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► **To cite this version:**

Saskia Gueller, Sigal Gery, Verena Nowak, Liqin Liu, Hubert Serve, et al.. Adaptor protein Lnk associates with Y568 in c-Kit. *Biochemical Journal*, 2008, 415 (2), pp.241-245. 10.1042/BJ20080102 . hal-00478952

HAL Id: hal-00478952

<https://hal.science/hal-00478952>

Submitted on 30 Apr 2010

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Adaptor protein Lnk associates with Y568 in c-Kit

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Short title: Lnk associates with Y568 in c-Kit

Key words: Lnk, c-Kit, adaptor protein, signal transduction, receptor tyrosine kinase, protein-protein interaction

Abbreviations used: APS, adapter protein with pleckstrin homology and Src homology 2 domains; CSF1R, colony-stimulating factor 1 receptor; DD, dimerization domain; EpoR, erythropoietin receptor; Grb2 and 7, growth factor receptor-bound protein 2 and 7; GST, glutathion-S-transferase; IVT, in vitro translation; JXM, juxtamembrane; Lnk, lymphocyte adaptor protein; Mpl, thrombopoietin receptor; PH, Pleckstrin homology domain; PI3K, phosphoinositide-3-kinase; PLC γ , phospholipase C gamma; PDGFR, platelet derived growth factor receptor; SCF, stem cell factor; SH2, Src homology domain 2; SH2-B, SH2B adaptor protein 1.

SYNOPSIS

The adaptor protein Lnk is expressed in hematopoietic cells and plays a critical role in hematopoiesis. Animal model studies demonstrated that Lnk acts as a broad inhibitor of signalling pathways in hematopoietic lineages. Lnk belongs to a family of proteins sharing several structural motifs, including a Src homology 2 (SH2) domain which binds phospho-tyrosines in various signal-transducing proteins. The SH2 domain is essential for Lnk-mediated negative regulation of several cytokine receptors (i.e. Mpl, EpoR, c-Kit). Therefore, inhibition of the binding of Lnk to cytokine receptors might lead to enhanced downstream signalling of the receptor and thereby to improved hematopoiesis in response to exposure to cytokines (i.e. erythropoietin in anemic patients). This hypothesis led us to define the exact binding site of Lnk to the stem cell factor receptor c-Kit. Pull-down experiments using GST-fusion proteins of the different domains of c-Kit showed that Lnk almost exclusively binds to the phosphorylated juxtamembrane domain. Binding of Lnk to the juxtamembrane domain was abolished by point mutation of the Y568 and was competed by peptides with a phospho-tyrosine in position 568. Co-immunoprecipitation with full-length wild-type or Y568F mutant c-Kit and Lnk confirmed these results, thus showing the importance of this phosphorylated tyrosine. Lnk bound directly to c-Kit without requiring other interacting partners. The identification of the binding site of Lnk to c-Kit will be useful to discover inhibitory molecules that prevent the binding of these two proteins, thus making hematopoietic cells more sensitive to growth factors.

INTRODUCTION

The receptor for stem-cell factor (SCF), c-Kit, belongs to the class III family of receptor tyrosine kinases that also includes the platelet-derived growth factor receptors (PDGFR) and the macrophage colony stimulating factor receptor (CSF1R) [1;2]. These receptors are structurally characterized by an extracellular region consisting of five immunoglobulin-like domains, a transmembrane, juxtamembrane and an intracellular tyrosine kinase domain. The latter is split into a proximal and a distal region separated by an insert. In general, c-Kit is expressed on hematopoietic stem cells and their progenitor cells; and expression is lost during further differentiation. It plays an important role in self-renewal, survival and growth of hematopoietic stem cells. Therefore, homozygous c-Kit knock-out mice are nonviable due to anemia; and heterozygous deletional mice have reduced c-Kit tyrosine kinase activity causing anemia and reduced fertility [3].

Binding of SCF to c-Kit results in receptor dimerization and activation of the tyrosine kinase, which subsequently causes autophosphorylation of specific tyrosine residues. These phospho-tyrosines can be recognized and bound by signal transduction molecules containing Src homology 2 (SH2) domains. Thereby, Grb2, Grb7, APS, Lnk, PI3K, PLC γ and other proteins can associate with activated c-Kit [4].

