Differential substrate specificity of ERK, JNK, and p38 MAP kinases toward Connexin 43

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ABSTRACT

Phosphorylation of connexin 43 (Cx43) is an important regulatory mechanism of gap junction (GJ) function. Cx43 is modified by several kinases on over 15 sites within its ~140 amino acid-long C-terminus (CT). Phosphorylation of Cx43CT on S255, S262, S279, and S282 by ERK has been widely documented in several cell lines, by many investigators. Phosphorylation of these sites by JNK and p38, on the other hand, is not well-established. Indeed, ERK is a kinase activated by growth factors and is upregulated in diseases, such as cancer. JNK and p38, however, have a largely tumor-suppressive function due to their stress-activated and apoptotic role. We investigated substrate specificity of all three MAPKs toward Cx43CT, both *in vitro* and in two cell lines (MDCK - non-cancerous, epithelial cells and porcine PAECs – pulmonary artery endothelial cells). Cx43 phosphorylation was monitored through gel-shift assays on an SDS-PAGE, immunodetection with phospho-Cx43 antibodies, and LC-MS/MS phosphoproteomic analyses. Our results demonstrate that p38 and JNK specificity differ from each other and from ERK. JNK has a strong preference for S255 and S279, while p38 readily phosphorylates S279 and S282. In addition, while we confirmed that ERK can phosphorylate all four serines (255, 262, 279, and 282), we identified T290 as a novel ERK phosphorylation site. This work underscores the importance of delineating the effects of ERK, JNK, and p38 signaling pathways on Cx43 and GJ function.

INTRODUCTION

Gap Junctions (GJs) are cell structures that uniquely allow for direct cell-cell communication within tissues, and it has been well established that GJ intercellular communication (GJIC) is crucial for a multitude of cellular outcomes; GJs regulate the coordination of development, cellular homeostasis, and tissue function.[1–4] In vertebrates, GJs are made up of integral membrane proteins termed connexins (Cx). Each Cx molecule spans the membrane bilayer four times, with both the N and the C-terminus in the cytoplasm. Six connexin monomers oligomerize into a half GJ termed connexon while trafficking through the secretory pathway. When connexons arrive at the plasma membrane, they interact with the connexons found in the neighboring cell's plasma membrane, forming a complete gap junction.[5,6]

It has been well-established that GJ oligomerization, forward trafficking, GJ channel formation and opening, as well as GJ closing, internalization and degradation, are precisely regulated through posttranslational modifications and interactions with many binding partners (protein chaperones, scaffolds, ubiquitination and endocytosis machinery).[1,7,8] The protein life cycle of Cx43 (the most ubiquitously expressed connexin), is regulated through phosphorylation/dephosphorylation events on over 15 sites by at least 9 kinases within the flexible and largely unstructured C-terminal tail. This includes mitogen-activated protein kinases (MAPKs), Src, protein kinases A and C (PKA and PKC), Akt, casein kinase 1 (CK1), and cyclin-dependent kinase 1 (CDK1/cdc2).[8]

MAPK family consists of three distinct signaling pathways: extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK).[9] Four MAPK phosphorylation sites were previously identified within the C-terminal region of Cx43: S255, S262, S279 and S282 (**Fig. 1A**). There is a large body of evidence of ERK phosphorylation at these four sites in multiple cell lines.[8,10–19] While some studies of Cx43 phosphorylation by JNK and p38 have been conducted, none focused on the identification of specific serine residues.[20–23] Even though these parallel MAPK pathways are highly conserved, they respond to distinct stimuli, leading to diverse, and often cell line-specific outcomes. ERK signaling is activated mostly through growth factor receptors, resulting in changes in cell proliferation and migration,[24] while JNK and p38 pathways respond to stress, such as osmotic shock, hypoxia, and reactive oxygen species.[9,25]

MAPKs are proline-driven kinases and recognize the following phosphorylation consensus motif in their substrates: P-X-**S/T**-P, with the phospho-acceptor serine or threonine at the [0] position (**Fig. 1B**).[1,26–28] Two sites (S279 and S282) in both the human and rodent Cx43 sequence fully conform to this phosphorylation motif, while S255 lacks the proline in the [-2] position but does have the proline in the [+1] position. In addition, S255 conforms to a cyclin dependent kinase motif [29] (S-P-X-R/K, **Fig. 1B**) and has been observed to be phosphorylated by a cyclin-dependent kinase cdc2 at the onset of mitosis.[30,31] More recently, S255 phosphorylation by MST1 (mammalian STE20-like kinase) was detected in endothelial cells during atherosclerosis[32] - a surprising finding given MST's strong preference against S/T residues that are followed by a proline in the +1 position.[33] Unlike S255, S279 and S282 sites, serine 262 in the human sequence of Cx43 is not a conserved site due to lack of proline at both [-2] and [+1] positions in the human sequence (**Fig. 1B** and **1C**). However, it is not surprising that S262 phosphorylation has been observed by many researchers because either a rodent cell line was used or a rodent (typically, rat) sequence of Cx43 was expressed in mammalian cells or studied *in vitro*.[12,34] Nonetheless, there is plenty of evidence that MAPKs can phosphorylate substrates that do not fully conform to the P-X-S/T-P motif, also relying on key docking site sequences that may be available both on the substrate and on the MAPK itself.[26–28,35,36] It is therefore important to systematically assess each MAPK's ability to phosphorylate these serine sites on Cx43 rather than relying on the presence of canonical phosphorylation motifs.

