INDUCTION OF CELL MEMBRANE PROTRUSIONS BY THE N-TERMINAL GLUTAREDOXIN DOMAIN OF A RARE SPLICE VARIANT OF HUMAN THIOREDOXIN REDUCTASE 1

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The human thioredoxin system has a wide range of functions in cells including regulation of cell proliferation and differentiation, immune system modulation, antioxidant defense, redox control of transcription factor activity, and promotion of cancer development. A key component of this enzymatic system is the selenoprotein thioredoxin reductase 1 (TrxR1), encoded by the *TXNRD1* gene. Transcription of *TXNRD1* is subjected to extensive alternative splicing, leading to a number of transcripts also encoding isoforms of TrxR1 that differ from each other at their N-terminal domains. Here we have studied the *TXNRD1_v3* isoform containing the atypical N-terminal glutaredoxin (Grx) domain. Expression of the transcript of this isoform was found predominantly in testis but was also detected in ovary, spleen, heart, liver, kidney and pancreas. By immunohistochemical analysis in human testis with antibodies specific for the Grx domain of TXNRD1_v3 the protein was found to be predominantly expressed in the Leydig cells. Expression of the TXNRD1_v3 transcript was also found in several cancer cell lines (HCC1937, H23, A549, U1810 or H157), and in HeLa cells it was induced by estradiol or testosterone treatments. Surprisingly, GFP fusions with the complete TXNRD1_v3 protein or with only its Grx domain localized to distinct cellular sites in proximity to actin and furthermore, had a potent capacity to rapidly induce cell membrane protrusions. Analyses of these structures suggested that the Grx domain of TXNRD1_v3 localizes first in the emerging protrusion and is then followed into the protrusions by actin and subsequently by tubulin. The results presented here thus reveal that TXNRD1_v3 has a unique and distinct expression pattern in human cells and suggest that the protein can guide actin polymerization in relation to cell membrane restructuring.

INTRODUCTION

The thioredoxin system involves several thioredoxins (Trx[1]) and thioredoxin reductases (TrxR) that are found in different cellular and subcellular locations where they participate in a wide range of functions in cells, including

protection from oxidative stress, synthesis of DNA precursors, regulation of cell proliferation and differentiation, control of apoptosis, modulation of the immune system, redox control of transcription factor activity and promotion of cancer development (1-6). The many functions of the thioredoxin system are mainly carried out by the interaction of thioredoxins with target proteins, most often involving redox reactions whereby thioredoxin reduces a disulfide in the target protein. For this purpose, the active site disulfide thioredoxin has first to be reduced to a dithiol, which is catalyzed by TrxR using NADPH, and hence, making TrxR a key player for essentially all downstream cellular functions regulated by the thioredoxin system.

In human cells, three genes encode thioredoxin reductases, namely *TXNRD1*, *TXNRD2* and *TXNRD3*. The classical TrxR1 enzyme, encoded by the *TXNRD1* gene, is predominantly cytosolic and is expressed in most, if not all, human cells, where Trx1 is believed to be its major substrate (4,5,7,8). The *TXNRD2* gene encodes mitochondrial TrxR2 that reduces mitochondrial Trx2 (9-11) although TrxR2 is also found in the cytosol (12) but seems to have non-ubiquitous lower expression level compared to TrxR1 (see (13) and www.proteinatlas.org). Both cytoplasmic and mitochondrial thioredoxin systems are essential for mammals, as shown by the embryonic lethal phenotypes of the respective knockout mice (14,15). Thioredoxin reductases TrxR1 and TrxR2 are both homodimeric selenoprotein oxidoreductases containing FAD and having a carboxylterminal –Gly-Cys-Sec-Gly active site motif (where Sec is selenocysteine), which is providing the thioredoxin-reducing activity (16-21). The thioredoxin reductase isoenzyme TGR, encoded by the *TXNRD3* gene, is similar to TrxR1 and TrxR2, but contains an additional monothiol Grx domain as an N-terminal addition to the TrxR-module. The TGR enzyme is mainly expressed in early spermatids in testis and is suggested to participate in the redox reactions required during maturation of sperm (22-24).

All three human thioredoxin reductase genes are subject to extensive splicing, mainly in the 5'-end, leading to a number of transcripts that in several cases encode different protein isoforms (7,8,12,25,26). In the present study, we have characterized further a rare alternative transcript derived from the *TXNRD1* gene that expresses an atypical N-terminal Grx domain fused to the TrxR1 module and encoded by alternative exons located upstream of the core promoter. Adhering to the generally proposed nomenclature for human genes, we originally named the transcript b1 and its encoded protein isoform TXNRD1_v3 (7) and will keep to this nomenclature in the present paper. For ease of readability, the TXNRD1_v3 protein is hereafter referred to as version three ("v3") of TrxR1. In **Scheme 1**, the exon organization of the 5'-region of the v3 transcript is depicted and compared to the other main isoforms of human TrxR1, with the localization of the different primers and probes used in this study also shown in the scheme.

The Grx domain of v3 has an atypical CTRC active site sequence (7,25), instead of the more common CPYC motif found in Grx's (27). This Grx domain of v3 was earlier found to lack activity in any of the classical Grx assays, except if the CTRC active site was mutated to the classical CPYC motif whereupon the protein gained typical Grx activity (25). The rare cDNA or EST clones found to be encoding v3 have thus far only been derived from testis (7,25). Interestingly, the transcripts encoding this isoform can be found only in human, chimpanzee and dog, but not in mouse or rat (25). Furthermore, expression of the v3 protein was recently detected in a human mesothelioma cell line using immunoblotting and mass spectrometry (28). To date, no information exists about the possible function(s) of v3 in human cells, which has been the focus of the present study.

EXPERIMENTAL PROCEDURES

Northern blot analysis

Human multiple tissue Northern blots with poly(A+) RNA from different tissues were purchased from Clontech. A PCR fragment composed of the three first exons of the Grx domain of the TXNRD1_v3 was labeled with [α-^{32}P]dCTP (Rediprime random primer labeling kit; Amersham Pharmacia Biotech) and hybridized at 65°C overnight in ExpressHyb Solution (Clontech) following the protocol. The blots were also hybridized with human glyceraldehyde-3-phosphate dehydrogenase as control. The blots were scanned and quantified with the Gel Pro

Analyzer program (Media Cybernetics).

PCR analyses

First strand cDNA panels from various tissues were obtained from Clontech. The β1 variant cDNA cloned into a pET vector (Novagen) served as a positive control when used as template in the PCR reactions. The cDNA of the different cancer cell lines used here was generously provided by Christopher Horst Lillig and Maria Lönn (Karolinska Institutet). The following primers were used: R5, 5'-GCA ACC CAC ATT CAC ACA TGT TCC TCC-3'; R2, 5'-GGA CCA TGG CCG CCT ATC TTT CTC TGT TTC ACA AAC AC-3'; FI, 5'-GAG TCC TGA AGG AGG GCC TGA TGT CTT CAT CAT TCT C-3'; and $F\beta_{-VIII}$, 5'-CAA GCA CAA AAT AAG GAA CAC ACA GAG ATT TAA ATA ACA G-3'. For PCR reactions 2.5 U/reaction Taq polymerase, 10 μM of each primer and 2-3 μl cDNA template were used. Reactions were heated to 96°C for 1 min before the first of 35 cycles (94°C for 30 sec, 58°C for 45 sec and 72°C for 2 min) followed by 72°C for 3 min after the final cycle and then paused at 4°C. PCR products were analyzed on 1.2% agarose gels with 5 μl sample loaded. Sequencing reactions of some DNA bands were performed to verify the sequence of PCR products.

