Supplementary data

Supplementary text

Characterization of CKIγ2 mutants with different subcellular localizations

When we analyzed the subcellular localization of GFP- and Flag-tagged CKIγ2 using fluorescence microscopy and cellular fractionation assays, we found that wild-type CKIγ2 resides in three subcellular compartments: cytoplasm, nucleus and the plasma membrane (Supplementary Figure S2, S5B-a and data not shown). The majority of CKIγ2 is in the cytoplasm, where it appears to be rapidly trafficking along microtubule-like structures (data not shown). A fraction of wild-type CKIγ2 resides in the nucleus as speckles. CKIγ2 is also known to be tethered to the plasma membrane through palmitoylation of its C-terminal tail region (Davidson et al., 2005, Supplementary Figure S5A and B-a).

To understand which pool of CKIγ2 is responsible for the inhibition of Smad3 activity, we decided to manipulate its intracellular localization by mutating/deleting specific regions of this kinase. Davidson et al (2005) reported that deletion of the last 20 amino acids of *Xenopus* CKIγ1, a region highly homologous to that of *Xenopus* and human CKIγ2, disrupts the membrane anchoring and leads to exclusively cytoplasmic localization of the kinase, indicating that palmitoylation occurs within this sequence. Consistent with this result, a bioinformatics search identified three consecutive, evolutionarily conserved cysteine residues (C399/400/401, Supplementary Figure S5A) as putative palmitoylation sites (http://bioinformatics.lcd-ustc.org/css_palm/). To verify this prediction, we mutated the three cysteines to alanines (designated as CKIγ2-3CA).

Indeed, this substitution abolished the plasma membrane association of CKIγ2 (Supplementary Figure S5B-c). Surprisingly, this CKIγ2-3CA mutant concurrently becomes highly enriched in the nucleus (Supplementary Figure S5B-c), suggesting that CKIγ2 might undergo active nuclear import.

Following this thought, we noticed that the C-terminal deletion mutant of XCKIγ1 reported by Davidson et al (2005) not only failed to associate with the plasma membrane but was also absent from the nucleus, indicating there might be a nuclear localization signal (NLS) within the same 20-amino-acid tail. In fact, we did find a stretch of positively charged, evolutionarily conserved residues (KRRKRK, a.a. 404-409) in this tail region of CKIγ2 that may very likely function as a typical NLS (Supplementary Figure S5A). As we expected, when this sequence was deleted by introducing a stop codon immediately before K404, the resulting mutant (CKIγ2-ΔNLS) was absent from the nucleus of almost all cells (Supplementary Figure S5A and B-b). This suggests that the KRRKRK sequence does function as an NLS, and to our knowledge this is the first evidence of CKIγ2 undergoing nucleocytoplasmic shuttling. This finding also explains the result of Davidson et al (2005) such that deletion of the last 20 amino acid of XCKIγ1 actually removes both the palmitoylation site and the NLS, therefore the mutant is exclusively cytoplasmic. Since the NLS is only three residues away from the CCC motif, the 3C→A mutation might have somehow enhanced the NLS activity, leading to the strong nuclear enrichment observed with the CKIγ2-3CA mutant.

We have also generated a further deletion of the C-terminal tail of CKIγ2 based on previous studies of CKIε tail function (Gietzen & Virshup, 1999; Waddell et al., 2004). This truncated CKIγ2 (designated as CKIγ2-ΔC, Supplementary Figure S5A) is not plasma membrane-bound due to the loss of the palmitoylation site (Supplementary Figure S5B-d). However, although the C-terminal NLS is also missing, CKIγ2-ΔC is primarily nuclear (Supplementary Figure S5A and B-d). This suggests that a second NLS exists in this mutant and/or a nuclear export sequence (NES) within the tail region (a.a. 354-403) has been removed from CKIγ2.

When compared to wild-type CKIγ2, the above mutants (ΔNLS, 3CA and ΔC) were expressed at similar levels (Supplementary Figure S6A and B). In MEFs, both CKIγ2-WT and CKIγ2-ΔNLS inhibited TGF-β/Smad3-induced expression of Integrin α5, JunB and CTGF to the same extent (Supplementary Figure S6A). In a parallel experiment, the two nucleus-enriched mutants, CKIγ2-3CA and CKIγ2-ΔC, also strongly repressed Smad3 activity (Supplementary Figure S6B). Importantly, all of the CKIγ2 mutants could strongly phosphorylate Smad3 as well as themselves (Supplementary Figure S6C). Therefore, phosphorylation of Smad3 by cytoplasmic or nuclear CKIγ2 can lead to the same result, i.e. an accelerated clearance of P-Smad3 and shortened TGF-β signaling duration. In contrast, β-catenin was only regulated by CKIγ2-WT and CKIγ2-ΔNLS but not the 3CA or ΔC mutants (data not shown), highlighting the importance of plasma membrane localization for CKIγ2 function in the Wnt pathway (Davidson et al., 2005). These latter findings also indicate that CKIγ2 regulates Wnt and TGF-β signaling through different mechanisms.

