**Title**

Tumor suppressor REIC/Dkk-3 interacts with the dynein light chain, Tctex-1

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**Key words:** REIC, Dkk-3, Tctex-1, Dynein, Endoplasmic reticulum, Two-hybrid screening.

**Running title:** REIC/Dkk-3 interacts with Tctex-1.

**Highlights**

►Tumor suppressor REIC/Dkk-3 interacts with dynein light chain Tctex-1 via the 136-157 amino acid region of REIC/Dkk-3.

►REIC/Dkk-3 and the dynein intermediate chain (DIC) share an amino acid motif [-E-X-G-R-R-X-H-] in their Tctex-1 binding region.

►REIC/Dkk-3 and Tctex-1 are co-localized around the endoplasmic reticulum in human fibroblasts.

**Abstract**

REIC/Dkk-3 is a member of the Dickkopf family proteins known as Wnt-antagonists, and REIC/Dkk-3 expression is downregulated in a broad range of cancer types. REIC/Dkk-3 acts as a tumor suppressor in multiple cancer cell lines by inducing apoptosis through endoplasmic reticulum (ER) stress signaling. However, the intracellular interaction partners of REIC/Dkk-3 have not been fully elucidated. By employing yeast two-hybrid screening, we identified the human dynein light chain, Tctex-1, as a novel interaction partner of REIC/Dkk-3. We further disclosed that the interaction involves the 136-157 amino acid region of REIC/Dkk-3 by using the mammalian two-hybrid system. Interestingly, this binding region of REIC/Dkk-3 with Tctex-1 contains an amino acid sequence motif [-E-X-G-R-R-X-H-] which was previously reported as the Tctex-1 binding domain of dynein intermediate chain (DIC). Immunocytochemistry demonstrated that both REIC/Dkk-3 and Tctex-1 were localized around the ER of human fibroblasts, and the similar distribution pattern of the proteins suggests that their interaction occurs around the ER. This is the first study showing the interaction of a Dickkopf family protein with a dynein motor complex protein. The link between REIC/Dkk-3 and Tctex-1 may be of significance for understanding the molecular functions of the proteins in ER stress signaling and intracellular dynein motor dynamics, respectively.

## Introduction

REIC (reduced expression in immortalized cells) is a tumor suppressor gene, and the REIC gene is identical to the Dickkopf-3 (Dkk-3) gene, which is a member of the Dickkopf gene family [1]. The REIC/Dkk-3 protein is ubiquitously expressed in mouse and human organs and in normal cells, but its expression is significantly downregulated in a variety of cancer cells [2]. Several investigators have previously demonstrated that overexpression of REIC/Dkk-3 induces apoptosis in multiple cancer cell lines [3-6]. The forced expression of REIC/Dkk-3 using a plasmid vector inhibited cell growth in HeLa and liver cancer cell lines [3]. An adenovirus vector carrying REIC/Dkk-3 selectively induced apoptosis in prostatic and testicular cancer cells, but not in non-cancer cells, through the activation of c-Jun-$NH_2$-kinase (JNK) and c-Jun [4,5]. A recent study using malignant mesothelioma cells indicated that adenovirus-mediated REIC/Dkk-3 overexpression triggers a significant induction of apoptosis via endoplasmic reticulum (ER) stress [6]. Ad-REIC treatment also inhibits the expression of Id-1, which is involved in cell cycle progression and anti-apoptosis [6]. Therefore, accumulating evidence suggests that the intracellular overexpression of REIC/Dkk-3 plays a distinct role against cancer growth.

It has been reported that ER stress signaling is activated in REIC/Dkk-3 induced apoptosis [7]. ER stress can be evoked, and plays a role in the induction of apoptosis, when particular glycosylated proteins are overexpressed and protein folding and secretion are impaired [8,9]. GRP78 protein (also called BiP), which is associated with protein folding at the ER and is a key signaling molecule of ER stress [10,11], is up-regulated during REIC/Dkk-3-induced apoptosis [6]. In addition, the induction of apoptosis by REIC/Dkk-3 could be regulated by (JNK) phosphorylation, accompanied with the ER stress [4,6]. It appears that the characteristics of the REIC protein itself or of the specific expressional process, involves apoptotic ER stress signaling, however, it has not yet been elucidated which molecules are directly associated with the REIC protein. In order to clarify the molecular mechanisms of REIC/Dkk-3-induced cellular responses, we have been searching for proteins that

interact with REIC/Dkk-3. We herein present evidence that Tctex-1, a 14 kDa light chain of the cytoplasmic dynein motor complex, is a novel REIC/Dkk-3 interactive protein.