Immunohistochemistry

Paraffin-embedded testis tissue slides (ProSci) were de-waxed and rehydrated by placing the slides in three changes of xylene for 3 min each time followed by three changes of ethanol (99.5% for 2x5 min, 95% for 2x4 min) 70% for 1x5 min) and then hydrated at 37°C for 3 min. For antigen retrieval, slides were immersed into a solution of pH 7.8 containing 0.1 g $CaCl_2$ and 0.1 g chymotrypsin (from bovine pancreas) in water at 37°C. After removing the slides, they were placed in cold water and rinsed twice with TBS containing 0.025% Triton X-100. Slides were blocked in 10% normal goat serum (DAKO) with 1% BSA in TBS for 2 h at RT, subsequently drained and wiped around the sections. Polyclonal rabbit antibodies, α-TXNRD1_v3 (α-v3), were made by AgriSera against the synthetic v3-derived peptide (NH_2-*C*KGKNGDGRRWSAKDHHPGK-COOH), with the underlined cysteine residue added for maleimide-linked conjugation of keyhole limpet hemocyanin protein as immunization carrier. The peptide sequence is encoded by exons β_{-VIII} and β_{-VI} (see **Scheme 1**). The antibodies were eluted at pH 7.0 from affinity purified rabbit IgG and kept in PBS with 20% glycerol (0.497 mg/ml) and used as primary antibodies. The slides were incubated overnight at 4°C with a 1:200 dilution of α-v3 primary antibodies in 200 μl TBS with 1% BSA. For the pre-absorption control, the same synthetic peptide antigen (500 μg) used for raising the antibodies was added to the primary antibody solution. Subsequently, the slides were rinsed for 2x5 min in TBS with 0.025% Triton X-100. Slides were incubated for 2 h at RT with biotinylated goat α-rabbit secondary antibody (DAKO) in a 1:200 dilution in TBS containing 1% BSA. Slides were rinsed for 2x5 min in TBS. Streptavidin antibody complexed with horseradish peroxidase (DAKO) were applied to the slides in TBS for 30 min at RT. Slides were rinsed 2x5 min with TBS and developed with chromogen (DAB, DAKO) for 10 min at RT. Following another rinse under running tap water for 5 min, slides were counterstained with Mayer's hematoxylin (DAKO) for 2 min. Then, slides were rinsed with water and counterstained with eosin for 2 sec, rinsed again with water and finally mounted using fluorescent mounting medium (DAKO).

Transfection experiments

TXNRD1_v3-GFP, v3(Grx)-GFP or TrxR1 module-GFP fusion constructs were made using a human EST clone for TXNRD1_v3 (Genbank accession number BG772375) as template for PCR. The following forward primers were used: Grx-TrxR1 GFP Forward, CTG AAT TCG CCA CCA TGG GCT GCG CCG or TrxR1 GFP Forward, CTG AAT TCG CCA CCA TGG ACG GCC CTG (both introducing an Eco RI site and designed for construction of a Kozac sequence) in combination with the following reverse primer: Grx/TrxR1 GFP Reverse, ATG GAT CCG CAG CCA GCC TGG AG (introducing a Bam HI site). Cloning of the final desired target constructs for transfection experiments was made using the pEGFP-N3 vector (Clontech), fusing the GFP at the carboxylterminal end of the protein domains and for the TrxR1 module-encoding transcripts excluding both the selenocysteine residue and the 3'-untranslated region of the cDNA, essentially as described previously (29). HEK293 and MCF7

cells were grown on glass cover slips until they reached ~80% confluency. Subsequently, cells were transfected with the constructs using lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. We used various concentrations of DNA ranging from 0.25 to 1 µg per well on a six well plate, without any differences observed. Cells were fixed 16 hours after transfection in 4% paraformaldehyde for 30 minutes at room temperature and then permeabilized with PBS-T (PBS with 0.1% Tween 20) for 3x5 min washes, followed by a blocking step using 5% normal goat serum (Jackson ImmunoResearch Laboratories) in PBS-T. Subsequently, after a short wash in PBS-T cells were incubated with a monoclonal anti-β-tubulin antibody (T4026, Sigma) diluted 1:200 in PBS-T for 1 hour followed by 3x5 min washes. The tubulin antibody was detected using a goat anti mouse antibody conjugated to Alexa Fluor 568 (Molecular Probes) diluted 1:500 while the actin network was visualized using phalloidin Alexa Fluor 647 (Molecular Probes) diluted 1:40 in PBS-T. After a 1-hour incubation cells were washed briefly and stained with DAPI (Molecular Probes) for 5 min and then washed 3x5 min in PBS-T before mounting using fluorescence mounting media (Callbiochem). For co-staining of ERα and ERβ, cells were treated with 10 nM estradiol and stained essentially as described before (29).

Fluorescence microscopy

For the imaging we employed a Leica AS MDW fluorescence microscope (Leica microsystems). DAPI, GFP and Alexa Fluor 568 were excited and fluorescence detected using a triple band-pass filter cube (Leica microsystems) with the excitation wavelengths/band-pass of 420/30, 495/12, 570/20 and the emission wavelengths/band-pass of 465/20, 530/30 and 640/40. Separation of the fluorophores was achieved by adjusting the excitation wavelength of the monochromator. For the phalloidin Alexa Fluor 647 a separate filter was used, a Cy5 filter (Chroma) with excitation and emission wavelengths of 620/60 and 700/75. Image stacks were obtained with a step size of 0.25 µm in the z-direction and then deconvolved using AutoQuant's blind deconvolution algorithm. Images were then pseudocolored and prepared in Photoshop software (Adobe). The live cell experiments were performed using the AS MDW as well, with the difference that the z-stacks obtained had a 0.5 µm step size. For production of the movie, the transfected cells were followed for 1 hour with a z-stack obtained every minute and were deconvolved as previously described and composed into a single file.

