The Oncogenic Potential of TCTEX1D4 is Modulated by the Phosphoprotein Phosphatase PP1

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Research Article

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Abstract

Protein phosphatase 1 (PP1) regulates several cellular events via interaction with multiple regulatory subunits. The human prostate proteome includes various PP1-interacting proteins; however, a very limited number of interactions is yet characterized and their role in prostate tumorigenesis remains poorly understood. Tctex1 domain-containing protein 4 (TCTEX1D4) was previously identified as a PP1-interacting protein, but its function, as well as the relevance of its interaction with PP1, are virtually unknown. In this study we addressed the role of the PP1/TCTEX1D4 complex in prostate tumorigenesis. We found distinct expression levels and subcellular distributions for TCTEX1D4 and PP1 γ in human prostate epithelial normal-like and malignant cells. Moreover, we showed that TCTEX1D4 participates in the regulation of cell proliferation and modulation of microRNAs expression and that its interaction with PP1 controls its function. Taken together, our study provides first evidence for the involvement of the PP1/TCTEX1D4 complex in prostate tumorigenesis.

1. Introduction

Protein phosphatase 1 (PP1) is a major serine/threonine protein phosphatase involved in the posttranslational regulation of several proteins. PP1 holoenzymes exhibit a highly conserved catalytic subunit (PP1c) that is encoded by three genes—*PPP1CA*, *PPP1CB* and *PPP1CC*. With the exception of PP1 γ 2 (resultant of a tissue-specific alternative splicing of *PPP1CC*), PP1c isoforms are ubiquitously expressed, albeit variations in their expression levels and subcellular localization are observed in different cell type and pathophysiological conditions [4]. PP1c binds to several regulatory subunits that modulate its activity, subcellular localization and/or substrate affinity [1].

In previous studies, we identified Tctex1 domain-containing protein 4 (TCTEX1D4)—a dynein light chain protein—as a PP1-interacting protein [2, 3]. The interaction occurs via a RVSF motif and it seems that TCTEX1D4 dephosphorylation is critical for the regulation of its activity [3–5]. However, the functions of TCTEX1D4 itself and of the PPP1/TCTEX1D4 complex remain to be elucidated.

In 2006, Meng et al. showed that TCTEX1D4 interacts with transforming growth factor- β (TGF β) receptors [6]. TGF β signaling counterbalances the mitogenic effects of androgens by inhibiting cell proliferation and activating apoptosis in prostate epithelial cells [7]. Its role in prostate carcinogenesis is, nonetheless, paradoxical, since it acts as a tumor suppressor in early stages, while promotes tumor progression in advanced stages [8].

The TGF β signaling cascade has been implicated in the biogenesis and maturation of microRNAs (miRNAs) [9]. In addition, treating cells with TGF β directly leads to alterations in the expression of several miRNAs, including miR-145 and miR-200b [9–11]. These miRNAs have been associated with prostate carcinogenesis [12, 13]. These and other observations indicate that deregulation of miRNAs may underlie prostate tumorigenesis and, therefore, the establishment of the microRNA expression signature in PCa cells has been extensively studied [14].

In this study, we investigated the expression and localization of PP1 γ and TCTEX1D4 in human normal prostate and PCa cell lines and found higher expression and differential localization of both proteins in PCa cells. Given the previous findings on the involvement of both proteins in the regulation of the TGF β signaling pathway [6, 15], we went further to analyze the role of TCTEX1D4 and PPP1/TCTEX1D4 complex in PCa cell proliferation and migration, as well as their influence in the expression of PCa-associated miRNAs. Our results provide a better understanding on the functions of TCTEX1D4 and its regulation by PP1 and unravels a novel PP1 complex in prostate carcinogenesis.

2. Materials And Methods

2.1. Antibodies

Primary antibodies against TCTEX1D4 and PP1 γ were produced in-house [3]. Mouse anti- β -tubulin was acquired from Zymed Laboratories Inc. (UK). Secondary antibodies IRDye 800CW goat anti-mouse and IRDye 680RD goat anti-rabbit were obtained from LI-COR Biosciences (USA). Texas Red goat anti-rabbit and Alexa488 goat anti-mouse were acquired from Life Technologies (USA).