Lnk is an adaptor protein expressed in hematopoietic tissues and has critical functions in hematopoiesis [5-10]. Together with APS and SH2-B, Lnk shares several structural motifs including a SH2 domain, a pleckstrin homology (PH) domain and a dimerization domain (DD). In response to ligand mediated autophosphorylation of tyrosine residues within either the erythropoietin receptor (EpoR) [11], thrombopoietin receptor (Mpl) [12] or stem cell factor

receptor (c-Kit) [6;8], Lnk associates with the receptors through its SH2 domain and subsequently inhibits their downstream signalling [13-15]. The SH2 domain is essential for the inhibitory activity of Lnk [6;11;12]. Proliferation and differentiation of hematopoietic precursors is enhanced by decreased expression of Lnk and is inhibited by increased levels of Lnk [6;8]. Therefore, inhibition of Lnk binding to cytokine receptors makes hematopoietic progenitor cells more sensitive to the growth enhancing factors. The binding site of Lnk in c-Kit has not yet been determined. We wished to determine this site of interaction because we want to develop an assay to screen for agents that prevent binding of Lnk to cytokine receptors and thereby enhance the effect of endogenous or exogenous cytokines.

EXPERIMENTAL

Cell culture and transfections

293T cells were grown in Dulbecco's modified Eagle's medium with 10 % FBS and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. 48 hours after transfection, cells were lysed in lysis buffer containing 0.5 % NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN).

Expression vectors

V5-tagged human Lnk cDNA was cloned into the pcDNA3.1 vector. The point mutation Lnk R392E was generated by PCR and confirmed by sequencing. DNA corresponding to the juxtamembrane (JXM, amino acids 544–577), proximal kinase (Kin1, amino acids 578–685), kinase insert (KI, amino acids 686–762), distal kinase (Kin2, amino acids 775–845), and carboxyl tail (CT, amino acids 926–976) domains of c-Kit cloned into pGEX-4T-1 were kindly provided by Johan Lennartsson (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden). The point mutations Y568F and Y570F were generated by site-directed mutagenesis of the JXM domain, and the sequences confirmed by nucleotide sequencing. The expression vector (pDSR α 22) for wild-type full-length c-Kit was kindly provided by Amgen. The point mutation Y568F was generated using the Quikchange Mutagenesis Kit (Stratagene, La Jolla, CA) followed by nucleotide sequencing.

GST-fusion proteins

The pGEX-4T-1 constructs were transformed into TKX1 competent cells (Stratagene, La Jolla, CA) for production of GST-fusion proteins with phosphorylated tyrosines and into *E. coli* DH5 α for generation of unphosphorylated GST-fusion proteins. Transformation, accumulation of the fusion proteins and induction of the tyrosine kinase were performed according to the manufacturer's manual. Briefly, after growing either TKX1 or DH5 α bacteria overnight, production of the GST-fusion proteins was induced by 0.1 mM isopropylthio- β -galactoside (IPTG; Invitrogen) for 2.5 hours. Thereafter, only TKX1 bacteria were resuspended in tryptophan-free medium containing 10 μ g/mL indoleacrylic acid (IAA; Sigma-Aldrich, St. Louis, MO) to activate the tyrosine kinase and incubated for another 2 hours. Harvesting and purification of phosphorylated and unphosphorylated GST-fusion proteins were carried out as described in the "GST Gene Fusion System" manual (Pharmacia Biotech, Uppsala, Sweden).

Cells were sedimented and disrupted by sonication, proteins were bound to Glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ) and were stored in PBS containing 15 % glycerol at -80°C until their use for pull-down experiments.

In vitro translation

In vitro translated (IVT) Lnk was synthesized from the pcDNA3.1-Lnk expression vector using the TNT[®] T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) according to the manufacturer's recommendations. Generation of proteins was carried out in reticulocyte lysate using the one-step transcription/translation system. Expression levels of IVT Lnk were determined by Western blot.