RESULTS

Expression of v3 in human tissues and cells

As the human v3 transcript has only been identified from rare EST clones and partial PCR amplifications (7,25), we first aimed to analyze the complete size of the v3 transcript and its expression pattern in different human tissues using Northern blot. For this purpose, we used a v3-specific probe containing the first three exons of the Grx domain which are unique to this variant (red in **Scheme 1**). This analysis showed a single transcript of ~4.5 kb size in testis, while all other analyzed tissues were negative (**Fig 1A**). This size fits very well with the expected size of the β1 transcript, encoding the TrxR1 module plus the N-terminal Grx domain. Next, using specific PCR analyses with first-strand cDNA, we also found v3 transcript in samples from ovary, spleen, heart, liver, kidney and pancreas in addition to that of testis, but could not detect the transcript in thymus, prostate, small intestine, colon, leukocyte, brain, placenta, lung or skeletal muscle (**Fig 1B**). The PCR product obtained corresponded to the expected size of 468 bp (see **Scheme 1**) and its specificity was verified by sequencing of the PCR products from testis and ovary. Given that testis was the tissue with by far the highest amount of v3 mRNA, we set up to analyze the v3 protein expression pattern in testis by using specific polyclonal antibodies raised against the N-terminal Grx domain of v3, encoded by exons $\beta_{\text{-VIII}}$ and $\beta_{\text{-VI}}$ (see **Scheme 1**). Immunohistochemical staining of human testis using these antibodies identified interstitial Leydig cells as the most prominent cell type expressing v3 (**Fig 1C**). This should be compared to the staining of Leydig cells, Sertoli cells and spermatids using a commercial antibody against the TrxR1 module as published by others (see (13) and www.proteinatlas.org; **Fig 1E and F**).

We subsequently analyzed the expression of the v3 transcript in human cancer cell lines using the same PCR strategy as described for normal tissue (see **Scheme 1**). Several cancer cell

lines were found to express v3, i.e. HCC1937, H23, A549, U1810, U1285, and H157 (**Fig 2A**). Other cell lines, such as U1285, HeLa or Jurkat, were negative in the analysis. Interestingly, HeLa cells that normally did not express v3 induced the transcript upon treatment with estradiol or testosterone, while other treatment such as serum starvation or hydroxyurea had no obvious effect on the expression (**Fig 2B**).

Localization of TXNRD1_v3 in transfected cells

Human TXNRD1_v2 has been shown to co-localize with the estrogen receptors ERα and ERβ in the nucleus of transfected cells in the presence of ligand (29). Because of the effect of estradiol and testosterone on v3 expression (**Fig 2B**) and since the last exon of the Grx domain of v3 is shared with a part of TXNRD1_v2 (**Scheme 1**), we analyzed whether v3 would also co-localize with estrogen receptors in transfected cells. As shown in **Figure 3A and 3B** we found no evidence of such co-localization in transfected HEK293 cells of a v3-GFP fusion protein with either the ERα (**Fig 3A**) or the ERβ (**Fig 3B**) nuclear receptors in the presence of ligand. However, performing these experiments we noted a strikingly dotted appearance of the v3-GFP fusion protein fluorescence (**Fig 3A and B**). We thus analyzed that pattern in further detail, using GFP fusion constructs either with the complete v3 protein, the TrxR1 module alone or the Grx domain of v3 alone, also comparing their respective pattern of localization to that of actin and tubulin. This approach showed that all constructs containing the Grx domain of v3 display a dotted cytosolic appearance as well as membrane associated fluorescence in the two cell lines used (HEK293 and HeLa) (**Fig 4A, C and F**). This could not be seen with neither the TrxR1 module alone in fusion with GFP, which showed a more diffuse cytosolic localization (**Fig 4D**), nor with only GFP alone, which was used as control and found in both cytosol and nucleus in these cells (**Fig 4B and E**). In a mitotic cell, fluorescence of the GFP-coupled Grx domain of v3 appeared strongly localized to the cell membrane (**Fig 4G**). Comparing the localization of the Grx domain of v3 fused to GFP with the staining patterns of actin and tubulin in the same cells, it was clear that the Grx domain of v3 (either when expressed as the complete protein carrying the TrxR1 module or in the form of the isolated Grx domain) closely resembled the staining of actin, but without a complete overlap. Comparing the green fluorescence of the v3-derived GFP fusion proteins with the red staining of actin and analyzing the merged figures, it seemed like the green fluorescence was more pronounced at the cell membrane, while in the case of the dotted cytosolic staining the actin-derived staining was more pronounced than the fluorescence of v3, although a clear overlap (yellow in the merged pictures) could be seen (**Fig 4A, C, F and G**).

Induction of cell membrane protrusions

While performing the localization experiments shown in **Figure 4**, we noted that the cells transfected with GFP in fusion with the Grx domain of v3 (either alone or followed by the TrxR module) not only showed a dotted or membrane-associated pattern of fluorescence, but also gained a novel phenotype consisting of a strong tendency to form cell membrane protrusions. Interestingly, these protrusions displayed green fluorescence, suggesting that the Grx domain of v3 is directly involved in driving the formation of these novel structures. Analyzing one of the protrusions in more detail (**Fig 4C, dashed white box, merged picture)**, we observed that the v3-derived fluorescence appeared to be present in the whole membrane protrusion (**Fig 5A**) similarly to what is seen with actin staining (**Fig 5B**), while tubulin staining was mainly detected at the base of the protrusion (**Fig 5C**). The merged figure clearly showed that a smaller protrusion at the side of the major protrusion was only fluorescent for v3 (**Fig 5D**). Using computer analyses of a three-dimensional isosurface model from a AS MDW fluorescence microscope, it appeared clear that the v3-derived green fluorescence in fact seemed to "lead" the formation of membrane protrusions, with the red stained actin following the protrusion and tubulin growing last into the newly formed structure (**Fig 5E**). Indeed, we noted that cells transfected with a construct expressing the Grx domain of v3, either alone or in fusion with the TrxR1 module, displayed rapid formation of dynamic cell membrane protrusions. This is further illustrated in a time-lapse movie (**Movie 1, provided as supplemental data on JBC On-line**).

DISCUSSION

Here we found that the third splice variant of human TrxR1, v3, exhibits a prominent expression in Leydig cells of the testis and is expressed in a few additional tissues including ovary and several cancer cell lines. Its expression in HeLa cells may be induced by treatment with steroid sex hormones. We also found that the Grx domain of v3 seems to induce actin and tubulin polymerization, thus promoting a prominent formation of cell membrane protrusions. These findings suggest that v3 is a uniquely specialized splice variant of TrxR1. This adds further complexity to the human thioredoxin system and gives rise to a number of questions regarding both the molecular mechanisms for regulation of expression of v3 and the potential role of this splice variant for human cell function.

The transcripts for mammalian thioredoxin reductase isoenzymes are known to display significant alternative splicing in the 5'-region, resulting in some cases in alternative N-terminal domains of the encoded protein (7,8,12,25,26,29-31). The capacity to induce cell membrane protrusions as found here is, however, the hitherto first demonstrated function of the Grx domain encoded by the v3 transcript. Prior knowledge of the v3 transcript included its expression in testis (7,25), which was here shown to be predominantly derived from the Leydig cells. It was also shown earlier that the v3 transcript is present in human, chimpanzee and dog (where also the encoded protein was demonstrated using testis extract) but it is not found in mouse or rat (25). The human v3 protein was also recently demonstrated to be expressed in human mesothelioma cells (28). The encoded Grx domain of v3 lacks classical glutaredoxin activity and, interestingly, it furthermore inhibits the inherent thioredoxin reductase activity of the TrxR1 module to which it is fused (25).