Supplementary materials and methods

Plasmid construction and primer sequences

All point mutations were generated following the QuikChange Site-Directed Mutagenesis protocol from Stratagene, Inc. The following primers were used to create the Smad3 Ser418 mutants:

S3 S418A-F 5'-*G ATG GGC TCC CCA GCA ATC CGC TGT TCC*-3',

S3 S418A-R 5'-*GGA ACA GCG GAT TGC TGG GGA GCC CAT C*-3',

S3 S418D-F 5'-*G ATG GGC TCC CCA GAC ATC CGC TGT TCC*-3',

S3 S418D-R 5'-*GGA ACA GCG GAT GTC TGG GGA GCC CAT C*-3'.

To generate GST-S3C(WT), the MH2 domain of human Smad3 (residues 229-425) was amplified by PCR using the following primers:

Smad3-MH2-F: 5'-*CGG AAT TCC CCG GCC TTC TGG TGC TCC ATC*-3'

Smad3-R: 5'-*CG TCT AGA CTA AGA CAC ACT GGA ACA GCG*-3'.

The PCR fragment was first sub-cloned into the pBlueScript II KS vector (Stratagene) and then into the pGEX-4T-3 (Amersham) vector between the EcoRI and NotI sites. GST-S3C(S418A) was generated by the same strategy using HA-Smad3(S418A) as template for PCR.

pcDNA3-CKIγ2 was generated by digesting pMOSBlue-CKIγ2 with HindIII and EcoRI and sub-cloning the CKIγ2 fragment into pcDNA3 (Invitrogen, Inc.). The CKIγ2 insert was alternatively amplified by PCR using pMOSBlue-CKIγ2 as template and the following primers:

CKIγ2-F: 5'-*CGG AAT TCC ATG GAT TTT GAC AAG AAA GGA G*-3',

CKIγ2-R: 5'-*CGG AAT TCC TCA CTT GTG TCG CTG CAG CGA T*-3'.

The PCR product was then sub-cloned into the EcoRI site of pFlag-CMV-2 (Sigma) and pEGFP-C1 (BD Clontech) to generate Flag- and GFP-tagged CKIγ2, respectively. The following primers were used to create the CKIγ2 mutants:

CKIγ2K75R (kinase-dead)-F: 5'-*GAA TAC GTG GCT ATC AGA TTG GAG CCG ATC*-3',

CKIγ2K75R-R: 5'-*GAT CGG CTC CAA TCT GAT AGC CAC GTA TTC*-3'.

CKIγ2-3CA-F: 5'-*GCC GAT GAA ACC AAA GCC GCG GCA TTC TTC AAG AGG AGA*-3',

CKIγ2-3CA-R: 5'-*TCT CCT CTT GAA GAA TGC CGC GGC TTT GGT TTC ATC GGC*-3';

CKIγ2-F403 STOP-F: 5'-*C TGC TGT TTC TTC TAG AGG AGA AAG AGA AAA*-3',

CKIγ2-F403 STOP-R: 5'-*TTT TCT CTT TCT CCT CTA GAA GAA ACA GCA G*-3';

CKIγ2-ΔC-F: 5'-*CCG GGA CAA ATA ATA GCC GCA CAG C*-3',

CKIγ2-ΔC-R: 5'-*GCT GTG CGG CTA TTA TTT GTC CCG G*-3'.

GST-CKIγ2-ΔN was generated by moving the DNA sequence of clone 137 isolated from a previously described yeast two-hybrid screen into pGEX-3X (Liberati et al., 1999; 2001). pSuper- and pSuperRetro-CKIγ2 were generated using the following targeting sequences (sense):

R1: 5'-*GCACCTGGAGTACCGGTTC*-3',

R2: 5'-*GCGCTACATGAGCATCAAC*-3'.

All constructs were confirmed by DNA sequencing.

In vitro binding assay

Purified proteins (GST proteins and His-CKIγ2) were incubated in 50μl of ULB^+ at room temperature for 30min. In the meantime, Protein G beads were coated with the anti-His antibody diluted in 5% BSA-ULB^+ (1:50) at room temperature for 30min. Beads were then washed 3 times in ULB and combined with the protein mixtures. After another 30min incubation at room temperature, beads were washed 3 times in ULB and boiled in sample loading buffer for western blot analysis.

Nuclear and cytoplasmic fractionation

Following the indicated treatment, HaCaT cells were washed twice with ice-cold PBS, gently scraped off the plate (6cm or 10cm) and collected by centrifugation at 4ºC. Cell pellets were resuspended in Buffer A (10mM HEPES pH 8.0, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.05% NP-40, plus phosphatase and protease inhibitors) and incubated on ice for 10 minutes. The nuclei were then pelleted by brief centrifugation and the supernatant (the cytoplasmic fraction) was transferred to a fresh tube. The nuclear pellet was washed 2X with Buffer A and then lysed in Nuclear Lysis Buffer (10mM Hepes pH 7.9, 250mM KC1, 0.1mM EDTA, 0.1mM EGTA, 0.1% NP-40, 0.1% glycerol, plus phosphatase and protease inhibitors) for 30 minutes on ice. Lysates were centrifuged for 20 minutes at 14000rpm at 4°C and supernatants (nuclear fraction) were transferred to a fresh tube.