## Materials and methods

### Yeast two-hybrid assay

All yeast two-hybrid screenings were conducted using the ProQuest two-hybrid system (Invitrogen, Carlsbad, CA). Initially, human REIC/Dkk-3 full length cDNA, which was amplified by using the primers: Forward 5'-ACGCGTCGACCATGCAGCGGCTTGGGGCCAC-3' and Reverse 5'-TTCCTTTTTTGCGGCCGCTAAATCTCTTCCCCTCCCA-3',
was cloned into the SalI and NotI sites of the bait vector, pDBLeu. The REIC/Dkk-3-expressing clone and the human heart cDNA library cloned into pPC86 (Invitrogen) were co-transfected into the *Saccharomyces cerevisiae* strain MaV203. Positive MaV203 clones were selected on appropriate selection plates supplemented with the ß-galactosidase substrate. Transformation, plasmid isolation, construct identification, and the preparation of yeast lysates were performed according to the manufacturer's instructions.

### Cell culture

The 293T cell line was provided by the American Type Culture Collection (Rockville, MD). The normal human fibroblast cell line (OUMS24) was established by the Department of Cell Biology of our university [1]. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), penicillin (50 IU/ml) and streptomycin (50 μg/ml) under a humidified atmosphere of 5% $CO_2$ at 37 °C.

### Immunoprecipitation

For the analysis of immunoprecipitation, the full length human REIC/Dkk-3 and Tctex-1 cDNA were cloned into pcDNA3.1/Myc-His(-)A and pcDNA3.2/V5/GW/D-TOPO plasmids (Invitrogen), respectively. Plasmid DNA was transiently transfected into 293T cells with Lipofectamine 2000 (Invitrogen). Cell lysates of the Myc-tagged REIC/Dkk-3 or V5-tagged Tctex-1 transfected 293T

cells were collected 48 hours after the transfection by using lysis buffer (20mM Tris–HCl, pH7.5, 1% Triton X-100, 150mM NaCl, 5mM EDTA and Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)). The lysates were diluted to 1 ml with protein G sepharose (Invitrogen) which had been preincubated with 1 mg nonspecific mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-Myc mouse monoclonal antibody (Invitrogen, Cat.No. R95025) in the lysis buffer. After incubation for 12 hours at 4°C, the beads were washed extensively, and boiled in SDS loading buffer. The precipitates were analyzed by a Western blotting analysis with the anti-V5 mouse monoclonal antibody (Invitrogen, Cat.No. R96025), as previously described [12].

Mammalian two-hybrid assay

For the mammalian cell two-hybrid assay, various lengths of human REIC/Dkk-3 and full length human Tctex-1 cDNA were cloned into the pM GAL4 DNA-binding domain cloning plasmid and pVP16 transactivation domain cloning plasmid (Clontech Laboratories, Mountain View, CA), respectively. The templates for each length of human REIC/Dkk-3 were generated by PCR amplification using appropriate primer pairs [13]. Human Tctex-1 full length cDNA was amplified by using the primers:

Forward 5'-CCGGAATTCATGGAAGACTACCAGGCTGC-3',

Reverse 5'- GGGAAGCTTTCAAATAGACAGTCCGAAGG-3'.

Approximately $2 \times 10^5$ 293T cells were co-transfected with 400 ng of pM, 400 ng of pVP16, 250 ng of pFR-Luc firefly luciferase reporter plasmid (Promega, Madison, WI), and 10 ng of phRL-TK *Renilla* luciferase reporter plasmid (Promega). The cells were harvested 48 hours after transfection, and the luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The transfection efficiency was normalized by measuring the *Renilla* luciferase activity by the co-transfection of phRL-TK.

Immunostaining

Immunocytochemical staining of REIC/Dkk-3 and Tctex-1 in the OUMS24 cells was carried out by co-staining the endoplasmic reticulum, as previously described [2]. The cells were plated and cultured to 30–40% confluence on coated 12 mm coverslips in 24 well plates. The cells were fixed with 4% paraformaldehyde in 100 mM phosphate buffer and blocked with 3% BSA in phosphate buffered saline (PBS). The cells were incubated with a rabbit polyclonal anti-REIC/Dkk-3 antibody (1:200 dilution in PBS, [4]) or a rabbit polyclonal anti-Tctex-1 antibody (1:100 dilution in PBS, Santa Cruz Biotechnology, sc-28537) for 2 hours at room temperature (RT) and then with an Alexa488 green fluorescence-conjugated anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 hour. To determine the distribution in the endoplasmic reticulum, the cells were incubated with Alexa546 red fluorescence-conjugated concanavalin A (Molecular Probes) for 15 minutes at RT. The fluorescent staining was visualized by a confocal microscope system (CSU10; Yokogawa Electric, Kanazawa, Japan).