The presently available results regarding v3 suggest that its functions in cells may be highly specialized. Based on the transfection experiments shown herein, the protein was found to catalyze cell membrane restructuring with promotion of actin polymerization. Such properties may reflect a role in certain cell type-related growth conditions and could, possibly, be related to cellular transport functions. The role of v3 may also be related to steroid sex hormone signaling considering the strong expression in Leydig cells and its induction in HeLa cells upon steroid treatment. Many questions are yet unanswered, but certain considerations can be made regarding the potential relations between v3 and the membrane protrusions here found to be provoked by this protein. It is well known that the polymerization and functions of actin or tubulin are redox regulated and earlier experiments have also demonstrated links to the glutathione and thioredoxin systems, shortly summarized as follows. For more than 15 years, tubulin has been recognized as a protein with reactive sulfhydryl groups, the assembly of which may be regulated by the thioredoxin (32,33) or glutaredoxin system (34). It was also shown that deglutathionylation of Cys-374 in actin increases its polymerization potency in relation to EGF signaling and that this deglutathionylation is catalyzed by glutaredoxin (35). This effect was further demonstrated to have a physiological function using siRNA for knockdown of Grx1, which hampered actin polymerization and translocation close to the cell membrane in NIH 3T3 cells (36). It should be noted that those results may be seen as resembling or strengthening the findings presented in our study, but that knockdown of Grx1 (36) should presumably not affect v3 expression. These results indicate that different glutaredoxin isoforms may have either redundant function in relation to actin polymerization or rather highly specified such functions in relation to cell type or growth stimuli. The structural basis for redox regulation of actin was also recently delineated in further detail, showing the importance of both Cys-374 and Cys-272 in actin and paving the way for more detailed studies of actin regulation in relation to redox active proteins (37). It should be emphasized that although we found in this study a prominent capacity of v3 to induce actin polymerization and cell membrane protrusions the protein is, as already mentioned, inactive in regular glutaredoxin assays (25). It may be possible that it has a restricted substrate specificity interacting only with actin or some actin-associated protein(s). We have tried to measure its activity *in vitro* with actin polymerization assays but hitherto without findings of any such activity (unpublished observations) and the molecular basis for the cellular effects reported here must

therefore await further studies.

It was surprising to us that v3 was expressed in the Leydig cells of the testis. Several unique members of the thioredoxin system are known to be expressed in testis, including TGR (22), Txl-2 3 (38), and Sptrx-1, -2, and -3 expressed specifically in sperm (39-41). None of these other proteins, however, are being prominently expressed in the Leydig cells (42). What could the function of v3 be in the Leydig cells? It could be noted that testicular macrophages and the Leydig cells seem to be functionally coupled with the macrophages stimulating testosterone secretion by the Leydig cells and physically interacting by membrane projections from the Leydig cells being located within membrane invaginations of the macrophages, at least in rat testis (43). Specific structures of actin are also involved in the clustering of the Leydig cells around blood vessels (44). Perhaps v3 could be involved in keeping the integrity or forming such structural components required for Leydig cell function. It is also noteworthy that we found v3 expression to be induced in HeLa cells by testosterone or estradiol, suggesting that v3 could also be involved in pathways related to the testosterone synthesis or secretion by the Leydig cells of the testis or similar sex hormone-related functions in the ovary. It should be noted, however, that v3 did not co-localize with estrogen receptors in the nucleus, in contrast to the second splice isoforms of TrxR1, TXNRD1_v2 (29). It should also be noted that, similarly to the earlier study of TXNRD1_v2 (29), we performed those analyses using constructs lacking the selenocysteine residue of the TrxR1 module. These localization studies lead us to the finding that the Grx domain of v3 by itself can promote cell membrane protrusions, thereby evidently being a function not dependent upon the integrity of the selenocysteine residue in the TrxR1 module of v3. It is not possible to exclude, however, that the Grx domain of v3 can interact with endogenous selenocysteine-containing TrxR1 expressed in the transfected cells, which needs to be analyzed in future studies. It is also possible that some naturally expressed transcripts indeed encode the Grx domain of v3 without fusion to the complete TrxR1 module, as may be suggested by many incomplete EST clones found in Genbank. This is another factor that should be analyzed in future studies.

The expression of v3 in a number of cancer cells as found here is of potential interest in relation to actin polymerization, with possible importance for cell mobility or invasiveness, which should also be the focus of future studies. Moreover, since the expression of v3 is regulated by an alternative promoter located upstream of the core promoter, which is otherwise guiding expression of the more common TrxR1 splice variants (7,31), this alternative promoter should be studied in terms of its transcriptional mechanisms.

To conclude, this study is the most detailed study thus far regarding the tissue specific expression of the third splice variant of human TrxR1, discovering the highest expression in Leydig cells of the testis and a steroid sex hormone-responsive induction in a cancer cell line otherwise not expressing this splice variant. Ovary and several other cancer cell lines also expressed the transcript, without hormone treatment. This study is also the first to show a function of the Grx domain of this v3 splice variant using transfection experiments, which induced a polymerization of actin and tubulin with formation of cell membrane protrusions. Based upon the findings presented here, we suggest that the v3 splice variant of human TrxR1 is likely to have a highly specialized expression regulation and functional role in specific human cells and tissues.

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[1] ABBREVIATIONS

α-v3, peptide antibodies raised against part of the Grx domain of v3; GFP, green fluorescent protein; Grx, glutaredoxin; TGR, human thioredoxin glutathione reductase encoded by the *TXNRD3* gene; Trx, thioredoxin; TrxR, thioredoxin reductase; TrxR1, thioredoxin reductase 1; TrxR2, human mitochondrial TrxR isoenzyme encoded by the *TXNRD2* gene; TXNRD1_v3, splice variant of human TrxR1 encoded by the ß1 transcript of the *TXNRD1* gene; v3-GFP, fusion construct of v3 fused at its N-terminal end to GFP; v3(Grx)-GFP, fusion construct of the Grx domain of v3 fused at its N-terminal end to GFP; v3, short form notation for the human TrxR1 splice variant protein TXNRD1_v3

FIGURE LEGENDS

Scheme 1. Overview of the alternatively spliced transcripts of the *TXNRD1* gene studied here. Transcription of the α-transcripts is initiated at the major core promoter located upstream of exon I whereas an alternative promoter upstream of exon β_{-VIII} regulates transcription of the β1 splice variant. The β1 transcript is translated into the third isoform of TrxR1 (TXNRD1_v3, or v3) containing an atypical N-terminal glutaredoxin (Grx) domain fused to the common TrxR1 module. Arrowheads connected by a dotted line indicate primer pairs used to identify the respective splice variant and the length of the amplified fragment is given in parentheses. The dashed part of exon I shown in this scheme in transcripts α7,α8 indicates an alternative splice donor site within that exon, resulting in either α7,α8 encoding TXNRD1_v1 or α1,α2 encoding TXNRD1_v2. Filled exons of larger width indicate the open reading frame (ORF) of each transcript. The common TrxR1 module is shown in green, the unique part of the Grx domain of the β1 transcript is indicated in red, and the sequences shared between the Grx domain and the N-terminal part of TXNRD1_v2 are hatched. The scheme is drawn approximately to scale, as indicated in the figure. Note that the complete TrxR domain is encoded by additional exons downstream of exon V. The scheme has been modified from an earlier report, to which the reader is referred for further information including sequences and accession numbers (7).