Mass spectrometry protein sequencing analysis

5μg GST-Smad3 purified from E. coli was phosphorylated by 0.3μg of His-CKIγ2 (Invitrogen, PV3499) under the same condition as described in Materials and methods,

except that only non-radiolabelled ATP was used. Samples were snap-frozen in liquid nitrogen and stored at -80ºC before dialysis in XX buffer. DTT was added to the reaction mixture to a final concentration 4 mM followed by incubation at 50ºC for 10 min. The reaction mixture was further incubated in the presence of 14 mM iadoacetamide for 30 min in the dark prior to the addition of DTT and Tris-HCl and $CaCl_2$ (Final concentration: 7mM DTT, 100mM Tris-HCl, 1mM $CaCl_2$). After adding 120ng of trypsin (Wako), the reaction mixture was incubated at 37ºC for 16 hr. The digestion mixture was stopped with formic acid (1% final concentration) and analyzed by a 4000 Qtrap (Applied Biosystems, Foster City, CA) interfaced with an Eksigent NanoLC-2D instrument (Eksigent, Dublin, CA) for nanoflow chromatography. The mass spectrum was acquired in the negative- and positive-ion modes. MS/MS were searched with MASCOT (version 2.0, MatrixScience, London, UK).

DNAP assay

The protocol for DNAP assay was described previously (Lo & Massagué, 1999; Frederick et al., 2004). Specifically, primers (purchased from Invitrogen) containing the SBE (5'-AGTATGTCTAGACTGA-3') or the MBE (5'-AGTATCATAGCGTTGA-3') sequence were annealed with the respective complementary primers (5' biotin-labeled). Total cell lysates in DNAP buffer (1mg for HaCaT cells and 400μg for MEFs) were incubated with 2μg of annealed probes for 16 hours at 4°C. The Smad3-SBE complex was then isolated using Streptavidin beads (Pierce) and the precipitates were washed with DNAP buffer and resolved by SDS-PAGE. SBE-bound Smad3 was examined by immunoblotting.

Fluorescence microscopy

MEFs grown on poly-D-lysine-coated glass coverslips were transfected with the indicated GFP-CKIγ2 constructs. Twenty-four hours later, cell were fixed with 4% paraformaldehyde/4% sucrose in PBS/$Ca2^{+}$. After permeabilization with 0.2% Triton X-100 for 5 min, cells were stained with DAPI for 10 min at room temperature to visualize nuclei. Confocal images of fixed cells were obtained using a Nikon spinning disc confocal microscope with a 60x 1.4 N.A objective. Images were analyzed using Metamorph software (Universal Imaging Corporation). The outlines of nuclei were automatically traced by Metamorph based on DAPI staining.

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Supplementary figure legends

Supplementary Figure S1. CKIγ2 binds the MH2 domain of Smad3.

A. *In vitro* binding of Smad3 and CKIγ2. The indicated purified GST fusion proteins (2μg) were incubated with purified His-CKIγ2 (0.5μg, Invitrogen) and the mixture was subjected to immunoprecipitation using a polyclonal anti-His antibody (2365, Cell Signaling). Input and precipitated proteins were analyzed by western blot using monoclonal antibodies against GST (Santa Cruz Biotechnology) and His-tag (2366, Cell Signaling). **B.** Flag-tagged Smad3, Smad2/3, Smad3/2 (Guo et al., 2008) and Smad3ΔC (Feng et al., 1998, kindly provided by Dr. Rik Derynck) were transiently expressed in 293T cells and the cell lysates were incubated with His-CKIγ2 for the co-immunoprecipitation assay.

Supplementary Figure S2. Effect of CKIγ2 overexpression on nuclear and cytoplasmic Smad2 and Smad3.

HaCaT stable lines treated with or without TGF-β (100pM) were fractionated and endogenous total Smad2 and Smad3 were blotted for each fraction. Note that in CKIγ2-overexpressing cells, nuclear Smad3 decreased more rapidly than in control cells. In the meantime, the reappearance of dephosphorylated Smad3 in the cytoplasm was delayed, indicating a net loss of the repertoire of Smad3 in the CKIγ2-WT cells (top blots). Nuclear and cytoplasmic CKIγ2 from the overexpressing cells was also shown (middle blot). β-tubulin (left) and Lamin C (right) were used as loading controls for the cytoplasmic and nuclear fractions, respectively. The bottom panel shows Smad2/3 levels in cells co-treated with 30μM MG-132 during the entire time course.

Supplementary Figure S3. The Smad3 phosphopeptide identified by mass spectrometry.