## Results

REIC/Dkk-3 interacts with Tctex-1.

In order to identify novel interacting partners for REIC/Dkk-3, we performed a yeast two-hybrid screening using the full-length human REIC/Dkk-3 protein as bait. The screening was performed using a human cDNA library derived from normal heart tissue, because a higher level of REIC/Dkk-3 expression was observed in mouse and human heart tissue compared to other tissues [1,2]. Among the cDNA clones that reproducibly activated the reporters, some were identified as being independent. The positive clones were verified by plasmid rescue, retransformation and growth on reporter-selective media (Figure 1A), and suggested that Tctex-1 interacts with REIC/Dkk-3.

To confirm the interaction between REIC/Dkk-3 and Tctex-1, we performed an *in vitro* pull-down assay by the immunoprecipitation method (Figure 1B). The lysates from 293T cells expressing Myc-REIC/Dkk-3 and the V5-Tctex-1 fusion protein were analyzed for the binding between REIC/Dkk-3 and Tctex-1. The binding with Tctex-1 was detected by a Western blotting analysis using an anti-V5 antibody. Definite Tctex-1 binding with REIC/Dkk-3 was disclosed in the Myc-immunoprecipitated sample from the cell lysates. Therefore, the interaction between REIC/Dkk-3 and Tctex-1 was demonstrated by both the yeast two-hybrid and immunoprecipitation assay systems.

The REIC/Dkk-3 and Tctex-1 interaction involves the 136-157 amino acid region of REIC/Dkk-3.

In order to determine the Tctex-1 binding region of REIC/Dkk-3, we performed mammalian two-hybrid assay. By co-transfection of the GAL4-plasmids coding for a series of different lengths of REIC/Dkk-3 cDNA and the VP16-plasmid coding for the full length Tctex-1 cDNA, the luciferase activity of the 293T cell lysates was determined as a result of REIC/Dkk-3 and Tctex-1 interaction. Significant Tctex-1 binding was detected for the REIC/Dkk-3 fragment of 20-146 amino acids, and the binding activity decreased with the increasing length of the REIC/Dkk-3 amino acid fragment

(Figure 2A). To further define the REIC/Dkk-3 region required for the binding to Tctex-1, the length of the REIC/Dkk-3 amino acid fragment was varied within a smaller range. A significant luciferase activity of Tctex-1 binding was detected to the REIC/Dkk-3 fragment of 20-146 amino acids, and was weaker for the fragment of 20-157 amino acids (Figure 2B). Only a weak background binding was observed to the fragment of 20-135 amino acids. These results indicate that the interaction between REIC/Dkk-3 and Tctex-1 involves the 136-157 amino acid region of REIC/Dkk-3.

Another previous study demonstrated that the dynein light chain, Tctex-1, bound the dynein intermediate chain (DIC) around the amino acid sequence region [$^{120}$ SDSELGRRLHKLGVSKVTQVDFL $^{142}$] of DIC [14]. Interestingly, the amino acid region of REIC/Dkk-3 for the binding with Tctex-1 is [$^{136}$ VGDEEGRRSHECIIDEDCGPSM $^{157}$] and a consensus sequence of [-E-X-G-R-R-X-H-] was identified when compared to the Tctex-1 binding region of DIC (Figure 3A). With regard to the other Dkk family members (Dkk-1, Dkk-2, Dkk-4), we could not find this amino acid motif in any of their sequences. On the other hand, previous studies indicated that Tctex-1 bound several different proteins through their specific amino acid sequence motif [-R/K-R/K-X-X-R/K-] [14-19]. We found that the amino acid residue of the REIC/Dkk-3 binding region contains only [-R-R-] and partly matches the binding motif. Therefore, it appears that the binding region of REIC/Dkk-3 is not typical in comparison to the other binding partners of Tctex-1 (Figure 3B).

REIC/Dkk-3 and Tctex-1 are co-localized around the endoplasmic reticulum (ER).

The significant interaction between REIC/Dkk-3 and Tctex-1 indicated above led us to consider the intracellular co-localization of the two proteins. The REIC/Dkk-3 protein has been reported to exhibit a punctate localization pattern in the cell cytoplasm [7], and our recent study demonstrated that the protein is predominantly localized to the ER in stably transfected cells [2]. We therefore performed co-staining experiments utilizing fluorescence-conjugated concanavalin A, a marker

specific for the ER, to confirm the localization of REIC/Dkk-3 and Tctex-1. As expected, we found that both REIC/Dkk-3 and Tctex-1 were mainly localized around ER of the fibroblasts, and the subcellular distribution pattern was similar between the two proteins (Figure 4). We therefore concluded that REIC/Dkk-3 and Tctex-1 are co-localized around the ER in human fibroblasts.

