SMURF1/2 are novel regulators of WNK1 stability

Ankita B. Jaykumar[1], Sakina Plumber[1$], Derk Binns[1], Chonlarat Wichaidit[1#], Katherine Luby-Phelps[2], Melanie H. Cobb[1*]

From the Department of Pharmacology[1] and Department of Cell Biology[2], UT Southwestern Medical Center, Dallas, USA
*Corresponding author: Melanie.Cobb@UTSoutherstern.edu

6001 Forest Park Rd., Dallas, Texas 75390-9041; Tel. 214 645-6122; Fax 214 645-6124

$ Current Affiliation: Department of Biological Sciences, Columbia University, New York, NY

Current Affiliation: University of Arizona, Tucson, AZ

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Abstract

Angiogenesis is essential for remodeling and repairing existing vessels, and this process requires signaling pathways including those controlled by transforming growth factor beta (TGF-β). We have previously reported crosstalk between TGF-β and the protein kinase With No lysine (K) 1 (WNK1). Homozygous disruption of the gene encoding WNK1 results in lethality in mice near embryonic day E12 due to impaired angiogenesis and this defect can be rescued by endothelial-specific expression of an activated form of the WNK1 substrate kinase OSR1. However, molecular processes regulated via a collaboration between TGF-β and WNK1/OSR1 are not well understood. Here we show that WNK1 interacts with the E3 ubiquitin ligases SMURF1/2. In addition, we discovered complex inter-regulation between WNK1 and SMURF1/2 and we demonstrate that WNK1 activity regulates TGF-β receptor levels, in turn, controlling TGF-β signaling.

Introduction

Transforming growth factor beta (TGF-β) signaling is involved in regulating several key physiological processes such as angiogenesis, among others [1]. Homozygous disruption of the gene encoding the protein kinase With No lysine (K) 1 (WNK1) results in a lethal developmental failure in mice around embryonic day E12, due to impaired angiogenesis [2,3]. WNK1 is the most broadly expressed of a family of four related multi-functional and atypical protein-serine/threonine kinases, notable for their unique catalytic lysine location [4]. WNK1 phosphorylates substrates, the best characterized of which are oxidative stress- responsive 1 (OSR1) and STE20/SPS-1-related proline-alanine-rich kinase (SPAK, *STK39*), critical for maintaining cellular ion homeostasis [5,6,7,8,9]. We have previously reported collaboration among WNK1, and players involved in regulating TGF-β signaling pathways [10,11].

TGF-β-induced endothelial-mesenchymal transition initiates cytoskeletal turnover and a drastic down-regulation and disintegration of tight junctions to promote migration in endothelial cells [12,13]. Upon TGF-β stimulation, the TGF-β receptor type II redistributes into tight junctions which leads to recruitment of the E3 Ubiquitin ligase SMURF (SMAD Ubiquitination Regulatory Factor) 1 to the activated complex to mediate dissolution of tight junctions [12,13]. Occludin is a tight junction protein; while occludin is not necessary for the formation of tight junctions, occludin is vital in regulating tight junction integrity [14,15,16]. Furthermore, recent studies have shown that occludin is also involved in endothelial neovascularization and angiogenesis [17]. In addition, the related E3 ubiquitin ligase SMURF2 downregulates TGF-β signaling by targeting the TGF-β receptor ALK5 and itself for degradation [18,19].

In this study, we identify multiple molecular events that underlie WNK1-mediated context specificity of TGF-β signaling. We show that interactions between WNK1 and SMURF1/2 and show that WNK1 forms discrete signaling microdomains (sometimes referred to as WNK bodies [20]) for reciprocal regulation of WNK1 and E3 ubiquitin ligase SMURF1/2. In addition, we discover complex inter-regulation between WNK1 and SMURFs during TGF-β signaling.

Results

WNK1 colocalizes with SMURF1/2: SMURF1/2 are WW-domain-containing enzymes belonging to the NEDD4 (Neural precursor cell Expressed, Developmentally Down-regulated 4) family of E3 ubiquitin ligases. SMURFs mediate endothelial-mesenchymal transition during cardiovascular development, and they also regulate TGF-β signaling by targeting the TGF-β receptor and SMAD transcription factors for degradation [18,19,21,22,23,24,25,26,27]. Previous work from our laboratory showed that WNK1 is involved in TGF-β pathway-dependent modulation of SMAD2/3 protein stability [10,11]. In epithelial cells, SMURF2 regulates steady-state levels of SMAD2/3 [22,24,25,26] and SMURF1 controls cellular responsiveness to the TGF-β/SMAD2 pathway [27]. Therefore, we examined actions of WNK1 on TGF-β-pathway-dependent functions of SMURF1/2 in endothelial cells. First, we tested whether either SMURF1 or 2 co-localize with WNK1 in primary human umbilical vein endothelial cells (HUVECs). Interestingly, we found that a fraction of WNK1 and SMURF1/2 were observed in large punctate structures [26] (**Figure 1A, 1B**).