GST-Smad3 phosphorylated by incubation with CKIγ2 was digested with trypsin. Tryptic peptides were analyzed by 4000 QTrap mass spectrometer. Phosphopeptides were identified by precursor ion scanning of -79 products in negative ion mode. The phosphopetide was then analyzed in positive ion mode using a method previously described for identifying phosphopeptides by scanning for peptides that underwent neutral loss of 98 Da (H_3PO_4) (Beausoleil et al., 2004). The dataset acquired was submitted to the Mascot search engine. Shown here is the Mascot search result of phosphopeptide VLTQMGSPpSIR. Note that there is a -18 Da change of Ser418 (from 87 to 69 Da, pink box) indicative of the loss of the phosphate group from pSer. The ion score for this peptide is 62, which is considered to be highly reliable assignment based on the Mascot search criteria (http://www.matrixscience.com/).

Supplementary Figure S4. CKIγ2 phosphorylation does not affect Smad3-DNA binding.

A. DNAP assay of endogenous Smad3. HaCaT cells were treated with MG-132 (20μM, 3 hours) either in the presence of SB431542 (10μM, 3 hours, "-") or TGF-β (100pM, 1 hour "+") before cell lysis in DNAP buffer containing a mixture of protease and phosphatase inhibitors. TGF-β-activated Smad3 did not bind the MBE control probe (data not shown). **B.** MEFs were transfected with the indicated Smad3 constructs and treated as in (A). **C.** MEFs expressing the indicated Smad3 constructs were co-treated

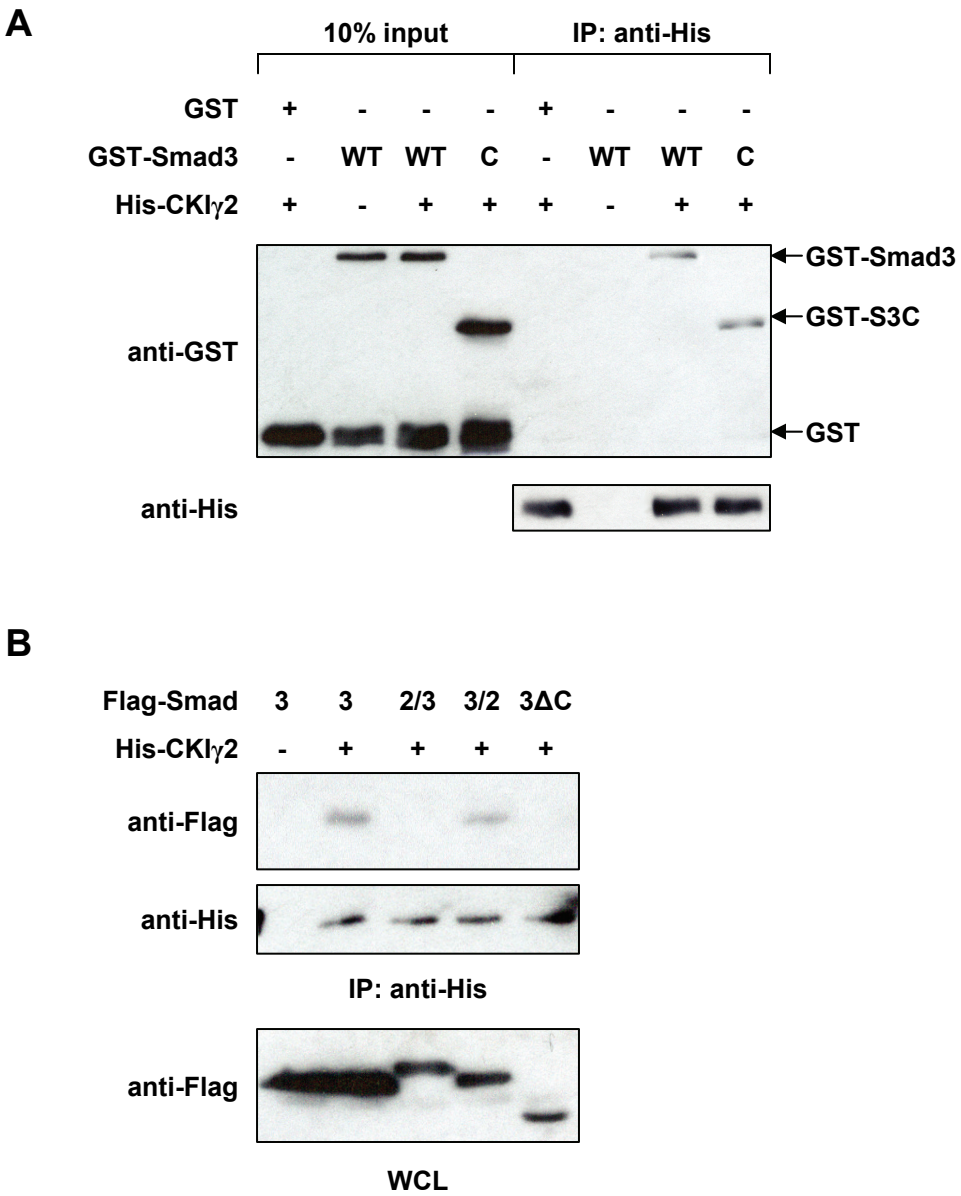
with MG-132 (20μM) and TGF-β (100pM) for 3 hours before being lysed in ULB^+ for co-precipitation assays.