## Discussion

The molecular partner(s) which interact with REIC/Dkk-3 have so far remained unknown. In order to disclose the relevant binding partners for REIC/Dkk-3, the yeast two-hybrid screening was performed using a cDNA library from human heart tissue. We demonstrated that Tctex-1 is an interacting partner of REIC/Dkk-3, and identified the specific Tctex-1 binding region of REIC/Dkk-3 by a mammalian two-hybrid assay. Confocal microscopy revealed that REIC/Dkk-3 and Tctex-1 are localized around the ER with a similar distribution pattern, suggesting that their interaction and functions occur around the ER. Thus, current findings have revealed a novel link between the tumor suppressor REIC/Dkk-3 and Tctex-1, a 14 kDa light chain of cytoplasmic dynein motor complex.

Cytoplasmic dynein is a microtubule-based molecular motor that plays an important role in various intracellular motile events, such as vesicle transport and mitotic spindle positioning [20]. The dynein motor is a multi-component protein and contains two heavy chains (- 530 kDa), two intermediate chains (- 74 kDa), four light intermediate chains (- 60 kDa), and several light chains (8, 14, 22 kDa) [20]. The dynein light chain and intermediate chain were reported to link the motor to vesicle-based cargoes by mediating the interaction between dynein and dynactin [21].

Tctex-1, a member of the dynein light chain, plays an important role as an intracellular adapter protein between cytoplasmic dynein motor complexes and specific vesicular cargos [14]. In particular, the interaction of Tctex-1 with the dynein intermediate chain (DIC) is essential for the functions of dynein complexes [14]. Besides the DIC protein, several other cellular molecules have been reported to interact directly with Tctex-1, such as the modulator of $Ca^{2+}$ dependent neurotransmitter release (DOC2) [15], lymphocyte surface membrane protein, CD5 [16], G protein beta-subunit (Gß1-5) [17], poliovirus receptor, CD155 [18], parathyroid hormone receptor (PTHR) [19], rod photoreceptor protein, Rhodopsin [22], and Fyn protein tyrosine kinase [23]. In most cases, the interaction between these proteins and Tctex-1 has been suggested to be critical for sorting these protein-bearing vesicular cargos for the dynein motor-based vesicle transport. Taken together with

the fact that vesicle transport is highly activated around the ER [24], the interaction between Tctex-1 and REIC/Dkk-3 and their predominant localization around ER suggest that the interaction may be implicated in the vesicular sorting and transport system around the organelle.

On the other hand, there is a possibility that the interaction between Tctex-1 and REIC/Dkk-3 modifies REIC/Dkk-3-related cellular signaling. The overexpression of REIC/Dkk-3 in multiple cancer cell lines induces apoptosis, and ER stress signaling is an important molecular pathway by which REIC/Dkk-3 induces apoptosis [3-7]. With regard to the physiological function of the intracellular REIC/Dkk-3 protein, the down-regulation of the REIC/Dkk-3 protein and its related signaling modifications may be attributed, at least partly, to the atypical cell differentiation or development of malignancy [2]. It is thus conceivable that the Tctex-1 protein could be implicated in cancer-related signaling through its association with REIC/Dkk-3. Future experiments are necessary to unravel the mechanistic details by which two proteins act on each other and whether the proteins cooperate in regulating their own functions.

The mammalian two-hybrid assay using different lengths of REIC/Dkk-3 indicated that the interaction with Tctex-1 involves the 136-157 amino acid region of REIC/Dkk-3. Previous studies demonstrated that several proteins bound Tctex-1 through a specific amino acid sequence motif, [-R/K-R/K-X-X-R/K-], in their Tctex-1 binding region [14-19]. However, the Tctex-1 binding site of REIC/Dkk-3 has limited homology with the previously reported consensus binding motif of the Tctex-1 targets. We showed that the amino acid residues of REIC/Dkk-3 contain only the [-RR-] motif and are not typical in comparison to the other binding partners of Tctex-1. It is thus possible that some different site or type of binding may be used in the REIC/Dkk-3 and Tctex-1 interaction. Interestingly, we observed that consensus amino acid motif of [-E-X-G-R-R-X-H-] is located in the Tctex-1 binding region of both the REIC/Dkk-3 and dynein intermediate chain (DIC) proteins. The significant homology in the Tctex-1 binding region clearly indicates that REIC/Dkk-3 binds Tctex-1, a member of the dynein light chain, in a similar manner as DIC. Since the interaction between

Tctex-1 and DIC plays an important role in the functions of the dynein motor complex, it would be interesting to determine whether intracellular REIC/Dkk-3 competes with DIC for Tctex-1 binding and induces some specific biological effects in terms of the dynein functions.