Figure 1. Expression of v3 in testis, in particular the Leydig cells. (*A*) A Northern blot using a v3-specific probe, containing the first three exons of the Grx domain unique to v3, identified a single transcript in testis while all other analyzed tissues were negative. (*B*) Specific analysis of first-strand cDNA using the $F\beta_{-VIII}$/R2 primer pair (see **Scheme 1**) showed a PCR-product corresponding to 468 bp with the strongest expression in testis and weak expression in heart, liver, kidney, pancreas, spleen and ovary. (*C-D*) Immunohistochemical staining of tissue sections from testis, derived from a donor in his mid 40's are shown. (*C*) A polyclonal antibody, α-v3, generated against a synthetic peptide contained in the unique part in the Grx domain of v3, showed a strong signal in the interstitial Leydig cells. *D* shows a pre-absorption control done with the synthetic peptide used to generate the antibody, diminishing the signal in Leydig cells and illustrating the specificity in the staining pattern. (*E-F*) Testis tissue sections taken from the Human Protein Atlas, according to its academic usage permission (see (13) and www.proteinatlas.org), are shown for comparison. Those testis sections were derived from a 26-year-old male (*E*) and a 68-year-old male (*F*) and stained with a commercial mouse monoclonal α-TrxR1 primary antibody (Santa Cruz, cat. # sc-28321) generated against amino acids 71-340, encoded by exon V (**Scheme 1**), of the TrxR1 domain. Hence, that antibody binds all three isoforms TXNRD1_v1-3 and staining is seen in Leydig cells as well as Sertoli cells and seminiferous tubules.

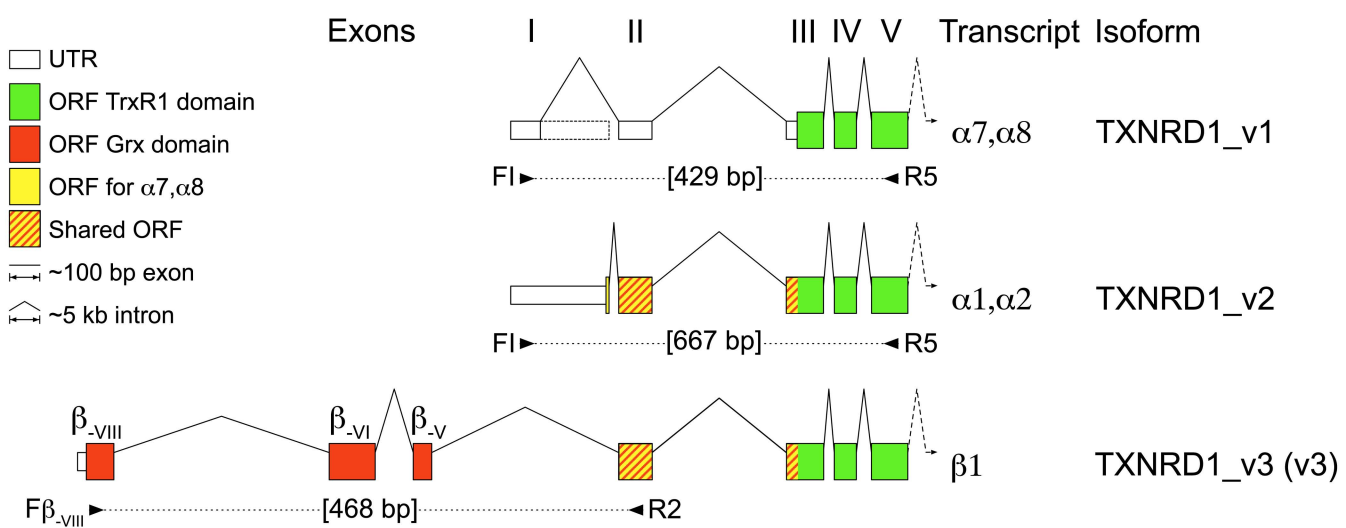
Figure 2. Expression of TrxR1 splice variant in different cancer cell lines. (*A*) First-strand RT-PCR of various cancer cell lines was performed to identify the potential concomitant transcription of different splice variants of TrxR1 (see **Scheme 1**). The β1 variant showed a distinct transcription pattern in some of the cell lines while the α7,α8 transcripts were expressed at a high level in all of the investigated cancer cell lines and α1,α2 was weakly but consistently detected. (*B*) PCR results using cDNA from treated HeLa cells showed an upregulation of β1 by estradiol or testosterone; *Estradiol* (1 μM); *Testost.*, testosterone (0.1 μM); *No FCS*, medium without FCS; *HU (10)*, hydroxyurea (10 μM); *HU (100)*, hydroxyurea (100 μM); *Pos. Ctrl.*, positive control; *Neg. Ctrl.*, negative control. The cDNA of v3 in a plasmid was used as a positive template control.

Figure 3. No co-localization of v3 with estrogen receptors. To analyze localization of v3 with respect to estrogen receptors (ER), HEK293 cells were transfected with either the complete isoform v3 fused to GFP (v3-GFP) and stained for ERα (*A*) or transfected with the Grx domain of v3 fused to GFP (v3(Grx)-GFP) and stained for ERβ (*B*) under treatment of estrogen as described in Materials and Methods. Cells were analyzed using an AS MDW fluorescence microscope.

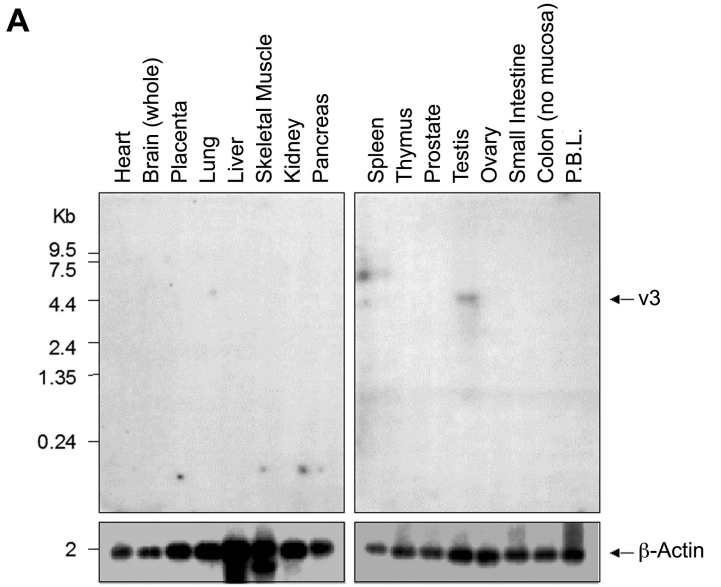
Figure 4. Dotted cytosolic and membrane-associated localization pattern of v3 compared to actin and tubulin. The fluorescence microscope pictures show HeLa (*A*-*B*) or HEK293 cells (*C*-*G*) transfected with either the complete isoform v3 fused to GFP (v3-GFP) or the Grx domain of v3 fused to GFP (v3(Grx)-GFP) and also stained for actin, tubulin and nuclei using DAPI. Cells transfected with a TrxR1-GFP construct or the GFP protein alone were used as control (GFP control).