Supplementary Figure S5. Characterization of CKIγ2 localization.

A. Alignment of human and *Xenopus* CKIγ2 C-termini and schematic representations of the CKIγ2 variants. **B.** Representative confocal images of MEFs expressing the indicated GFP-CKIγ2 constructs. White arrows indicate CKIγ2 on the plasma membrane (edge) of cells. Scale bar: 10μm.

Supplementary Figure S6. Functional analysis of CKIγ2 mutants in TGF-β responses.

A and **B.** MEFs were transfected with a control vector (-) or the indicated forms of CKIγ2. At 20 hours post-transfection, 50pM TGF-β was added as shown and total cell lysates were collected for immunoblotting. **C.** Flag-tagged CKIγ2 variants were expressed in 293T cells and immunoprecipitated with an anti-Flag antibody (M2) for *in vitro* kinase assay using GST-Smad3(WT) as substrate.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His-CKIγ2 + - + + + - + +
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GST-Smad3
GST-S3C
GST
anti-His
B
Flag-Smad 3 3 2/3 3/2 3ΔC
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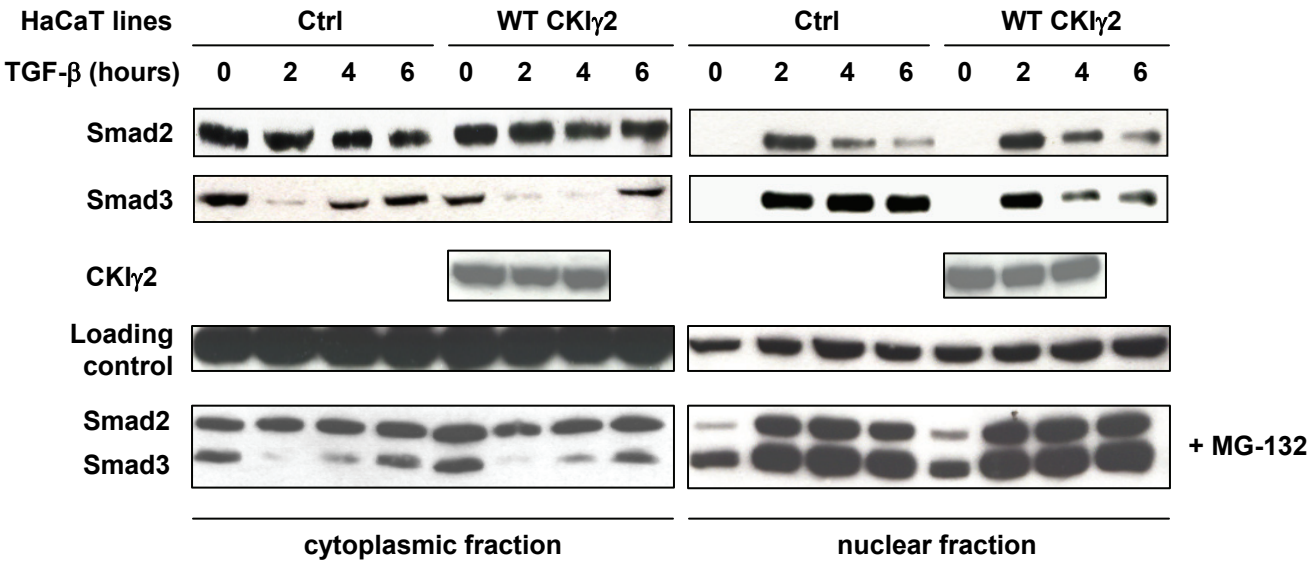
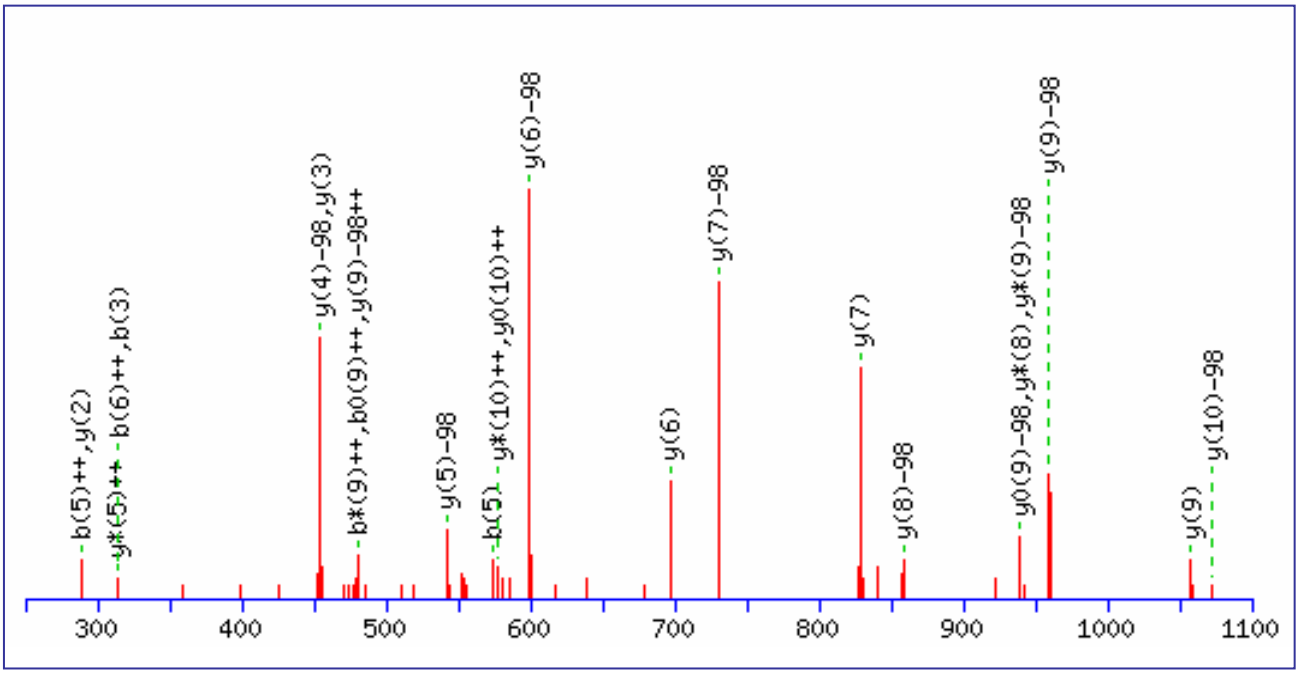
WCL