This is the first study showing the interaction of a Dickkopf family protein and a dynein motor complex protein. Taken together with the fact that Tctex-1 has critical functions as an adapter between the dynein motor complexes and cytoplasmic vesicles, our study suggests the involvement of the REIC/Dkk-3 protein in the ER-related vesicular trafficking system. A link between REIC/Dkk-3 and Tctex-1 may therefore be of significance for understanding the molecular functions of the proteins in ER stress signaling and the intracellular dynein motor dynamics, respectively.

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**Figure legends**

Figure 1. The interaction between the REIC/Dkk-3 and Tctex-1 proteins.

(A) The yeast two-hybrid analysis was conducted using pPC86(AD)/full length human Tctex-1 and pDBLeu(BD)/full length human REIC/Dkk-3 plasmids. The blue colonies indicate an interaction between the two proteins. (B) For the analysis of the immunoprecipitation (IP), the full length human REIC/Dkk-3 and Tctex-1 cDNAs were cloned into pcDNA3.1/Myc-His(-)A or pcDNA3.2/V5/GW/D-TOPO plasmids, respectively. Cell lysates from the Myc-tagged REIC/Dkk-3- and/or V5-tagged Tctex-1-transfected 293T cells were collected and analyzed. Immunoprecipitation of Myc-tagged REIC/Dkk-3 was done using an anti-Myc antibody. The Western blot analysis was done using anti-Myc or anti-V5 antibodies.

Figure 2. The determination of the Tctex-1 binding region of REIC/Dkk-3.

(A) The interaction of various lengths of REIC/Dkk-3 and full length Tctex-1 was examined in a mammalian two-hybrid assay. The left panel depicts various lengths of REIC/Dkk-3 fragments without the signal peptides. The numbers correspond to the amino acids in REIC/Dkk-3. Two-hybrid constructs of each REIC/Dkk-3 fragment and full length Tctex-1 were co-transfected into 293T cells as described in the Materials and Methods section, and the luciferase activity (arbitrary unit: A.U.) was measured. The binding activities with Tctex-1 are shown in the right panel. The results are given as the means ± SE (n = 3). The region of REIC/Dkk-3 was indicated as; a: signal peptide, b: coiled-coil structure, c: N-glycosylation site (putative), d: Cysteine-rich domain. (B) A tighter analysis was done to determine the REIC/Dkk-3 region between amino acids 78 and 157. The results are given as the means ± SE (n = 3).

Figure 3. The sequence alignment analysis of selected Tctex-1 binding regions.

(A) The amino acid sequence of the Tctex-1 binding regions of REIC/Dkk-3 (determined in this work) and dynein intermediate chain (DIC) [14]. The alignment analysis revealed a consensus amino acid motif of [-E-X-G-R-R-X-H-] in the Tctex-1 binding regions (shown in color letters). (B) The amino acid sequence of the Tctex-1 binding regions of REIC/Dkk-3 (determined in this work) and the previously reported Tctex-1 binding proteins [14-19]. The alignment analysis was done by focusing on the reported consensus amino acid motif of [-R/K-R/K-X-X-R/K-] in the Tctex-1 binding regions (shown in colored letters).

Figure 4. The intracellular localization of the REIC/Dkk-3 and Tctex-1 proteins.

(A) The co-localization of REIC/Dkk-3 and concanavalin A (an endoplasmic reticulum marker) was examined by double fluorescence staining in OUMS24 human fibroblast cells. The cells were imaged by confocal microscopy. Images in green and red show the subcellular localization of REIC/Dkk-3 and concanavalin A, respectively. The areas of overlap between REIC/Dkk-3 and the endoplasmic reticulum are shown in yellow. (B) The co-localization of Tctex-1 and concanavalin A was examined by double fluorescence staining. The areas of overlap between Tctex-1 and the endoplasmic reticulum are shown in yellow.