GST pull-down

GST pull-down assays were carried out with equivalent amounts of GST and GST-fusion proteins immobilized on glutathione-sepharose beads in lysis buffer. After 16-hour incubation at 4°C with whole 293T cell lysates or IVT Lnk, respectively, precipitated proteins were washed with PBS, eluted with SDS sample buffer and resolved by SDS-PAGE. After transfer to PVDF membranes, the Lnk protein was detected with V5-tag antibodies (Applied Biological Materials Inc., Vancouver, Canada), phosphorylation of the GST-fusion proteins with phospho-tyrosine antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and equal loading was confirmed either with GST-antibodies (Santa Cruz Biotechnology), Ponceau staining or Coomassie staining of the gel. Phosphorylation of TKX1-derived GST-fusion proteins was verified by Western blot using phospho-tyrosine antibodies (Santa Cruz Biotechnology).

Co-immunoprecipitation

For co-immunoprecipitation of full-length c-Kit and Lnk, 293T cells were co-transfected with c-Kit wild-type or Y568F mutant and Lnk wild-type cDNA. Before lysis, cells were serum-starved overnight and treated with 50 ng/mL human SCF (Peprotech, Rocky Hill, NJ) for 30 minutes. 500 μg of protein was precipitated using 1 μg of V5-tag (Invitrogen) or c-Kit (Santa Cruz Biotechnology) antibodies and 20 μL Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C for 16 hours, then washed with PBS, eluted with SDS sample buffer and resolved by SDS-PAGE. Precipitated proteins were detected with c-Kit or V5-tag antibodies.

Competition assay

Synthetic peptides corresponding to the tyrosine-containing part of the JXM domain of c-Kit were purchased from Quality Controlled Biochemicals (Hopkinton, MA) including different phospho-tyrosines: GKGKGKEEINGNNYVYIDPTQL-OH (Y568/570), GKGKGKEEINGNN[pY]VYIDPTQL-OH (pY568), GKGKGKEEINGNNYV[pY]IDPTQL-OH (pY570) and GKGKGKEEINGNN[pY]V[pY]IDPTQL-OH (pY568/570). Competition pull-down assays were carried out in lysis buffer using a 10- or 100-fold molar excess of the peptides compared to the amount of GST-JXM fusion protein and 5 μg of protein from 293T cells overexpressing Lnk. The samples were incubated at 4°C for 2.5 hours and then washed and processed as described for the pull-down assays.

RESULTS

Lnk binds to the phosphorylated juxtamembrane domain of c-Kit

Phosphorylated GST-fusion proteins consisting of the five different domains of c-Kit were isolated from TKX1 bacteria, and expression and phosphorylation were confirmed by Western blot (Figure 1A). Some of the GST-proteins showed low expression, so that the amounts of GST input were adapted according to their expression to get equal loading. In pull-down experiments using the different c-Kit domains that were incubated with whole cell lysates of 293T cells overexpressing wild-type Lnk, we found that Lnk mainly bound to the juxtamembrane (JXM) domain of c-Kit (Figure 1B). As expected, Lnk did not bind to either the unphosphorylated JXM fusion protein or the control GST without fusion product.

Mutation of Y568 abolishes binding of Lnk

The SH2 domain of various adaptor proteins recognize and bind to phosphorylated tyrosine residues of their binding partners. Therefore, we tested if Lnk binds to a tyrosine residue in c-Kit. The JXM domain of the receptor carries two tyrosine residues, that are potential binding sites for Lnk: Y568 and Y570. By site-directed mutagenesis, we created vectors containing the sequences for the JXM domain of c-Kit containing either Y568F or Y570F point mutations, respectively. Pull-down experiments were carried out with wild-type or point mutated GST-fused JXM domains and whole lysates derived from 293T cells overexpressing Lnk. We found that the Y570F mutation did not influence the binding of Lnk, but the Y568F mutation abolished binding of Lnk to the JXM domain (Figure 2A). We conclude that Lnk selectively binds to the phosphorylated Y568 tyrosine residue in the JXM domain of c-Kit. To confirm the specificity of binding of Lnk to Y568 of c-Kit, competition assays using peptides with different phosphorylation patterns as competitors for GST-JXM were performed. The peptides were utilized in a 10- or 100-fold molar excess as compared to GST-JXM. As shown in Figure 2B, binding of Lnk to GST-JXM was competed in a dose-dependent manner by peptides harbouring a phospho-tyrosine in position 568 (pY568/570 and pY568), whereas the other peptides did not affect binding. Furthermore, co-immunoprecipitation assays were performed to prove the specific binding site in full-length c-Kit. With V5-tag or c-Kit antibodies, respectively, proteins from 293T lysates overexpressing either wild-type or Y568F mutant c-Kit and V5-tagged wild-type Lnk were precipitated and analyzed by SDS-Page. Figure 2C shows that SCF-induced phosphorylation of tyrosine residues in wild-type c-Kit expressing cells led to binding of Lnk to the receptor. The mutation Y568F completely abolished the interaction, thus showing the importance of this tyrosine as a docking site for Lnk.