Guo et al., Supplementary Figure S2





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Matches (Bold Red): 25/168 fragment ions using 25 most intense peaks

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2	213.1598	107.0835					L	**1071.5615**	536.2844	1054.5350	527.7711	1053.5510	527.2791	10
3	**314.2074**	157.6074			296.1969	148.6021	T	**958.4775**	**479.7424**	**941.4509**	471.2291	**940.4669**	470.7371	9
4	442.2660	221.6366	425.2395	213.1234	424.2554	212.6314	Q	**857.4298**	429.2185	840.4032	420.7053	839.4192	420.2133	8
5	**573.3065**	**287.1569**	556.2799	278.6436	555.2959	278.1516	M	**729.3712**	365.1892	712.3447	356.6760	711.3606	356.1840	7
6	630.3280	**315.6676**	613.3014	307.1543	612.3174	306.6623	G	**598.3307**	299.6690	581.3042	291.1557	580.3202	290.6637	6
7	717.3600	359.1836	700.3334	350.6704	699.3494	350.1783	S	**541.3093**	271.1583	524.2827	262.6450	523.2987	262.1530	5
8	814.4128	407.7100	797.3862	399.1967	796.4022	398.7047	P	**454.2772**	227.6423	437.2507	219.1290	436.2667	218.6370	4