2.2. Cell culture

Human prostate epithelial cells RWPE-1 and human prostate cancer cells—LNCaP (androgen-dependent) and PC3 (androgen-independent)—were purchased from American Type Culture Collection (Spain). Pre-neoplastic PNT-2 cells were kindly provided by Dr. Ricardo Pérez-Tomás (Cancer Cell Biology Research Group, University of Barcelona, Spain). RWPE-1 cells were cultured in keratinocyte serum-free medium supplemented with 0.05 mg/mL BPE, 5 ng/mL EGF, 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin solution (5,000 U/mL). PNT-2, LNCaP and PC3 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin solution (5,000 U/mL). All media and supplements were from Gibco® (Life Technologies, USA). Cells were routinely maintained at 37°C in a humidified incubator with 5% CO₂ atmosphere.

2.3. Quantitative real-time PCR

Total RNA was isolated from cells using the RNeasy Midi Kit (Qiagen, Germany) and cDNA was synthesized using the RT² First Strand kit (Qiagen, Germany), according to manufacturers' instructions. Placenta tissue was used as control. For expression analysis, the following set of primers were used: *TCTEX1D4* (f:5'–gtgaggggggagtccaattct–3', r:5'–cagacacttatttattgggatgtga–3'); *PPP1CC* (f:5'–aacggctgctggaagtga–3', r: 5'–cagacacttatttattgggatgtga–3'); and *GAPDH* (f:5'–gacagtcagccgcattcttct–3', r:5'–gcgccaatacagaccaaactc–3'). qPCR occurred in the LightCycler®480 System (Roche, Switzerland) as follows: 95°C for 5 min, 95°C for 10 sec (45 cycles), 60°C for 10 sec, and 72°C for 10 sec. Results were analyzed using the Relative Expression Software Tool–Multiple Condition Solver–version 2.

2.4. Immunoprecipitation

Cells were washed in 1×PBS and lysed in RIPA lysis buffer (Merck Millipore, USA) with protease inhibitors [1 mM PMSF, 5 µM pepstatin A, 2 µM leupeptin, 10 mM benzamidine, 1.5 µM aprotinin, and 1 mM EGTA] (Sigma-Aldrich, USA). Lysates were centrifuged at 16,000×*g* for 15 min at 4°C and supernatants were pre-cleared with 20 µL Dynabeads protein G (Life Technologies, USA). Samples were then incubated overnight with 5 µg anti-TCTEX1D4 antibody, with rotation, at 4°C. The complex protein-antibody was collected by incubation with Dynabeads protein G for 1.5 hours, with rotation, at 4°C. After careful washing steps, Dynabeads protein G were resuspended in 1% SDS and boiled for 5 min prior to western blot analysis.

2.5. Western blot

Total protein extracts were prepared by treating cells with boiling 1% SDS. The total amount of protein in each extract was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Next, 60-100 µg of each lysate were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% non-fat milk in 1×TBST for 1 h at room temperature, washed with 1×TBST three times and incubated with the appropriate primary antibody. After being washed three times with 1×TBST, membranes were incubated with the secondary antibody. All antibodies were previously diluted in 3% non-fat milk in 1×TBST. Detection of the secondary antibodies was achieved using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA). The expression levels of PP1 and TCTEX1D4 was assessed by quantification of the intensity of bands using Quantity One® 1-D analysis software (Bio-Rad, USA). β-tubulin was used as loading control.

2.6. Slot blot

A nitrocellulose membrane was incubated in 10% methanol for activation. Then, 10-20 µg of each cell extract were applied in the Bio-Dot® SF microfiltration units (Bio-Rad, USA) and transferred onto the membrane. Membranes were then treated as previously described for western blot. The expression level of TCTEX1D4 was assessed using Bio-Rad Quantity One 1-D Analysis Software by quantification of the intensity of the bands using β-tubulin as loading control.