Figure 5. The isoform v3 leads formation of cell membrane protrusion and is followed by actin and tubulin. (*A*-*D*) An enlargement of a cell membrane protrusion of a HEK293 cell, transfected with the v3(Grx)-GFP construct, is given (area indicated in *Fig. 4C* by a dashed white box) whereas (*E*) shows a three-dimensional isosurface model. v3(Grx) is shown in green, actin in red and tubulin in blue.

Movie 1. TXNRD1_v3 provokes rapid formation of cell membrane protrusions. HEK293 cells transfected with v3-GFP were followed as live cells for one hour with a z-stack obtained every minute, deconvolved and then combined into a single file to compose this visual recording. (the enclosed figure is a still image from a movie provided as supplemental data on JBC On-line).


Exons
I
II
III
IV
V
Transcript
Isoform
UTR
ORF TrxR1 domain
ORF Grx domain
ORF for α7,α8
Shared ORF
~100 bp exon
~5 kb intron
α7,α8
TXNRD1_v1
FI
[429 bp]
R5
α1,α2
TXNRD1_v2
FI
[667 bp]
R5
β-VIII
β-VI
β-V
β1
TXNRD1_v3 (v3)
Fβ-VIII
[468 bp]
R2

Scheme 1


A
Heart
Brain (whole)
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small Intestine
Colon (no mucosa)
P.B.L.
Kb
9.5
7.5
4.4
2.4
1.35
0.24
v3
2
β-Actin
B
Heart
Brain
Placenta
Lung
Liver
Skel. Muscle
Kidney
Pancreas
Pos. Ctrl.
Neg. Ctrl.
Spleen
Thymus
Prostate
Testis
Ovary
Sm. Intestine
Colon
Leukocyte
Pos. Ctrl.
Neg. Ctrl.
468 bp