Figure 1

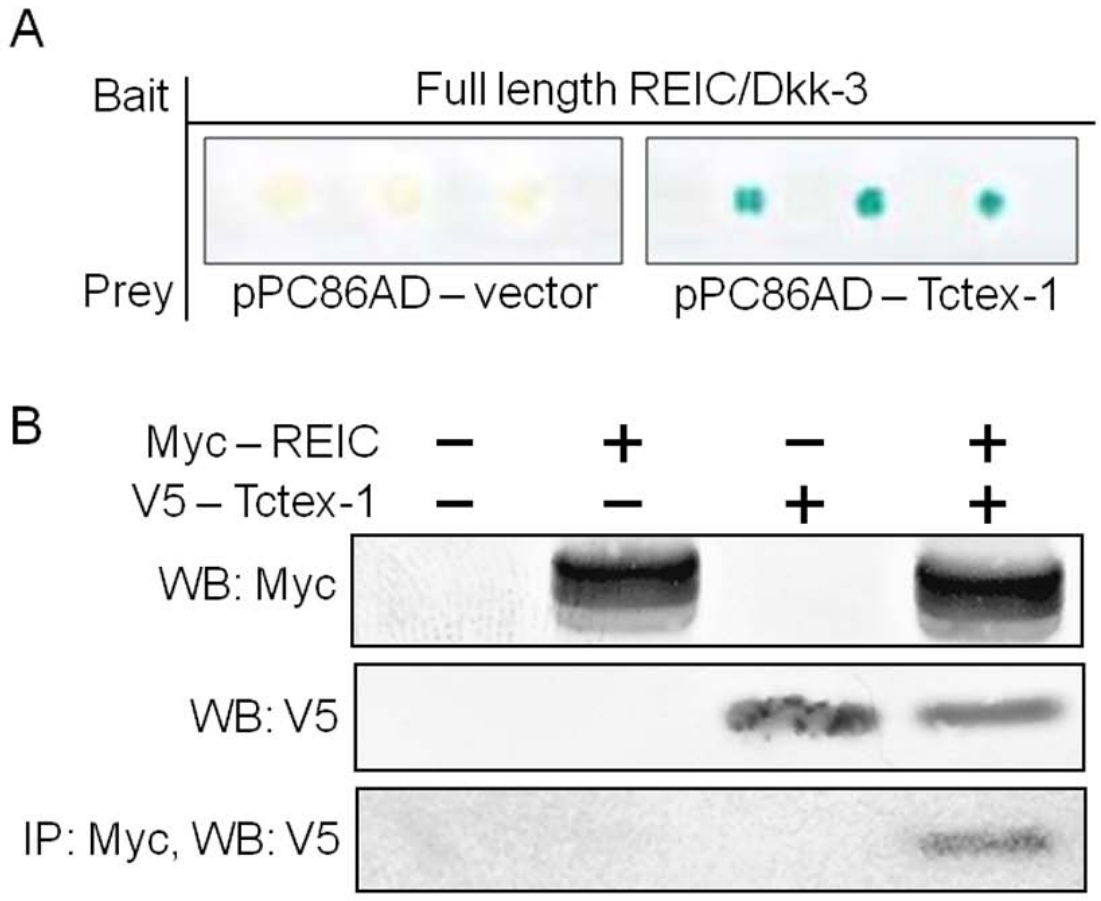

A
Bait
Full length REIC/Dkk-3
Prey
pPC86AD – vector
pPC86AD – Tctex-1
B
Myc – REIC
V5 – Tctex-1
WB: Myc
WB: V5
IP: Myc, WB: V5