2.7. Transient transfection

The synthesis of the expression plasmids Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA was previously described [3]. In Myc-TCTEX1D4-RVSA, the mutation of the last amino acid of the RVSF motif in TCTEX1D4 cDNA to an alanine was shown to decrease the interaction between TCTEX1D4 and PP1 by about 35% [3]. pCMV-Myc was used as mock transfection control. Confluent 6-well plates were transfected with Lipofectamine® 2000 (Life Technologies, USA) in Opti-MEM Reduced Serum Medium (Life Technologies, USA), according to manufacturer's protocol. Cells were harvested for subsequent assays 24 hours after transfection.

2.8. Proliferation and migration assays

Transfected cells were seeded into 96-well plates and were let to adhere overnight. Proliferation assays were performed using AlamarBlue Cell Viability Reagent (Life Technologies, USA) according to

manufacturer's instructions. The viability reagent was aseptically and directly added to the culture medium to a final concentration of 10%. After incubation for 48 hours, absorbance at 570 nm and 600 nm was measured in Infinite 200 PRO microplate reader (Tecan, Switzerland). The experiment was performed in triplicate (four replicates per experiment). For migration assays, transfected cells were seeded in serum-free medium into the upper chamber of HTS Transwell96 Permeable Supports with an 8 µm-pore size membrane (Corning, USA). After incubation for 24 hours, cells that did not migrate were wiped out, and AlamarBlue was added to the reservoir plate as described for proliferation assays. The experiment was performed twice (three replicates per experiment).

2.9. Isolation and relative quantification of miRNA

Transfected cells were collected for miRNA extraction using the GRS microRNA Kit (GRiSP, Portugal), according to manufacturer's conditions. The concentration and purity of miRNAs were measured at 260 nm and 280 nm using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). First-stranded cDNA was synthesized using the Applied Biosystems TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) with sequence-specific stem-loop primers for each miRNA. The thermal conditions were as follows: 16°C for 30 min, 42°C for 60 min, and 85°C for 10 min. For relative quantification, miRNA expression levels were analyzed by qRT-PCR using the Applied Biosystems StepOne Real-Time PCR (Thermo Fisher Scientific, USA). Reactions contained 1× master mix, 1× probes [TaqMan MicroRNA Assays miR-221 (002096), miR-222 (002097), miR-145 (002149), miR-7 (002314), miR-200b (002251)], the synthesized cDNA, and RNU48 endogenous control (TaqMan MicroRNA Assay 001006). Data analysis was carried out using the Applied Biosystems StepOne Software v2.2 (Thermo Fisher Scientific, USA) with the same baseline and threshold set for each plate, to generate threshold cycle values (Ct) for all miRNAs analyzed in each sample. The experiment was performed in duplicate.

2.10. Immunofluorescence

The day before immunofluorescence assay, cells were seeded on coverslips coated with polyLornithine (0.1 mg/ml). Cells were washed with 1×PBS and fixed using 4% paraformaldehyde for 15 min. After that, cells were permeabilized with 0.2% Triton X-100/1×PBS for 15 min, blocked with 3% BSA/1×PBS for 1 h at room temperature and incubated with the adequate primary antibody, previously diluted in the blocking solution, for 1 h. Cells were then washed with 1×PBS, incubated with Texas red-labeled anti-rabbit secondary antibody in the dark for 1 h. After being washed three times with 1×PBS, coverslips were mounted in slides using Vectashield Mounting Medium with DAPI (Vector Laboratories, USA), and cells were visualized in Olympus IX-18 motorized inverted microscope.

2.11. Statistical analysis

Statistical significance of the results was assessed using the Mann-Whitney U test or the Kruskal-Wallis test. To compare miRNA relative quantifications, we used the $2^{-\Delta\Delta Ct}$ along with t-test. Significance level was set to 0.05. The statistical analyses were performed using IBM SPSS Statistics 20.0 software or GraphPad Prism 6.