Binding of human Lnk to human c-Kit depends on its SH2 domain

Binding of murine Lnk to the murine c-Kit receptor is abolished by point mutation of the arginine R364 of Lnk [6]. We tested if the same arginine residue in the conserved SH2 domain of human Lnk is responsible for its binding to human c-Kit. R392 is the arginine residue in human Lnk which corresponds to R364 in murine Lnk. A point mutation R392E was inserted into the Lnk vector and it was expressed in 293T cells. Pull-down experiments were performed using GST-JXM of c-Kit and either wild-type or mutant Lnk lysates. As shown in Figure 3, R392E completely abolished binding of Lnk to c-Kit.

Binding of Lnk to c-Kit JXM is direct

Studies have shown that Lnk binds to c-Kit [6], but no one elucidated if the binding is direct or if Lnk needs co-partners to interact with c-Kit. Therefore, pull-down assays were carried out using in-vitro translated protein as a source of Lnk instead of whole cell lysates. Similarly to Lnk from 293T lysates, in vitro translated Lnk was able to bind to GST-JXM. Y570F point mutated GST-JXM still bound Lnk, whereas the Y568F point mutation abolished binding (Figure 4). These data demonstrate that Lnk binds to c-Kit directly and independently of other binding partners.

DISCUSSION

In this study, we demonstrated for the first time that the adaptor protein Lnk binds to the juxtamembrane domain of c-Kit and specifically to the tyrosine Y568. These results are in agreement with data that showed that APS, a family member of Lnk, binds to the same tyrosine residue in c-Kit [16]. In contrast to APS, Lnk binds to only one tyrosine and does not bind to the carboxy-tail of c-Kit; whereas APS binds to both the JXM domain, as well as the carboxy-tail. Both adaptor proteins lead to inhibition of downstream signalling of c-Kit upon stimulation with SCF. The juxtamembrane domain of c-Kit is known to have an autoinhibitory function. Transphosphorylation of Y568 and Y570 are the first steps that occur after ligand mediated dimerization of the receptor. As a result, the juxtamembrane segment no longer immobilizes the receptor in a static configuration, but allows conversion of the activation loop of c-Kit from a compact inactive form to an extended activated receptor. Subsequently, transphosphorylation of Y823 in the activation loop stabilizes the active form of the tyrosine kinase [17]. The SH2-domain containing phosphatases 1 and 2 (SHP-1 and SHP-2) are known to bind to phosphorylated Y568 and Y570 and terminate the SCF signal by dephosphorylating the two tyrosine residues and thereby restoring the inactive conformation of the receptor [18]. Thus, the juxtamembrane domain plays a major role both in activating as well as inhibiting c-Kit, which makes its tyrosine residues very important regulating elements. This is also reflected by the fact that several signal transduction molecules influencing the activity of the receptor (i.e. Src kinases, Chk and Shc) are known to bind to the same domain. We showed that binding of Lnk to this site is independent of binding of other proteins, but Lnk might still be an important factor that mediates binding of other regulating proteins to the receptor. APS has been shown to bind c-Cbl, a ubiquitin ligase which targets growth factor receptors for degradation in lysosomes [19]. Thereby, loss of binding of APS to PDGFR β leads to decreased recruitment of c-Cbl and to reduced degradation of the receptor, followed by enhanced c-fos activation. In contrast, low levels of c-Cbl had no effect alone on levels of the receptor, but in the presence of APS, enhanced recruitment of c-Cbl occurred resulting in degradation of the receptor [20]. The C-terminal part of Lnk contains a c-Cbl binding site similar to APS, so the same mechanism could lead to its inhibitory function, whereas c-Cbl can also bind directly to c-Kit [21]. Nevertheless, further exploration is necessary to elucidate the interaction between Lnk and c-Kit.