Figure 2

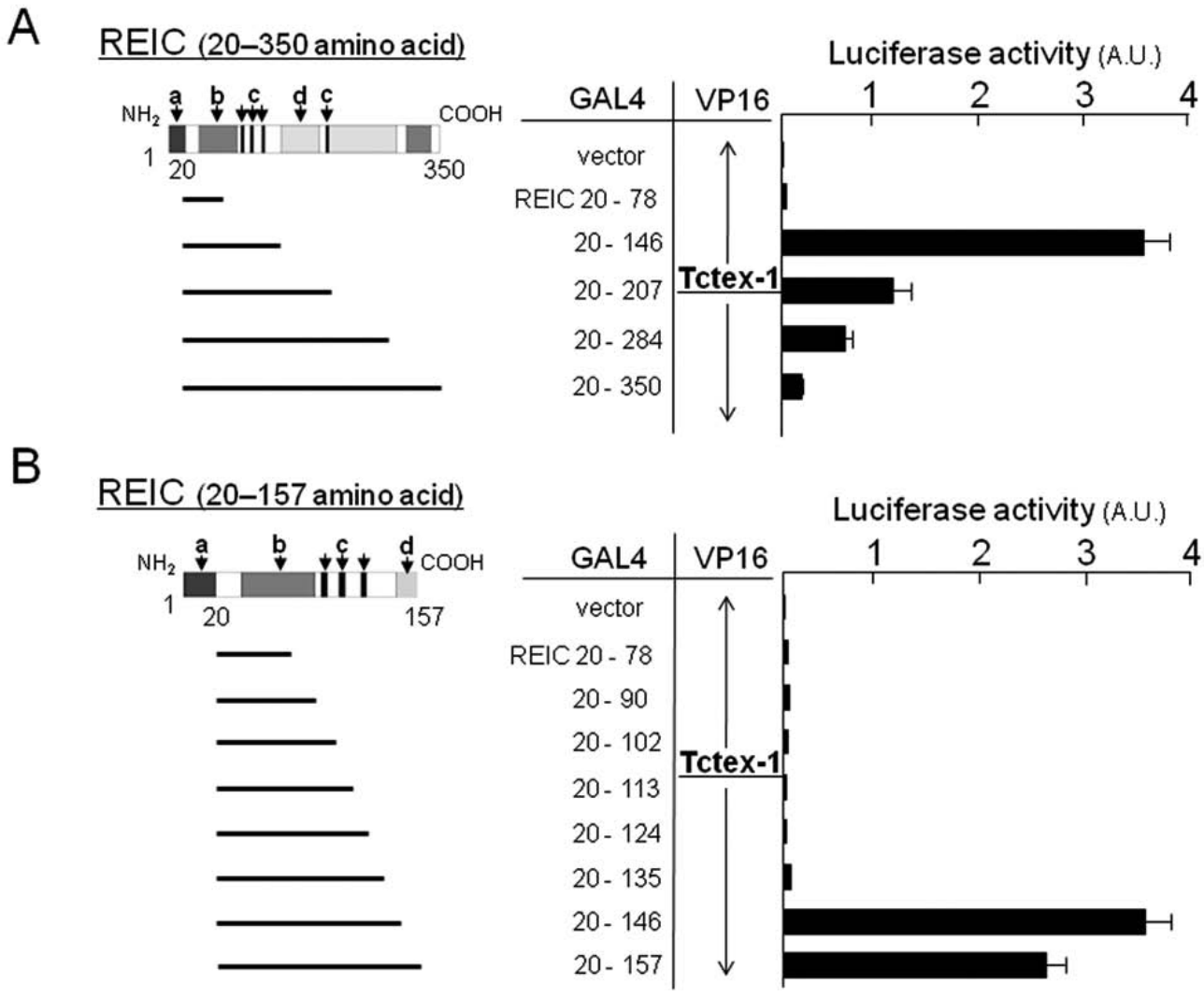

A
REIC (20–350 amino acid)
NH2
a
b
c
d
c
COOH
1
20
350
GAL4
VP16
Luciferase activity (A.U.)
1
2
3
4
vector
REIC 20 - 78
20 - 146
20 - 207
20 - 284
20 - 350
Tctex-1
B
REIC (20–157 amino acid)
NH2
a
b
c
d
COOH
1
20
157
GAL4
VP16
Luciferase activity (A.U.)
1
2
3
4
vector
REIC 20 - 78
20 - 90
20 - 102
20 - 113
20 - 124
20 - 135
20 - 146
20 - 157
Tctex-1