3. Results

3.1. TCTEX1D4 and PP1 γ protein expression levels are increased in PCa cells, but their mRNA levels decrease with malignancy progression

The expression levels of TCTEX1D4 and PP1 γ were assessed in RWPE-1, PNT-2, LNCaP and PC3 cell extracts. TCTEX1D4 and PP1 γ were found to be expressed at higher levels in PCa cells than in normal-like prostate epithelial cells (Fig. 1A and 1B). Due to the very low levels of TCTEX1D4 in normal-like human prostate cell lines, the presence of TCTEX1D4 in all cell lines was further confirmed by immunoprecipitation (Fig. 1A). The comparison between RWPE-1 and PCa cells showed an increase of about 2 to 3 times in the expression of TCTEX1D4 and 2 to 4 times in the expression of PP1 γ in LNCaP and PC3 cells, respectively (Fig. 1B). Our results also showed a clear tendency towards the increase of TCTEX1D4 protein levels with the progressive aggressiveness of PCa cells (Fig. 1C). To understand if this tendency relied on transcriptional differences, TCTEX1D4 and PP1 γ mRNA levels were quantified in PCa cells by RT-qPCR. However, a slight decrease in TCTEX1D4 mRNA levels was observed, whereas a significant reduction in PP1 γ mRNA levels was detected in PC3 cells (Fig. 1D).

3.2. TCTEX1D4 and PP1 γ are differentially localized between normal-like and PCa cells

TCTEX1D4 and PP1 γ localizations were analyzed by immunocytochemistry to uncover potentially different distribution patterns in PCa cells. As shown in Fig. 2, both TCTEX1D4 and PP1 γ displayed a fine punctuated pattern within the cytoplasm and nucleus of normal-like prostate epithelial cells. In PCa cells, PP1 γ and TCTEX1D4 localizations were clearly distinct, with TCTEX1D4 strictly localized into large aggregate-like clusters within the cytoplasm and PP1 γ mostly localized in the nucleus (Fig. 2).

3.3. TCTEX1D4 regulates cell proliferation and this function depends on its interaction with PP1

To determine the function of TCTEX1D4 in cell proliferation and its potential role in PCa, we overexpressed TCTEX1D4 in PNT-2, LNCaP and PC3 cell lines. Overexpression of TCTEX1D4 significantly reduced the proliferation of PNT-2 cells, while increased the proliferation of LNCaP cells (Fig. 3A). To address the relevance of PP1 in TCTEX1D4 function, we transfected cells with a mutant TCTEX1D4 that had an impaired PP1-docking motif [3]. PNT-2 and LNCaP cells transfected with mutant TCTEX1D4 exhibited higher proliferation rate than wild-type TCTEX1D4-transfected cells (Fig. 2A). No significant alterations were found for PC3 cells (Fig. 2A). No alteration was observed regarding cell migration for none of the cell lines (data not shown).

3.4. TCTEX1D4 modulates microRNAs expression patterns

The expression levels of miR-7/-145/-200b/-221/-222 were quantified in wild-type and mutant TCTEX1D4-transfected cells by qRT-PCR. The expression levels of the selected miRNAs were all upregulated in PNT-2 cells overexpressing wild-type TCTEX1D4 and, in a less extent, mutant TCTEX1D4 (Fig. 3B). In PCa cells, only miR-145 and miR-222 showed significant differences upon plasmids transfection (Fig. 3B). TCTEX1D4 overexpression upregulated the expression of miR-222 in LNCaP cells, while upregulated miR-145 and downregulated miR-222 in PC3 cells (Fig. 3B). On the other hand, overexpression of mutant TCTEX1D4 upregulated miR-145 and downregulated miR-222 in LNCaP cells, whereas upregulated miR-222 in PC3 cells (Fig. 3B).