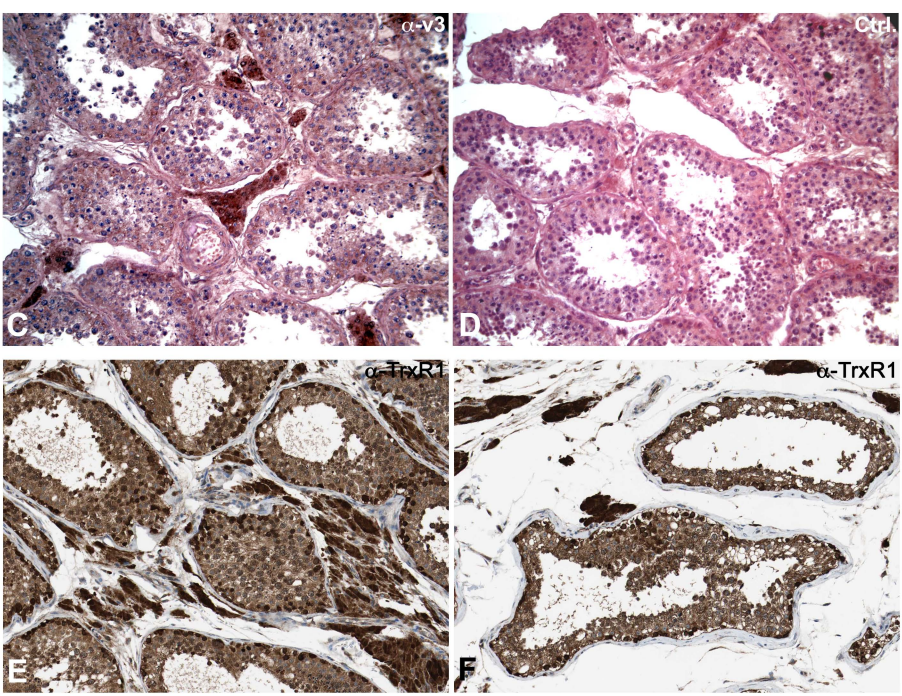

α-v3
Ctrl.
C
D
α-TrxR1
α-TrxR1
E
F

Figure 1

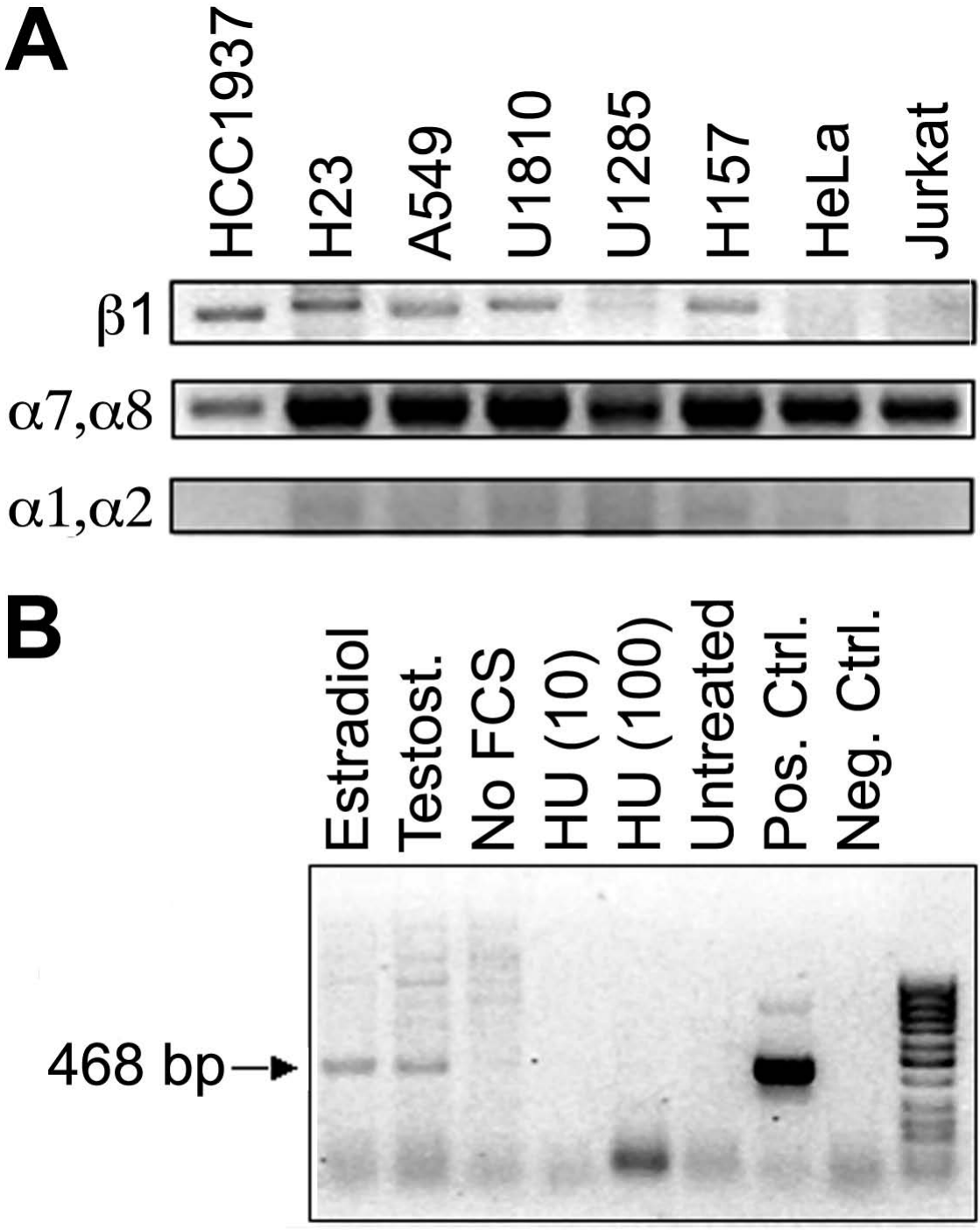

A
HCC1937
H23
A549
U1810
U1285
H157
HeLa
Jurkat
β1
α7,α8
α1,α2
B
Estradiol
Testost.
No FCS
HU (10)
HU (100)
Untreated
Pos. Ctrl.
Neg. Ctrl.
468 bp