Our data identifying the binding site of Lnk to c-Kit can help to develop an assay to screen for “small inhibitor molecules” to prevent the binding of these two proteins; and thus, potentially make hematopoietic cells more sensitive to either endogenous or exogenous hematopoietic growth factors offering a novel approach to stimulate blood cell production.

ACKNOWLEDGEMENTS

This work was supported in part by the Deutsche Krebshilfe (SG) and the Tower Cancer Research Foundation Fellowship (SG).

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FIGURE LEGENDS

Figure 1 Lnk binds to the juxtamembrane domain of c-Kit

(A) Expression (IB: GST, upper panel) and phosphorylation (IB: PY, lower panel) of GST-fusions of various domains of c-Kit: JXM (juxtamembrane), Kin1 (proximal kinase), KI (kinase insert), Kin2 (distal kinase), CT (carboxy-tail). GST indicates the GST control protein without fusion. The proteins obtained from the *E. coli* strain TKX1 are phosphorylated which is not the case for these proteins produced by regular DH5 α *E. coli*. IB: immunoblot. Bands below the stars (*) highlight target protein fragments. (B) Lnk pull-down from a lysate of 293T cells overexpressing Lnk. The input lane shows Lnk expression in the lysate. The anti-V5 immunoblot (IB: Lnk, upper panel) reveals strong interaction of Lnk with the phosphorylated JXM domain of c-Kit. Coomassie staining of the SDS gel (lower panel) shows the relative amount of GST fusion proteins in each lane.

Figure 2 Phosphorylated Y568 is the binding site of Lnk in c-Kit

(A) GST pull-down of Lnk with mutated c-Kit JXM proteins. Input lane shows Lnk expression in the lysate. GST indicates the GST control protein without fusion. The anti-V5 immunoblot (IB: Lnk, upper panel) reveals strong interaction of Lnk with the phosphorylated wild-type (WT) and Y570F point mutated (570) JXM domain of c-Kit. Interaction is abolished by the Y568F (568) point mutation. Phosphorylation and equal loading of the GST-fusion proteins is confirmed by reprobing the blot with phospho-tyrosine antibodies (IB: PY) and Coomassie staining (lower panels). (B) Binding of Lnk to phosphorylated GST-JXM (TKX1) can be competed by peptides harbouring a phospho-tyrosine in position 568. For competition pull-down experiments, peptides containing the two tyrosine residues of c-Kit JXM with different phosphorylation patterns were used: phospho-tyrosines in position 568 and 570 (pY568/570), only in position 568 (pY568) or 570 (pY570) or without any phospho-tyrosines (no pY). Pull-downs were carried out without competition (no comp.) or with 10- or 100-fold excess (10x or 100x) of the indicated peptides. Competition of peptides with a phospho-tyrosine in position 568 is dose-dependent, whereas peptides without pY568 does not compete. Ponceau staining shows equal loading of GST-JXM. (C) Co-immunoprecipitation (IP) of Lnk with wild-type and Y568F mutant full-length c-Kit. Lnk interacts with wild-type c-Kit (WT), but not with Y568F mutant c-Kit (568). "Input" shows expression of Lnk (IB: Lnk) or c-Kit (IB: c-Kit) in lysates from 293T cells that were used for IP with either V5-tag, c-Kit or isotype IgG antibodies. Precipitated proteins were detected with either c-Kit or V5-tag antibodies.