4. Discussion

As a dynein light chain protein, TCTEX1D4 determines cargo binding and specificity, regulating the retrograde transport of vesicles and components of signaling pathways inside cells [16]. Besides this role, common to all members of its protein family, TCTEX1D4 roles are yet to be determined. The analysis of TCTEX1D4 sequence revealed many putative serine phospho-sites, but no threonine or tyrosine phospho-sites [3], suggesting a major role for serine phosphorylation in the regulation of its activity. Accordingly, we have previously identified TCTEX1D4 as a PP1-interacting protein in a yeast two-hybrid screening using a human testis cDNA library [3]. The binding involves a consensus RVSF motif located at the N-terminus of TCTEX1D4 and the interaction was found to occur mainly in the nucleus and the microtubule-organizing center (MTOC) [3]. Here we show that TCTEX1D4 is expressed at very low levels in human normal-like prostate epithelial cells, but its expression levels, as well as the expression levels of PP1 γ , are increased in PCa cells (Fig. 1). Despite the increased protein content of both proteins, their mRNA levels decrease with malignancy, which is indicative of differential regulation of both proteins in normal and cancer cells (Fig. 1). Hence, posttranscriptional and/or posttranslational mechanisms might be involved in their regulation.

Our results also demonstrate that TCTEX1D4 and PP1 γ are localized within nucleus and cytoplasm in normal-like prostate epithelial cells, but their localizations change with malignancy. TCTEX1D4 becomes mainly confined to large cytoplasmic aggregate-like agglomerates in PCa cells, while PP1 γ is mostly restricted to the nucleus (Fig. 2). A possible compromised degradation of TCTEX1D4 may facilitate its aggregation as observed for other proteins. Therefore, whilst in normal-like prostate cells TCTEX1D4 and PP1 γ share subcellular compartments, in PCa cells their main localizations are distinct, leading us to hypothesize that a possible interaction between PP1 γ and TCTEX1D4 may be lost during PCa progression.

Meng et al. suggested that TCTEX1D4 was able to inhibit TGF β signaling by compromising the internalization of membrane receptors [6]. Hence, it was reasonable to expect that its overexpression, at least in normal cells, would lead to enhanced proliferation. In fact, other dynein light chain proteins have already been associated with the regulation of cell proliferation, as it is the case of dynein light chain 1 in human breast cancer cells [17]. Here, we corroborate the ability of TCTEX1D4 to regulate cell proliferation and establish an essential role for PP1 in regulating TCTEX1D4 function. Overexpression of wild-type

TCTEX1D4 downregulates the proliferation of PNT-2 cells, while upregulates the proliferation of LNCaP cells (Fig. 3A). This contradictory behavior might be partially explained by the redistribution of PP1 to the nucleus and consequent dissociation, at least in part, of the complex. The mutation of the PP1-docking motif in TCTEX1D4 by one amino acid was previously demonstrated to decrease the binding of TCTEX1D4 to PP1 in about 35% [3]. Mutant PNT-2 and particularly LNCaP cells showed improved proliferation rates (Fig. 3A), probably due to the enhanced inhibitory role of TCTEX1D4 on the TGF β pathway. On the other hand, neither TCTEX1D4 itself nor the PP1/TCTEX1D4 complex are relevant for the proliferation of castration-resistant PC3 cells (Fig. 3A). Taken these results together, two hypotheses can be further explored: on the one hand, PP1 may 'switch off' the inhibitory role of TCTEX1D4 on the TGF β signaling pathway, thereby restoring TGF β -inhibitory growth effect; on the other hand, TCTEX1D4 may be responsible for the translocation of PP1 into the nucleus, where it catalyzes the dephosphorylation of other substrates, as AR dephosphorylation (Ser⁶⁵⁰) and, consequently, promotes AR-mediated transcriptional activity (Fig. 4) [18].

It is also known that the TGF β signaling directly interferes with the expression of several miRNAs which, in turn, have been implicated in tumorigenesis [19]. Our results suggest a potential role for TCTEX1D4 in the modulation of the expression of a panel of miRNAs known to be involved in PCa, particularly in PNT-2 cells (Fig. 3B). These results provide further evidence for the activity of the TGF β signaling pathway in normal-like prostate cells, since the expression of miR-145 can be induced after activation of Smads [20]. However, upon disruption of the TCTEX1D4/PP1 complex, only miRs-145/-221/-222 were significantly upregulated (Fig. 3B). In PCa cells, only miR-145 and miR-222 were shown to be differentially regulated (Fig. 3B). miR-145 has tumor suppressive roles, inducing cell cycle arrest and apoptosis, while inhibiting cell motility and metastasis formation [21]. On the other hand, responses to miR-222 are controversial and both oncogenic and tumor suppressive functions have been described [13, 22]. The role of TCTEX1D4 and TCTEX1D4/PP1 in regulating miR-222 might be explained by alterations in the MAPK signaling [23], which is known to be negatively regulated by PP1 [24].