Figure 2

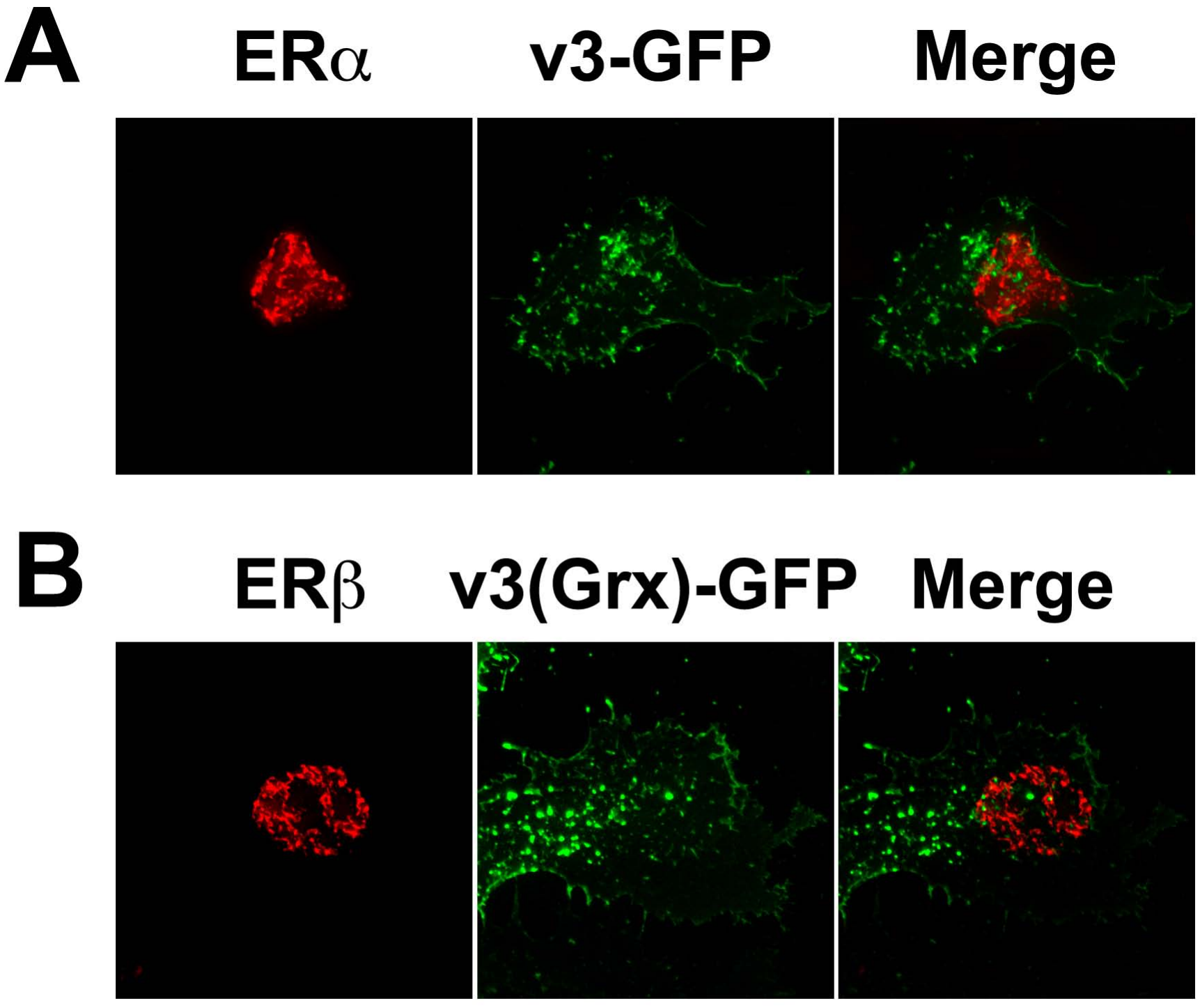


Figure 3

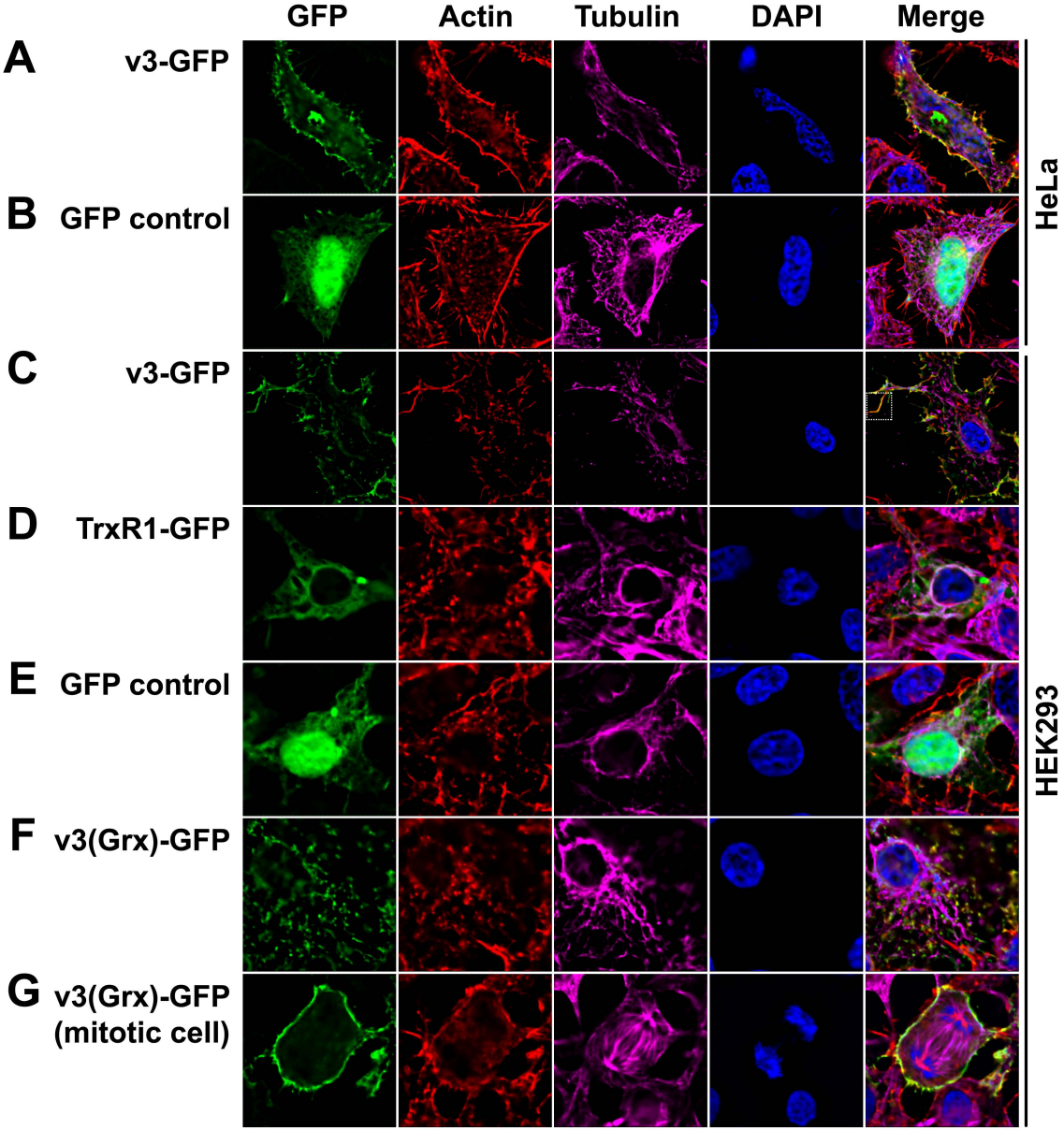

GFP
Actin
Tubulin
DAPI
Merge
A
v3-GFP
B
GFP control
C
v3-GFP
D
TrxR1-GFP
E
GFP control
F
v3(Grx)-GFP
G
v3(Grx)-GFP
(mitotic cell)
HeLa
HEK293

Figure 4

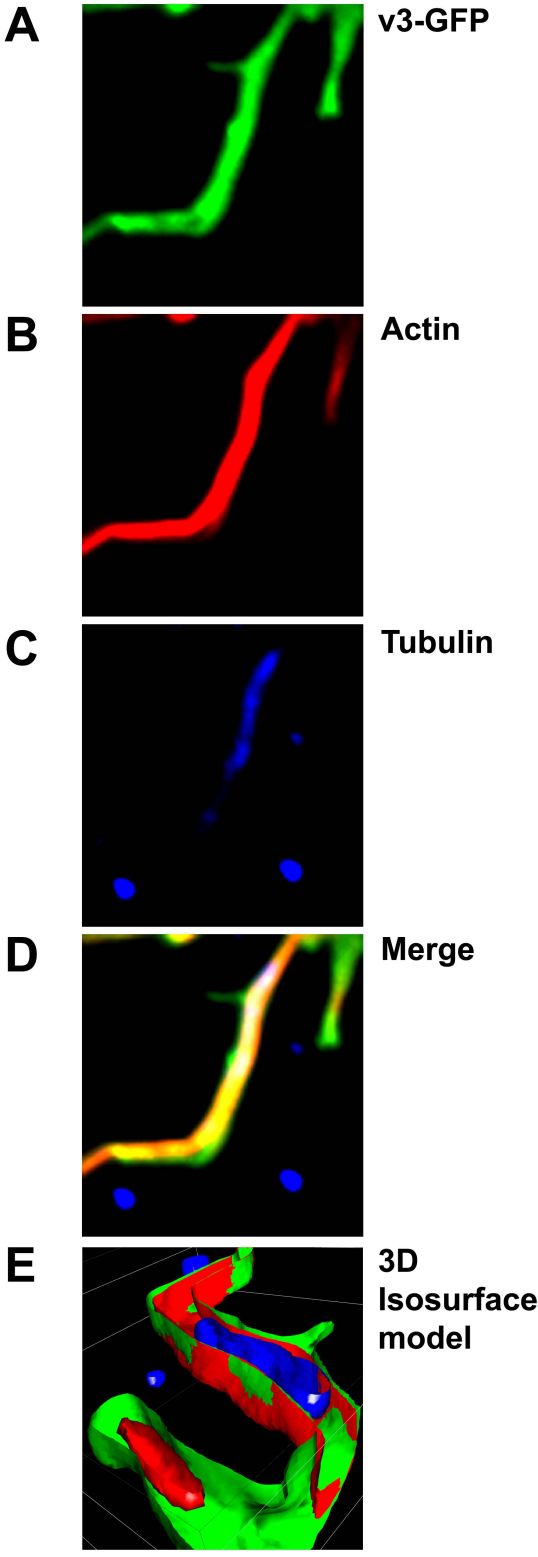
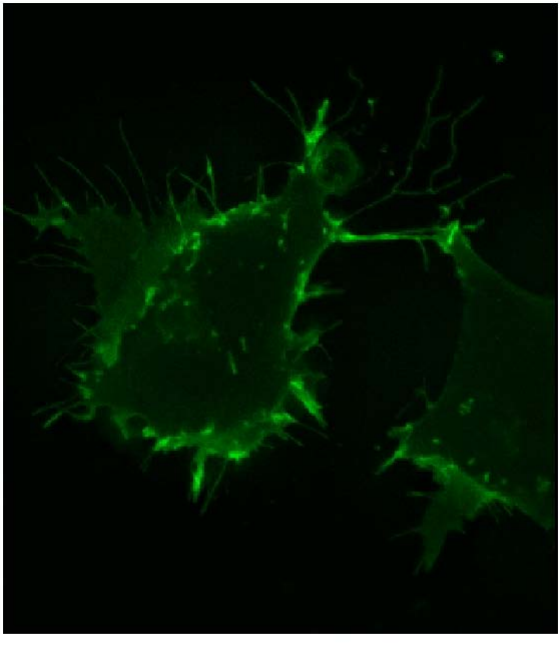


Figure 5



Movie 1