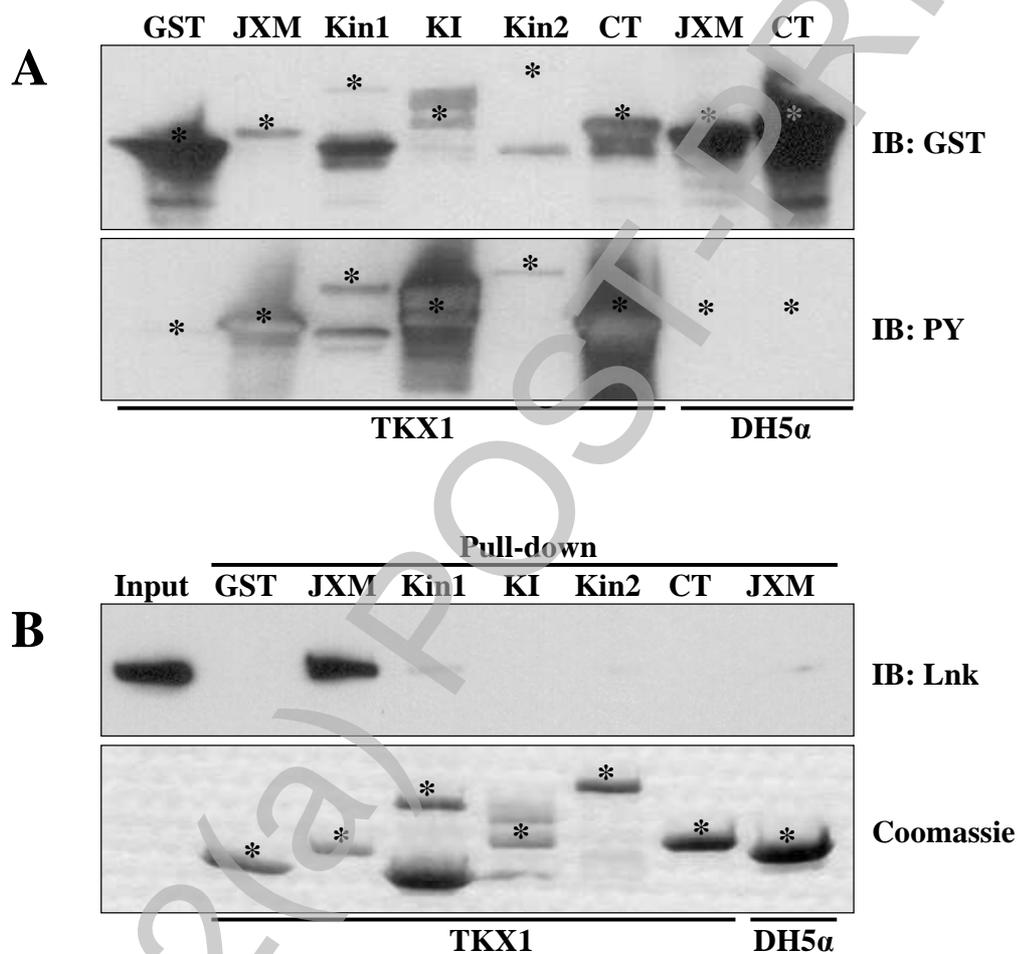
Figure 3 Binding of Lnk depends on R392 in its SH2 domain

Pull-down experiments were performed with wild-type c-Kit JXM domain and either wild-type or R392E mutated Lnk lysates from 293T cells, respectively. Human Lnk with a point mutation of the SH2 domain lost the ability to bind to c-Kit. Input shows expression of Lnk proteins in the lysates, Ponceau staining indicates equal amounts of GST-JXM.

Figure 4 Lnk binds to c-Kit directly without requirement of co-partners

Pull-down assays were performed using in-vitro translated (IVT) protein as a source of Lnk. IVT Lnk bound to phosphorylated (TKX1) wild-type (WT) and Y570F mutated (570) GST-JXM, but not to either unphosphorylated (DH5 α) or Y568F mutated (568) GST-JXM. Input shows expression of IVT Lnk, Coomassie staining confirms equal amounts of GST-fusion proteins.