WNK1 kinase activity regulates association of WNK1/OSR1 with SMURF2: Given the colocalization between WNK1 and SMURF1/2, we asked whether WNK1 interacts stably with SMURF1/2 in HUVECs and found SMURF2 in endogenous immunoprecipitates of WNK1 (**Figure 2A**). We found that the WNK1-regulated kinase OSR1 also weakly co-immunoprecipitated with SMURF2 (**Figure 2B**). OSR1 contains a conserved C-terminal (CCT) domain, which can bind substrates and other proteins via short conserved RFxV motifs [28,29]. We then asked whether this interaction is mediated via the OSR1 CCT domain and these motifs in SMURFs. We found marginal decreases in OSR1 co-immunoprecipitating with SMURF2 in primary HUVECs upon co-incubation with a blocking peptide which blocks OSR1 CCT interactions with RFxV motifs (**Figure 2B**). We previously found that WNK1/OSR1 regulate the turnover of tight junctions [10]. Therefore, we asked whether SMURF2 interacts with the tight junction protein occludin and found that the interaction between occludin and SMURF2 was enhanced upon inhibition of human dermal microvascular endothelial cells (HDMECs) with the pan-WNK inhibitor WNK463 [10]. This interaction was also diminished upon co-incubation with the blocking peptide (**Figure 2C**). These observations suggest that OSR1 interacts with occludin and weakly with SMURF2 via the CCT domain and that WNK activity regulates the interaction between WNK1/OSR1 and SMURF2.

WNK1/OSR1 regulates SMURF1/2 and vice versa: Given the interaction between WNK1 and SMURFs, we then asked if WNK1 impacts amount of SMURFs. We found that depletion of WNK1

decreased steady state SMURF2 protein (Figure 3A, 3B, 3D, 3E). In addition, depletion of WNK1 prevented the expected decrease in steady-state SMURF1 protein. However, upon co-treatment with the proteasomal inhibitor MG132, we observed a decrease in SMURF1 protein (**Figure 3A, 3C**). Treatment of cells with the SMURF1 inhibitor A01 [30] lead to a significant increase in WNK1 expression (**Figure 3D, 3F**). The amount of SMURF2 protein expressed in the absence of added ligands or inhibitors was related to WNK1 expression (**Figure 3D, 3F, 3G**). In contrast, depletion of OSR1 caused no differences in amounts of either SMURF1 or SMURF2 (**Figure 3A, 3B, 3C, 3G, 3H**). Decreased SMURF2 was observed only in OSR1-depleted cells that were also treated with either the proteasomal inhibitor MG132 or the SMURF1 inhibitor A01 (**Figure 3A, 3B, 3C, 3G, 3H**). In contrast, SMURF1 increased in OSR1-depleted cells that were also treated with MG132 (**Figure 3A, 3C**). These results suggest a complex inter-regulation among WNK1, OSR1 and SMURF1/2.

WNK1 kinase activity mediates SMURF2-dependent regulation of ALK5: We asked whether the kinase activity of WNK1 is important for regulation of SMURF2 and found that treatment with WNK463 decreased SMURF2 protein, similar to the effect of WNK1 knockdown (**Figure 4A**). We also found that baseline SMURF2 was enhanced by treatment with the proteasomal inhibitor MG132; WNK463 co-treatment efficiently decreased SMURF2 protein (**Figure 4B, 4C**). These results suggest that the observed effects on SMURFs are dependent on WNK1 activity. Previously, we found that WNK463 decreased expression of the type 1 TGF-β receptor ALK1 [10]. Degradation of the TGF-β type I receptor, ALK5, is facilitated via recruitment of the SMURF2 complex [12,13,18,19]. Given the regulation of WNK1 by SMURF2 and effects of WNK inhibition of ALK1, we asked whether WNK1 kinase activity also regulates ALK5. We found that treatment with WNK463 did, in fact, decrease ALK5 protein as well (**Figure 4D, 4E**). We also found that knockdown of WNK1 decreased SMURF2 levels (**Figure 4F**). As expected, knockdown of SMURF2 increased ALK5 (**Figure 4G, 4H**). Interestingly, SMURF2 knockdown also resulted in a corresponding increase in the phosphorylated active form of OSR1, suggestive of enhanced WNK1 activity (**Figure 4G, 4I**). Given the regulation of ALK5 by WNK1, and that of WNK1 by SMURF2, these data suggest that the increase in ALK5 by SMURF2 knockdown may, in part, result from increased WNK1 activity. Overall, these results suggest that WNK1 kinase activity and SMURF1/2 reciprocally regulate each other to affect responses to TGF-β (**Figure 4J**).