5. Conclusion

Overall, our results suggest new roles for TCTEX1D4 and the PP1/TCTEX1D4 complex in prostate tumorigenesis. PP1 is known to bind and dephosphorylate intermediate chains of dynein complexes, so binding to TCTEX1D4 is most likely to facilitate the access to intermediate chains, thus regulating the dynein complex function. Whether PP1 is capable of directly dephosphorylate TCTEX1D4 is still to be determined. While PCa progresses, the complex might be lost and TCTEX1D4 might be reallocated towards other signaling pathways, organelles or molecular complexes, or it may simply lose its function and undergo aggregation. Considering its function as a dynein light chain protein, it is also possible that TCTEX1D4 is responsible for the retrograde movement of PP1-interacting proteins in normal cells and that the loss of its function promotes the relocalization of PP1 γ . Subsequent studies should improve this characterization, as well as identify additional interacting partners that may explain the cellular outputs of TCTEX1D4 overexpression.

Declarations

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Competing Interests

The authors have declared that no conflict of interest exists.

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Author Contributions

Juliana Felgueiras, Luís Sousa, Luís Korrodi-Gregório, Rui Medeiros and Margarida Fardilha contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juliana Felgueiras, Luís Sousa, Ana Luísa Teixeira, Bárbara Regadas, Luís Korrodi-Gregório, Ann-Kristin Ahlers and Georg Luers. The first draft of the manuscript was written by Juliana Felgueiras and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

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Figures

Figure 1

TCTEX1D4 and PP1γ protein expression in prostate cell lines. A) Basal protein expression of TCTEX1D4 and PP1γ were assessed in RWPE-1, PNT-2, LNCaP and PC3 cell extracts by western blot (WB). TCTEX1D4 expression was also assessed by slot blot (SB) and immunoprecipitation (IP). β-tubulin was used as loading control in WB and SB experiments. B) Mean intensity of TCTEX1D4 and PP1γ expression normalized to the respective β-tubulin content. C) Relative expression of TCTEX1D4 and PP1γ tend to increase with increasing aggressiveness of cells (in comparison to the results obtained for RWPE-1 cells). D) mRNA expression of PP1γ and TCTEX1D4 in prostate cancer cells. Expression ratios were calculated using placenta as control of the experiment. Error bars: 95% confidence interval. * $p < 0.05$, ** $p < 0.01$. Note: the lanes on western blot were rearranged into the presented order.

Figure 2

Subcellular localization of TCTEX1D4 and PP1 γ in prostate cell lines. RWPE-1, PNT-2, LNCaP and PC3 cells were fixed, permeabilized and assessed for the basal localization of TCTEX1D4 and PP1 γ . DAPI (blue) was used to counterstain the nucleus. Cells were observed under an Olympus IX-81 motorized inverted epifluorescence microscope. Representative figures from three independent experiments are shown. Scale bars equal 20 μ m.

Figure 3

Roles of TCTEX1D4 and TCTEX1D4/PP1 complex in cell proliferation and miRNA expression. PNT-2, LNCaP and PC3 cells were transfected with Myc-TCTEX1D4 and Myc-TCTEX1D4-RVSA plasmids to overexpress wild-type TCTEX1D4 and mutant TCTEX1D4 (mutated PP1-docking motif). A) Cell proliferation was assessed 72 h after transfection using AlamarBlue. The experiments were done in four replicates and repeated in three independent experiments. B) Cells were collected 24 h after transfection to quantify the expression levels of miR-7, miR-145, miR-221, miR-222 and miR-200b. Error bars: 95% confidence interval. * $p < 0.05$; ** $p < 0.01$.

Figure 4

Schematic representation of the working hypothesis for the PPP1/TCTEX1D4 complex.