatus but do not display any overlap at the plasma membrane (Cao et al., 2001). It has been suggested that dynamin only performs its direct activating function towards eNOS in the Golgi localization, whereas it is indirectly required, for example, following stimulation with bradykinin, when eNOS is redistributed from the plasma membrane to vesicular structures (Chatterjee et al., 2003; Thuringer et al., 2002). In CHO-eNOS cells lacking endogenous NOSTRIN we could hardly detect any co-localization of eNOS with dynamin-2-GFP, whereas co-expression of NOSTRIN resulted in a strong co-localization of the two proteins. Under these conditions, NOSTRIN may assist in recruiting a crucial number of dynamin molecules required to drive vesicle fission and endocytosis of eNOS. It appears that dynamin, together with NOSTRIN, rather has a role in endocytosis of eNOS than in regulation of its enzymatic activity since NO release was only marginally affected by expression of dynamin-GFP, both in the absence and presence of NOSTRIN (A.I. and K.S., unpublished).

Recently, it was reported that the mouse ortholog of NOSTRIN interacts with Disabled-2 (Dab2) through docking of its SH3 domain to a proline-rich region of Dab2 (Choi et al., 2005). The multi-domain protein Dab2 has been proposed to function as an endocytic adaptor by linking cargo proteins, the endocytic adaptor complex-2 (AP-2) and clathrin (Mishra et al., 2002; Morris and Cooper, 2001). Thus, the reported interaction between NOSTRIN and Dab2 is well in line with a role for NOSTRIN in endocytosis, although this issue was not tackled by the authors (Choi et al., 2005).

Our results herein establish that N-WASP represents another important interaction partner of NOSTRIN in CHO-eNOS cells. When transfected alone, N-WASP was dispersed throughout the cytoplasm and the nucleus. Upon co-expression of NOSTRIN, we observed N-WASP to be associated with elongated structures running along actin filaments, where it co-localized with NOSTRIN. This finding suggests that NOSTRIN can activate N-WASP which would in turn recruit the Arp2/3 complex, thereby initiating actin filament polymerization. For full-blown activation of N-WASP, other SH3-containing proteins have been shown to cooperate with either small GTPases or $PIP_2$ (Caron, 2002; Ho et al., 2004). Since NOSTRIN, like Toca-1, contains an HR1 motif, it may analogously recruit a member of the Rho family of GTPases to enhance activation of N-WASP. So far, the identity of the putative NOSTRIN-interacting Rho GTPase remains elusive. It should be noted that RhoA appears to be involved in targeting of eNOS to caveolae, in a process stimulated by low density lipoprotein (Zhu et al., 2003); however, the question of the molecular link between eNOS and RhoA has not been addressed to date. Following these lines, we currently analyze the interaction of NOSTRIN with various small GTPases.

Growing experimental evidence indicates that, during pinching off of endocytic vesicles, dynamin action and actin polymerization are tightly coupled (Merrifield, 2004). Thus, endocytic effectors such as SH3-containing proteins would be required to interact simultaneously with both dynamin and N-WASP. To perform this function, they would either have to contain multiple SH3 domains, as found in intersectin and Grb2 (Carlier et al., 2000; Yamabhai et al., 1998), or to oligomerize, as was reported for pacsins/syndapins (Kessels and Qualmann, 2004) and NOSTRIN (this study). Similarly to NOSTRIN, the chicken ortholog of pacsin/syndapin (i.e. FAP52) mainly occurs as a trimer in vitro and in vivo (Nikki et al., 2002). Against this background, one might speculate about potential heteromer formation among members of the PCH family; however, we failed to observe a relevant interaction between NOSTRIN and pacsin-1, i.e. the human ortholog of FAP52.

One of the major issues remaining to be solved concerning NOSTRIN-mediated internalization of eNOS is what type of endocytosis we are dealing with. For other PCH proteins,

current knowledge exclusively indicates a role in clathrin-mediated endocytosis (Kessels and Qualmann, 2004). Importantly, eNOS is associated with caveolae at the plasma membrane by direct binding to caveolin-1 (Feron et al., 1996; Ghosh et al., 1998; Ju et al., 1997). Although association with caveolin has so far only been considered a mechanism of enzymatic inhibition, it is tempting to speculate that caveolae may also play a role in the internalization of eNOS. For caveolar endocytosis, dynamin recruitment as well as actin rearrangement has been shown to be essential (Pelkmans et al., 2002). In line with this, our data support a role for NOSTRIN as a multivalent adaptor protein linking eNOS to dynamin-mediated vesicle fission and to reorganization of the actin cytoskeleton by N-WASP.

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