Discussion

Unanticipated findings revealed that WNK1 and SMURFs reciprocally regulate each other to fine tune TGF-β signaling. Aggregated structures containing SMURF1/2 and WNK1 in our study are similar to those observed with respect to SMURF2 and clustering of SMURF2 is suggested to regulate its E3 ubiquitin ligase activity. [18,26]. We show that depletion or inhibition of WNK1 decreases SMURF2. The amount of SMURF2 protein present upon inhibition of SMURF1 was dependent on WNK1 protein amount. One possible explanation is that SMURF1 regulates SMURF2 protein, and this is dependent on the relative expression of WNK1. SMURF2 inhibits its own ubiquitinase activity and is thereby stabilized. Therefore, it is possible that inhibition of WNK1 may enhance the activity of SMURF1/2 and thereby lead to downregulation of itself, and ALK5 as observed in this study. Future studies will focus on addressing the mechanistic details of this potential mode of regulation.

The WW-domains of SMURF/NEDD4 E3 ligases generally recognize and bind proline-rich sequences such as PY-motifs on substrate proteins [26]. Interestingly, PY-motifs are also found in WNK1 and WNK1 binds to and phosphorylates NEDD4-2, another member of the NEDD4 family of E3 ubiquitin ligase [20,31,32]. NEDD4-2 negatively regulates TGF-β signaling by ubiquitin-mediated degradation of SMAD2 and TGF-β type I receptor [33]. Therefore, regulation of NEDD4-2 by WNK1 may also be involved to precisely control TGF-β signaling output to modulate cellular processes underlying angiogenesis.

Signal transduction pathways regulated by TGF-β control a diverse array of cellular processes including angiogenesis [34]. Signaling specificity and versatility are present in the TGFβ signaling pathway and importantly, it exhibits differential, sometimes opposing responses depending on the cellular context [35,36,37,]. This occurs through diverse regulatory mechanisms and cross-connections among several TGF-β mediators [34,35,36,37]. The duration and intensity of TGF-β signaling are tightly regulated by various processes, including proteasome-mediated degradation involving E3 ubiquitin ligases such as SMURF1/2, phosphorylation by several protein kinase signaling pathways, among others [34,35,36,37]. These observations suggest that dynamic convergence of inputs via individual TGF-β signaling nodes may fine-tune TGF-β signaling pathways to trigger diverse cellular outcomes. Consistent with this notion, our results suggest that WNK1 regulates multiple nodes of the TGF-β signaling pathway and affects TGF-β signaling output in a context-dependent manner.

In this study, we show that WNK1 collaborates with downstream TGF-β signaling component SMURF1/2 to regulate and fine-tune processes involved in TGF-β signaling such as turnover of TGF-β receptor in a context-dependent manner. Therefore, we propose that expression and activity of WNK1 may contribute, in part, to the context-specificity in TGF-β signaling.

Methods

Cell lines: Primary Human Dermal Microvascular Endothelial Cells (HMEC-1: ATCC, CRL-3243) were grown in complete MCDB media (Fisher Scientific, MT15100CV) supplemented with 10% fetal bovine serum (Sigma-Aldrich, F0926), 1% L-glutamine, 1% penicillin and streptomycin, 1µg/mL hydrocortisone (Sigma Aldrich, H0888 or H6909), and 10 ng/mL epidermal growth factor (EGF: Cell Signaling Technology, 8916SC). Human Umbilical Vein Endothelial Cells (HUVEC: ATCC, PCS-100-013) were grown in complete VascuLife® EnGS media kit (Fisher Scientific, 50-311-891) supplemented as per manufacturer's instructions. All cells were maintained at 37°C and 5% CO_2.

Co-immunoprecipitation: Cells were lysed in 1X lysis buffer (50mM HEPES, 0.1M NaCl, 0.5mM EDTA, 0.1% SDS) supplemented with protease inhibitor cocktail, PMSF, and phosphatase inhibitors (PhosStop). Cell extracts were harvested and cleared by centrifugation. 1X IP buffer (50mM HEPES, 0.1M NaCl, 0.5mM EDTA, and 1% CHAPS (Sigma Aldrich, C3023) supplemented with protease inhibitor cocktail, PMSF, and phosphatase inhibitors (Sigma Aldrich, 4906837001) was added in a 2:1 ratio to the cell lysate. Samples were incubated with primary antibody (control sample incubated with rabbit IgG primary antibody) overnight at 4°C and then with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, sc-2003) beads for 1 hour with head-to-tail rotation. This was performed either in the absence or presence of CCT blocking peptide peptide SAGRRFIVSPVPE (United Biosystems). Samples were then washed three times with 1X IP buffer before adding 5X SDS buffer (0.25% bromophenol blue, 0.5M DTT, 50% glycerol, 10% SDS, 0.25M Tris-Cl) and heating at 90°C for 2 minutes. Samples were then run on 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, 4568096) or 12% polyacrylamide home-made gels before being transferred to PVDF membranes. Membranes were then washed in TBS-T before being blocked with TBS-based blocking buffer (LI-COR). Membranes were incubated with primary antibodies and then washed again before being incubated with species-specific, light chain-specific secondary antibodies (Jackson ImmunoResearch Labs, 115-655-174 and 211-622-171) and imaged using LI-COR imaging.