Figure 1



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Figure 2

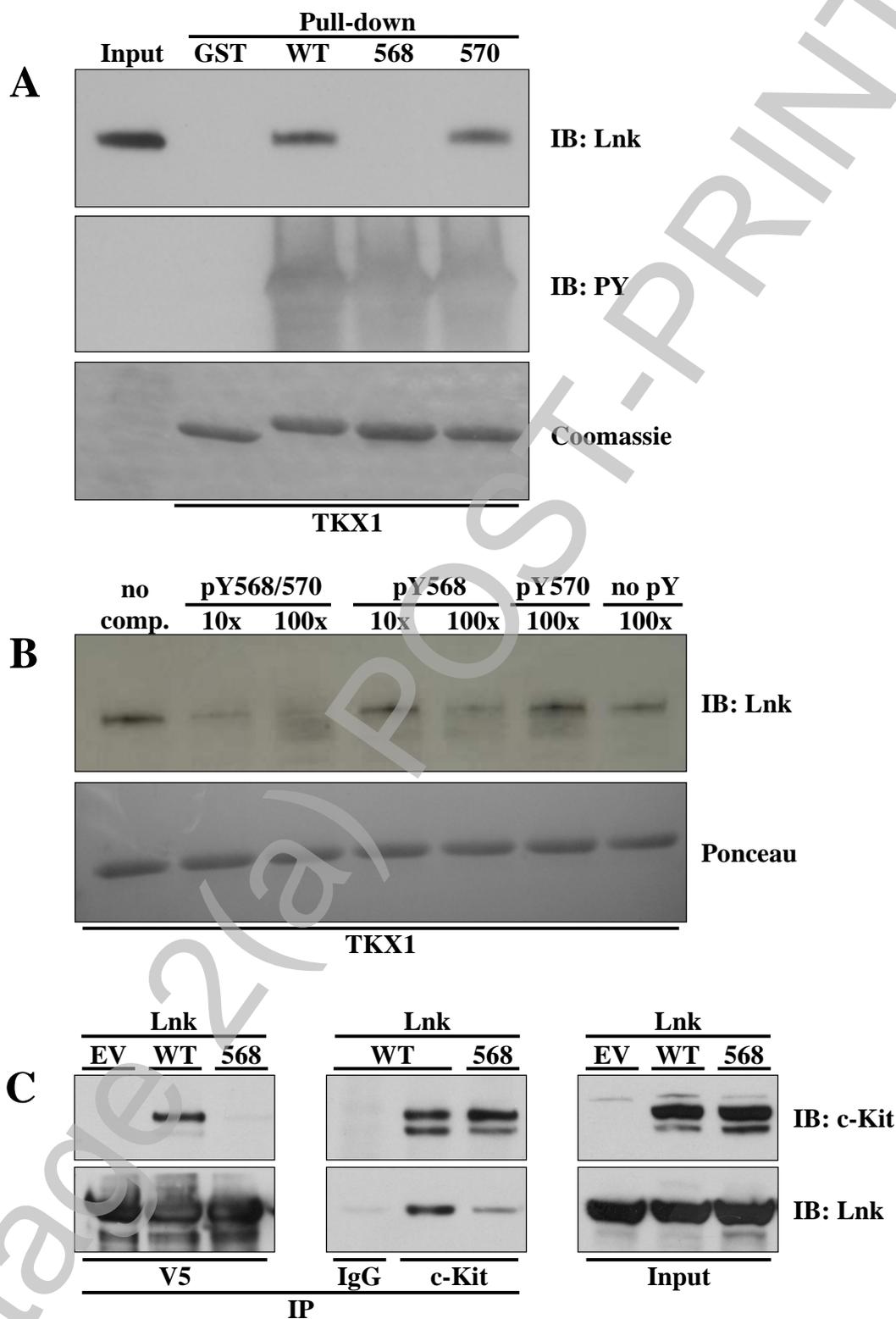
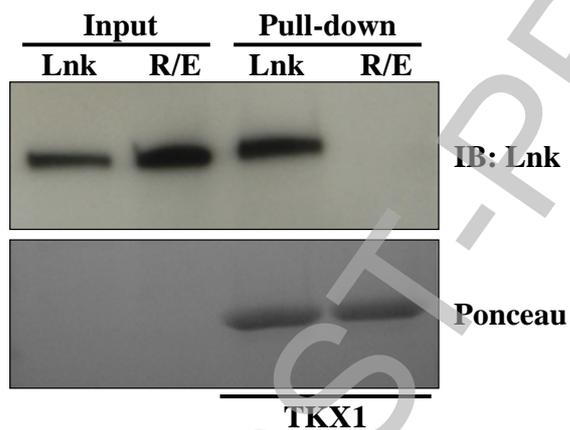


Figure 3



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