Figure 3

A


REIC 136 VGDEEGRRSHECIIDEDCGPSM 157
DIC 120 SDSELGRRLHKLGVSKVTQVDFL 142

B

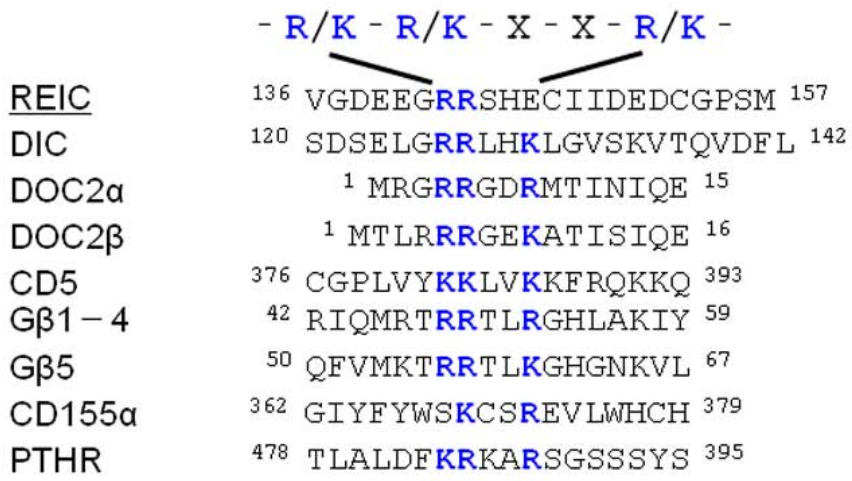
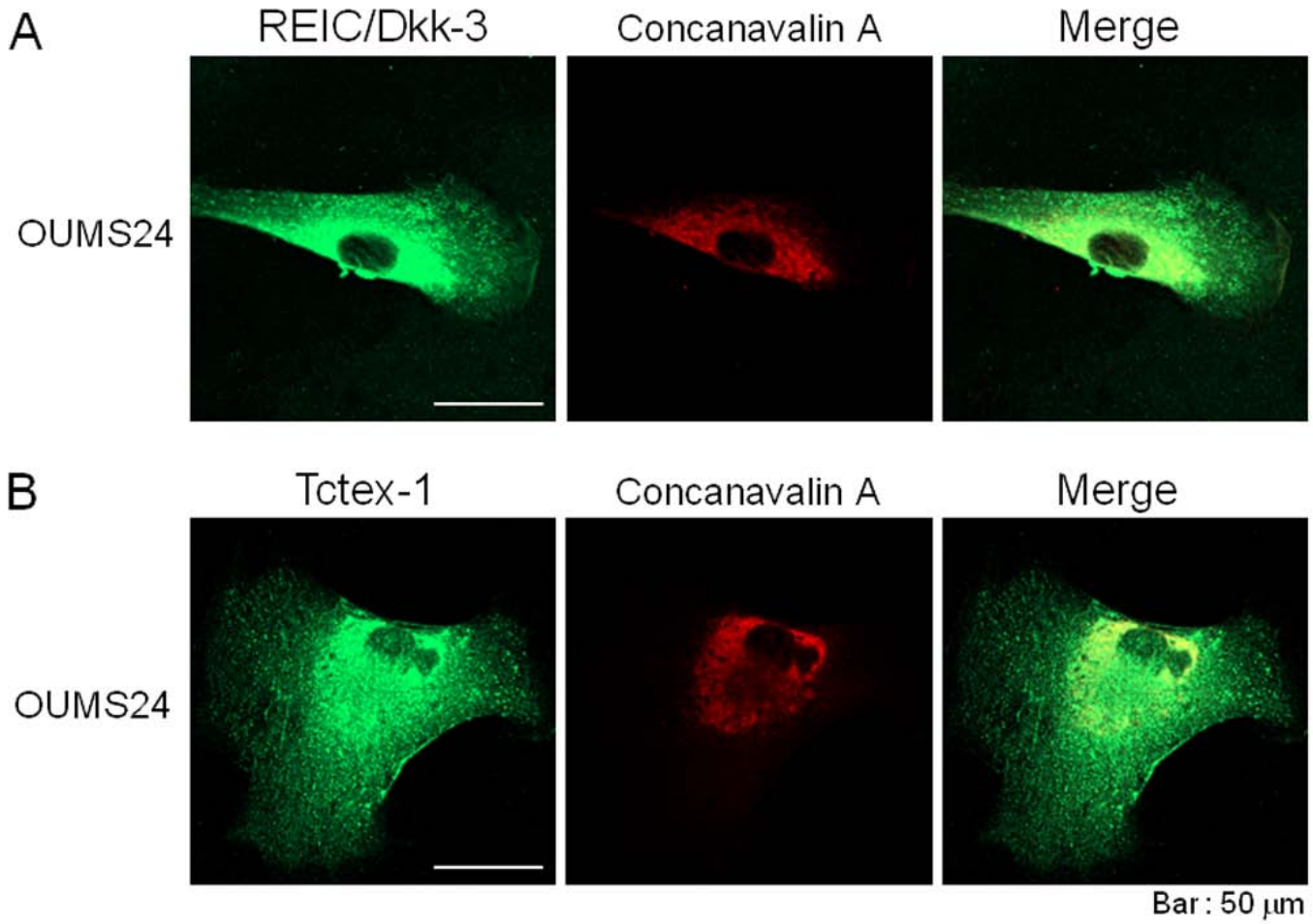

- R/K - R/K - X - X - R/K -
REIC 136 VGDEEGRRSHECIIDEDCGPSM 157
DIC 120 SDSELGRRLHKLGVSKVTQVDFL 142
DOC2α 1 MRGRRGDRMTINIQE 15
DOC2β 1 MTLRRRGEKATISIQE 16
CD5 376 CGPLVYKKLVKKFRQKKQ 393
Gβ1 – 4 42 RIQMRTRRTLRGHLAKIY 59
Gβ5 50 QFVMKTRRTLKGHGNKVL 67
CD155α 362 GIYFYWSKCSREVLWHCH 379
PTHR 478 TLALDFKRKARSGSSSYS 395

Figure 4


A
REIC/Dkk-3
Concanavalin A
Merge
OUMS24
B
Tctex-1
Concanavalin A
Merge
OUMS24
Bar : 50 μm