Immunofluorescence: HUVEC cells were fixed on glass coverslips (Fisher Scientific, 12-545-80) with 4% paraformaldehyde for 20 minutes at room temperature. Coverslips were washed with sterile PBS and blocked in 5% normal goat serum (Life Technologies, 50-062Z) before incubating with primary antibodies for 1 hour at room temperature. Coverslips were washed with 1X PBS. Subsequently, cells were incubated with an Alexa Fluor® 488 conjugated goat-anti-mouse secondary antibody (Thermo Fisher Scientific, A11029) and Alexa Fluor® 594 conjugated goat-anti-rabbit secondary antibody (Invitrogen, A11037) for 30 min at room temperature in dark, and the slides were mounted with DAPI Fluoromount-G (Thermo Fisher Scientific, 00-4959-52). Immunofluorescence images were acquired using a Zeiss LSM880 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were deconvolved using AutoQuant® software (Media Cybernetics, USA).

siRNA knockdown: Oligonucleotides encoding siRNA for human WNK1 (siWNK1: 5' CAGACAGUGCAGUAUUCACTT 3'), control siRNA (Thermo Fisher Scientific, 4390844) as in [99], OSR1 siRNA (Thermo Fisher Scientific, s19303 Silencer® Select), and SMURF2 siRNA (sc-41675, Santa Cruz Biotechnology). HDMEC cells were transfected with 20 nM siRNA using Lipofectamine RNAiMax reagent (Thermo Fisher Scientific, 13778150). After 24-72 hours of transfection, cells were provided with their respective treatments and were then harvested in 1X SDS buffer (0.05% bromophenol blue, 0.1M DTT, 10% glycerol, 2% SDS, and 0.05M Tris-Cl) with 5% β-mercaptoethanol.

Immunoblotting: Cell lysates containing 1X SDS buffer were homogenized with 27-G syringe and whole lysates were run on 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, 4568096) or 6/10/12% home-made polyacrylamide gels before being transferred to PVDF membranes (Bio-Rad, 1620177). Membranes were then washed in TBS-T before being blocked with TBS-based blocking buffer (LI-COR). Membranes were incubated with primary antibodies and then washed again before being incubated with species-specific secondary antibodies and imaged using LI-COR imaging.

Reagents: WNK463 (Selleck Chemicals, S8358), SMURF1 inhibitor A01 (Sigma Aldrich, SML1404), MG132 (Sigma Aldrich, M7449), TGF-β1 (Cell Signaling Technology, 8915LC), anti-Vinculin antibody (Sigma Aldrich, V9131), anti-pOSR1 antibody (EMD Millipore, 07-2273), anti-OSR1 polyclonal antibody (Cell Signaling, 3729S), anti-OSR1 monoclonal antibody (VWR, 10624-616), anti-WNK1 antibody (Cell Signaling, 4979S), anti- GAPDH antibody (Cell signaling Technology, 97166L), anti-SMURF1 antibody (Santa Cruz Biotechnology, sc-100616), anti-

SMURF2 antibody (Santa Cruz Biotechnology, sc-393848), anti-flag antibody (Sigma-Aldrich, F1804), Q256 WNK1 antibody was homemade as in [7], Optimem (Invitrogen, 51985-034), Lipofectamine 2000 (Life Technologies, 11668019), bumetanide (Sigma Aldrich, B3023), 96-well plates (Corning, 3904 or Greiner, 655090).

Statistics and Reproducibility**:** The data are presented mean±SEM from at least three independent experiments with similar results. All presented micrographs (immunofluorescence images) are representative images from three representative experiments as indicated in the figure legends. For the quantification of immunofluorescence images, the number of cells used for each representative experiment is indicated and p values between two groups were determined using unpaired t-tests. Results are expressed as mean ± SEM. Single intergroup comparisons between 2 groups were performed with 2-tailed Student's *t*-test as specifically mentioned in each case. $p < 0.05$ was considered statistically significant.

Data sharing:

We will follow all NIH policies with respect to sharing reagents, materials, and information with other investigators. Detailed protocols are provided to everyone who requests them. Upon publication, this manuscript will be submitted to the National Library of Medicine's PubMed Central as outlined by NIH policy.

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Author contribution: ABJ: Conceptualized, supervised, designed and performed experiments, performed analysis, wrote manuscript, generated initial figures; **DB**: performed experiments; **CW**: Analyzed data; **KLP**: Discussed data; **MHC**: Supervision, acquired funding, edited manuscript.