9	883.4342	442.2207	866.4077	433.7075	865.4236	433.2155	S	357.2245	179.1159	340.1979	170.6026	339.2139	170.1106	3
10	996.5183	498.7628	979.4917	490.2495	978.5077	489.7575	I	**288.2030**	144.6051	271.1765	136.0919			2
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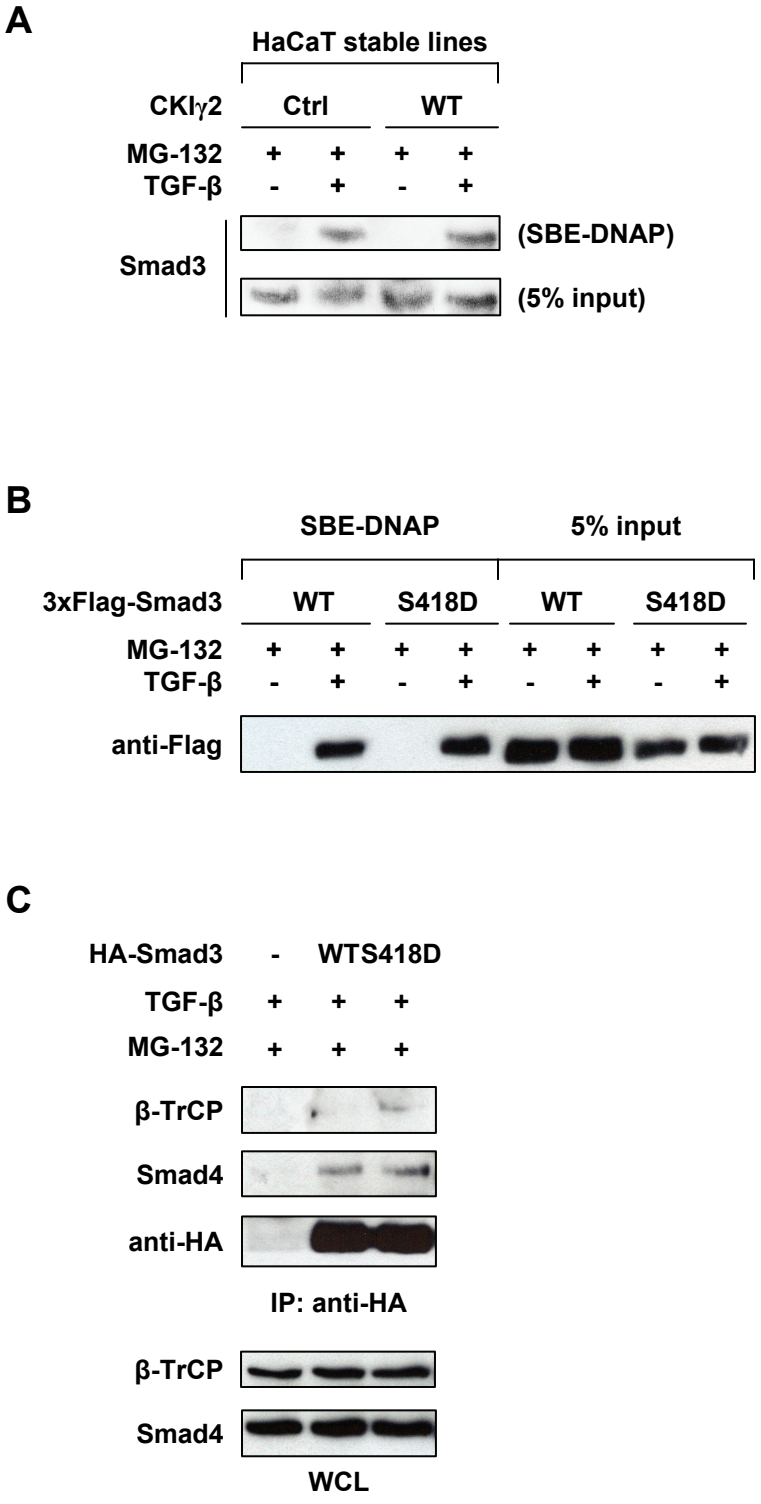
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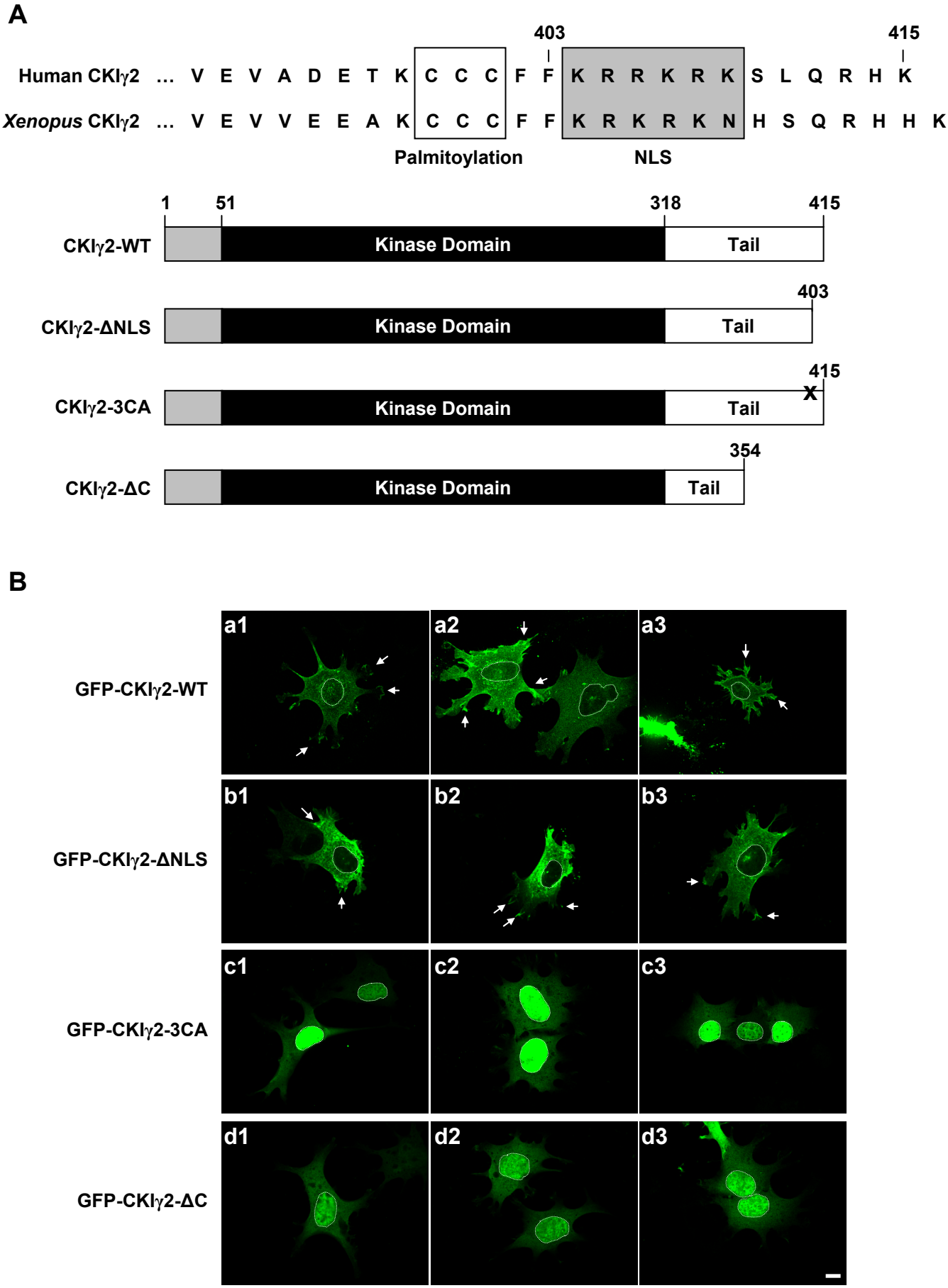
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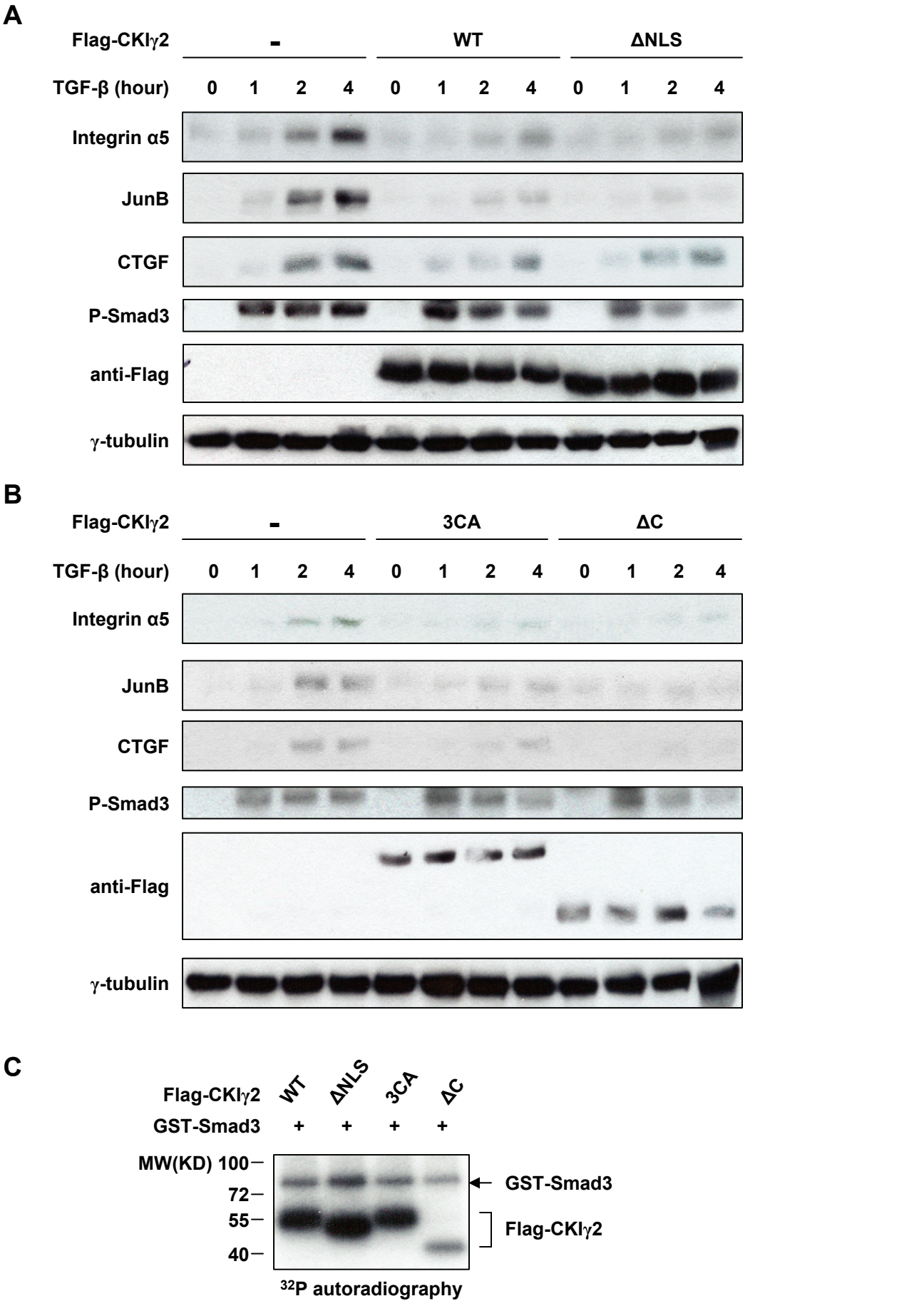
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