Declaration of interests: The authors declare no competing interests.

Figure Legends

1. WNK1 colocalizes with SMURF1/2: A and B) Representative confocal images of immuno-fluorescently labeled endogenous WNK1 (red), SMURF1/2 (green) and nucleus (DAPI: blue) in primary HUVECs upon 1-2 h TGF-β (10 ng/ml) stimulation. Merged panel (yellow) shows co-localization between WNK1 and SMURF1/2. Scale bar = 20 μm; n=3.

2. WNK1 kinase activity regulates association of WNK1/OSR1 with SMURF2: A) Representative Western blot show endogenous co-immunoprecipitation of SMURF2 with WNK1 show interaction between SMURF2 and WNK1 in HUVECs; n=3. **B**) Representative Western blot show endogenous co-immunoprecipitation of OSR1 and SMURF2 in HUVECs which is diminished upon co-incubation with the blocking peptide SAGRRFIVSPVPE (100 μM); n=3. **C**) Representative Western blot show endogenous co-immunoprecipitation of OSR1 and SMURF2 with occludin in HUVECs upon treatment with WNK463 (1 μM) which is diminished upon co-incubation with the blocking peptide SAGRRFIVSPVPE (100 μM); n=3.

3. WNK1/OSR1 regulates SMURF1/2 and vice versa: A) Representative Western blot show SMURF1/2 expression upon WNK1 or OSR1 depletion by siWNK1 or siOSR1, respectively in HDMECs. It shows increase in SMURF1 levels upon treatment with proteasomal inhibitor MG132 (10μM) for 6 hours which is prevented upon WNK1 or OSR1 depletion. **B**) Corresponding quantification of 'A' show decreased SMURF2 levels upon WNK1 and OSR1 depletion compared to siControl; n=3. **C**) Corresponding quantification of 'A' show decreased SMURF1 levels upon WNK1 and OSR1 depletion compared to siControl; n=3. **D**) Representative Western blot show SMURF1/2 expression upon WNK1 depletion in HDMECs co-treated with SMURF1 inhibitor A01 (2 μM). **E**) Corresponding quantification of 'D' show decreased SMURF2 levels upon WNK1 depletion similar to treatment with the SMURF1 inhibitor A01 (2μM) alone compared to DMSO or siControl; n=3. **F**) Corresponding quantification of 'D' show increased WNK1 upon treatment with SMURF1 inhibitor A01 (2μM) alone compared to DMSO or siControl; n=3. **G**) Representative Western blot show SMURF1/2 levels upon siRNA depletion of OSR1 in HDMECs. **H**) Corresponding quantification of 'G' show decreased SMURF2 levels with siOSR1 treatment followed by SMURF1 inhibitor A01 (2μM) treatment overnight compared to siControl or DMSO

control; n=3. Data are represented as Mean±SE; analyzed by unpaired two-tailed Student's t-test or one-way ANOVA. *p<0.05, **p<0.005.

4. WNK1 kinase activity mediates SMURF2-dependent regulation of ALK5: A) Representative Western Blot showing SMURF2 protein levels upon WNK463 overnight treatment (1 μM). **B**) Representative Western Blot showing SMURF2 protein levels upon overnight WNK463 (1 μM) ± MG132 (10 μM). **C**) Corresponding quantification of 'B' showing decreased SMURF2 levels upon co-treatment with WNK463 and MG132; n=3. **D**) Representative Western Blot showing ALK5 levels upon DMSO or WNK463 (1 μM) in HDMECs. **E**) Corresponding quantification of 'D' showing decreased ALK5 levels upon WNK463 (1 μM) treatment; n=3. **F**) Quantification of ALK5 in HDMECs treated with siControl or siWNK1; n=6. **G**) Representative Western Blot showing ALK5 and pOSR1 levels upon siControl or siSMURF2 co-treated with DMSO or WNK463 (1 μM) in HDMECs. **H**) Corresponding quantification of 'G' showing increased ALK5 levels upon siSMURF2 treatment; n=21. **I**) Corresponding quantification of 'G' showing increased pOSR1 levels upon siSMURF2 treatment; n=21. **J**) Model representing inter-regulation among WNK1, SMURF1/2 and ALK5. Data are represented as Mean±SE; analyzed by unpaired two-tailed Student's t-test or one-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005.

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Figure 1: WNK1 colocalizes with SMURF1 and SMURF2

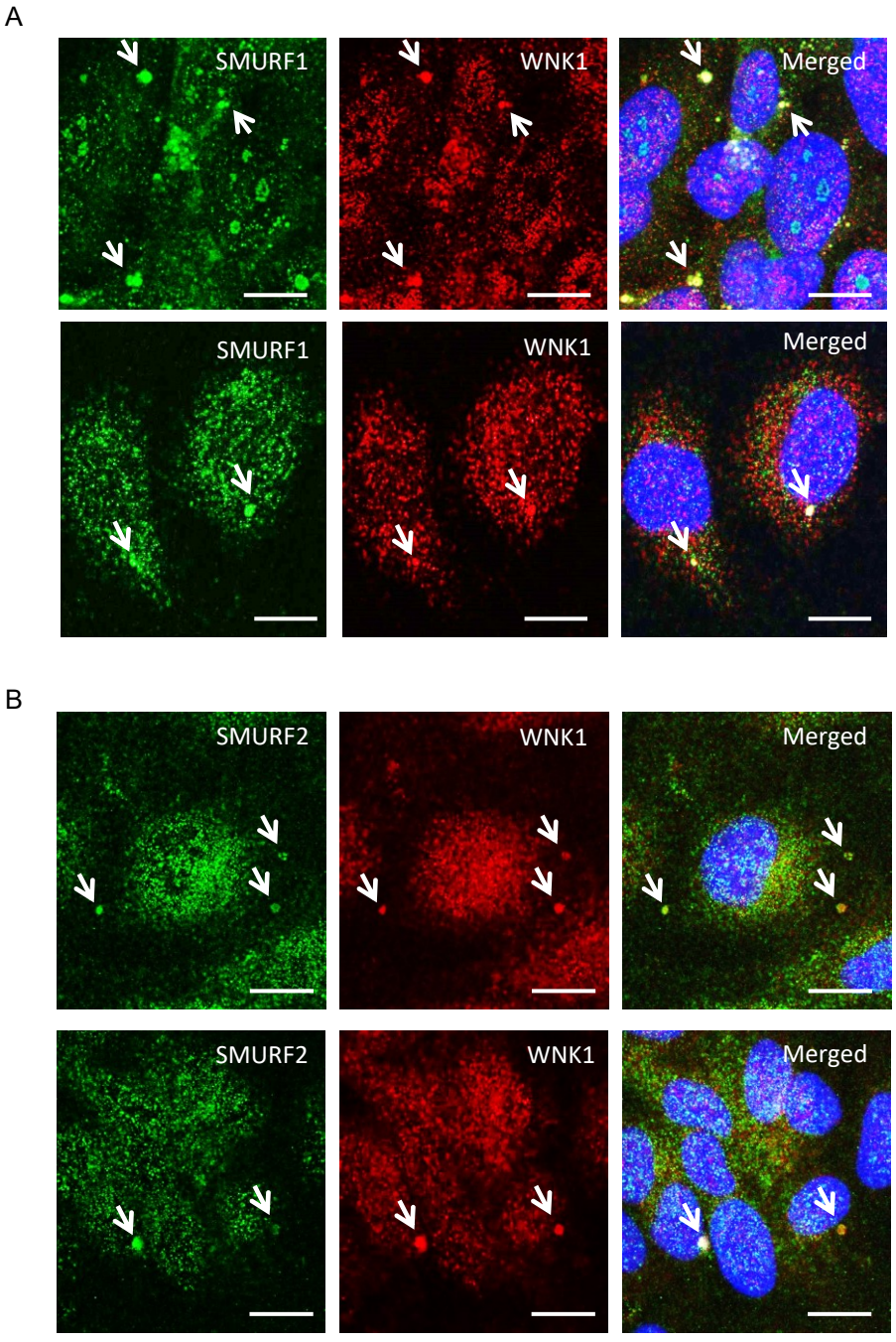
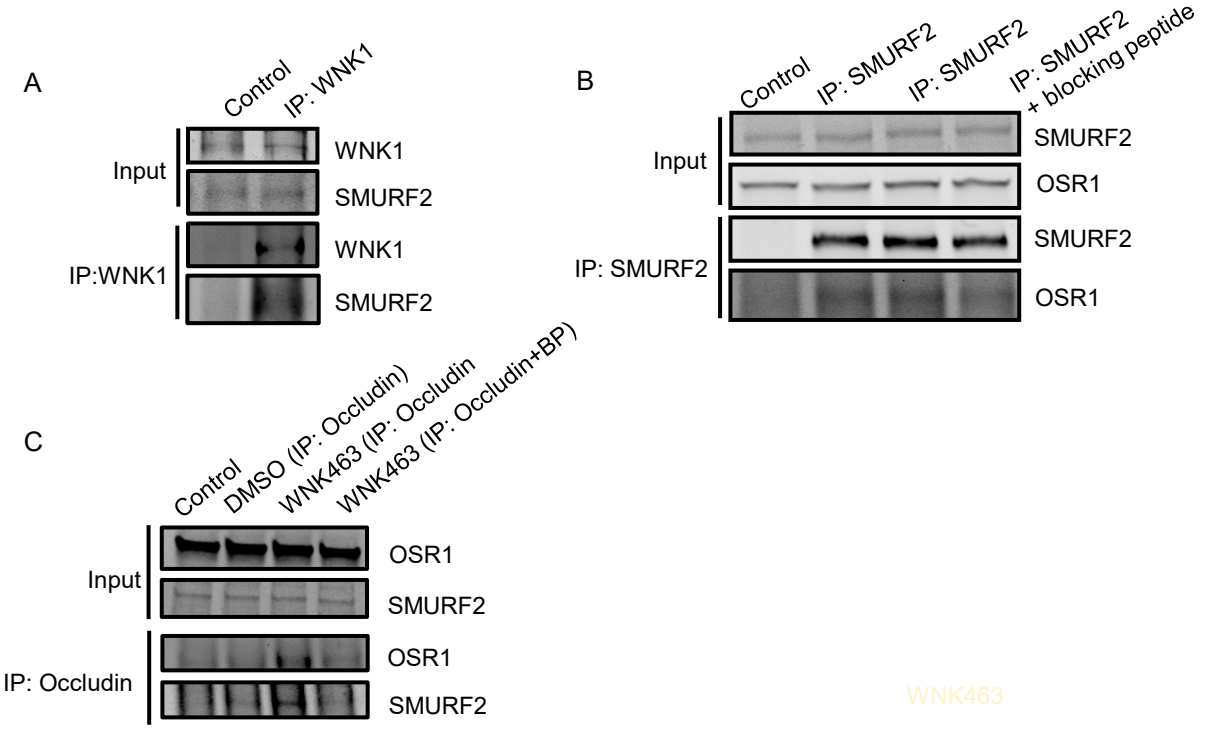


Figure 2: WNK1 kinase activity regulates association of WNK1/OSR1 with SMURF2



WNK463

DMSO

Figure 3: WNK1/OSR1 regulates SMURF1/2 and vice versa

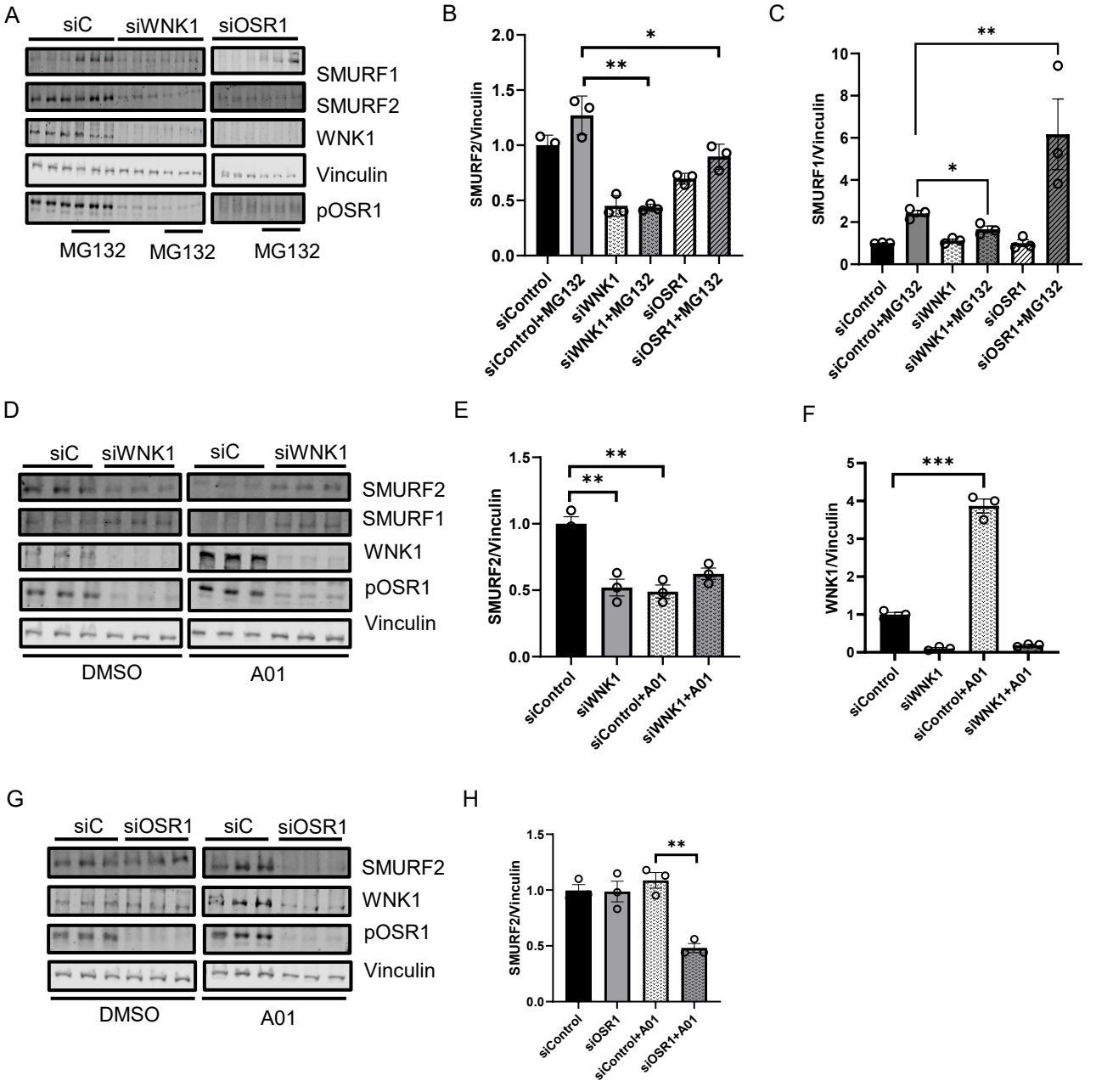
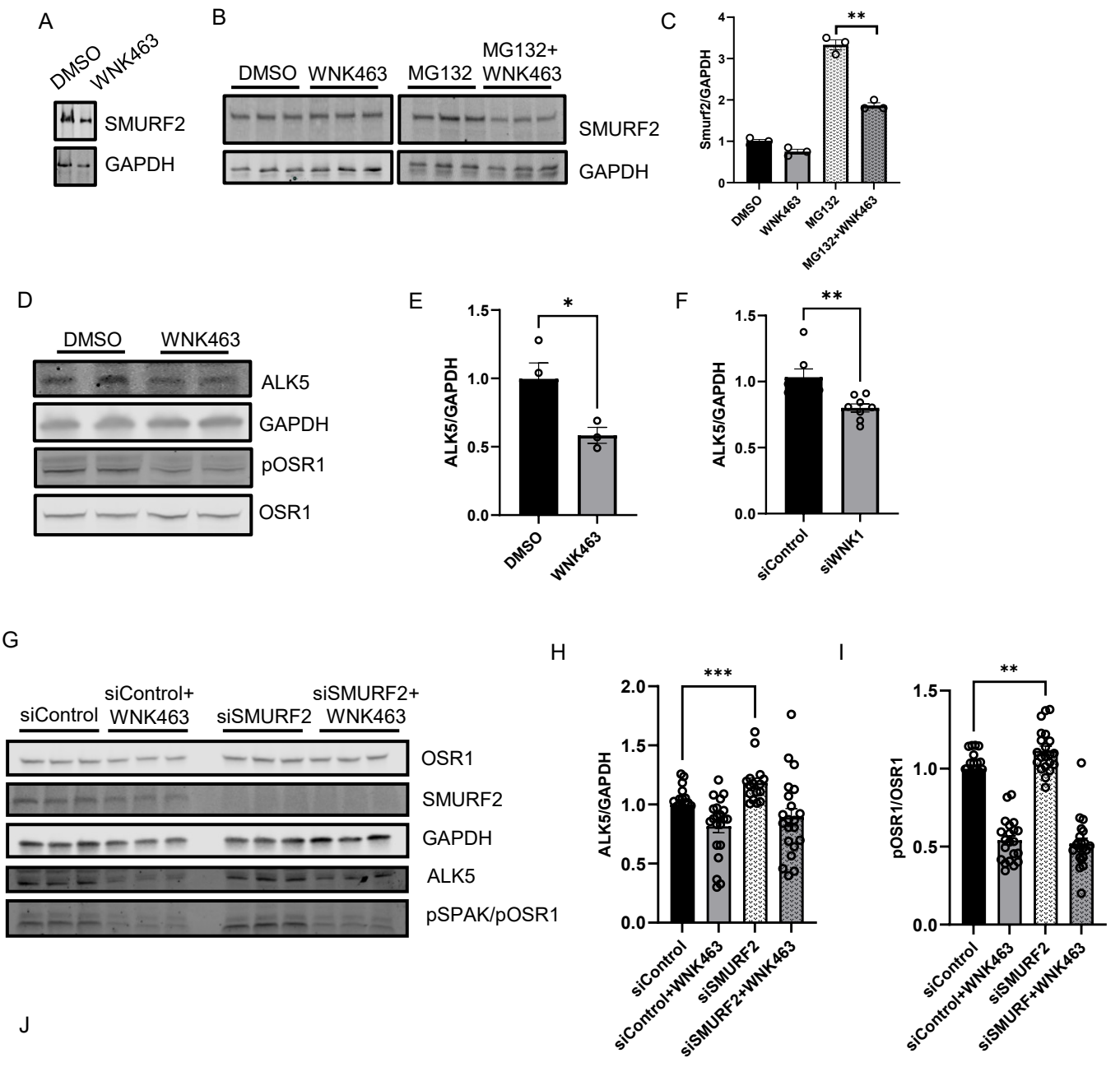



Figure 4: WNK1 kinase activity mediates SMURF2-dependent regulation of ALK5