Phosphospecific antibodies against serine residues on Cx43 have been successfully developed, commercialized, and used by many researchers.[10–12,37] In addition, it has been well-established that Cx43 undergoes changes in electrophoretic mobility when phosphorylated at specific serine residues (**Fig. 1D**). At least three electrophoretic forms have been observed: P0, P1, and P2, where unphosphorylated Cx43 migrates in the P0 isoform, with phosphorylation by some kinases shifting to P1, P2, (even to P3 in some cell lines) or remaining at P0.[1,7,23,38] These shifts have also been recapitulated by replacing serine sites with phosphomimetic mutations. For example, a triple phosphomimetic mutant (S255D/S279D/S282D) of recombinantly expressed and affinity purified Cx43 C-terminus demonstrates a shift to the P1 form, while single and double S255D/S262D remain in P0.[39] Thus, shifts to slower-migrating forms (when combined with the use of phospho-specific antibodies and serine site mutants) represent a valuable readout of Cx43 phosphorylation (**Fig. 1D**). Despite the utility of phosphospecific antibodies, users are limited to

observing only the phosphorylation sites recognized by a given antibody. Moreover, phosphoantibody performance can vary, with occasional non-specific binding to other proteins. Given these shortcomings, we sought to complement gel shifts and phosphoantibody usage with mass spectrometry-based phosphoproteomics - an unbiased discovery method.

We systematically evaluated the differences in substrate specificity of each MAPK toward Cx43. First, we treated purified GST-Cx43CT mutants with purified and active MAPKs through *in vitro* kinase assays. Moreover, in a hybrid approach, we generated active kinases in mammalian cells and treated purified GST-Cx43CT proteins with these lysates. Finally, we assessed full-length Cx43 phosphorylation directly in mammalian cells. We measured Cx43 phosphorylation through three complementary techniques: SDS-PAGE gel mobility shifts, immunoblotting with phosphoCx43-specific antibodies, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) phosphoproteomics. Importantly, we utilized phosphomimetic and phospho-dead mutants at MAPK sites as positive and negative controls, respectively, taking advantage of Cx43's ability to undergo electrophoretic mobility shifts, even when using phosphomimetic mutations (S to E). Our findings across all approaches map out phosphorylation preferences of each MAPK toward S255, S262, S279 and S282 on Cx43, indicating that each MAPK has a differential specificity reproducible in two mammalian cell lines. In addition, we identified T290 as a novel ERK phosphorylation site on Cx43.

RESULTS

In vitro kinase assays between active MAPKs and GST-Cx43CT proteins indicate differences in phosphorylation preference.

Recombinant ERK2, p38α, and JNK2α2 were purified from *E.coli* in their active form by coexpression with their upstream activators. Histidine-tagged ERK was coexpressed with a constitutively active MKK1. Robust ERK activation was seen in samples that contained both ERK and MKK1, while active ERK antibodies detected significantly lower levels of active ERK when MKK1 was absent. Total ERK expression levels were also detected with histidine tag antibodies and were highest in IPTG-treated samples (**Fig. S1A**). Milligram amounts of ERK were purified from the soluble fraction through nickel affinity chromatography and ERK retained its activity throughout the purification process (**Fig. S1B**). Active, GST-tagged p38α was produced through coexpression with its upstream activator, MKK6. The highest levels of active GST-tagged p38

were seen in the soluble protein fraction **(Fig. S1C**). GST-tagged, active p38α was purified by glutathione-affinity chromatography (**Fig. S1D**). Active, histidine tagged JNK2α2 was produced by expression with its two upstream activators: a constitutively active form of MEKK (MEKKC) and MKK4. Robust expression of active JNK was seen in the soluble *E.coli* fraction induced with IPTG (**Fig. S1E**). Histidine-tagged, active JNK was of high purity and retained its activity throughout the purification process (**Fig. S1F**).

To conduct *in vitro* kinase assays between each MAPK and Cx43, we expressed GST-tagged Cx43 C-terminus in *E.coli* (WT, phospho-dead and phospho-mimetic single, double and triple mutants: S255A, S255E, S279A/S282A, S279E/S282E, S255A/S279A/S282A, S255E/S279E/S282E) and purified them using glutathione affinity chromatography (**Fig. S1G**) to be used as substrates in phosphorylation reactions with active ERK, JNK, and p38 (**Fig. 2A**).

GST-tagged WT Cx43CT was tested with active ERK to identify kinase assay conditions. We initially incubated 6μM WT GST-Cx43CT in a time-dependent (0-2hrs) and ERK concentration-dependent (0-1μM) manner, establishing that 1 hour of Cx43 incubation with 0.5 μM of active ERK (**Fig. 2B**, **S2A**), active p38, and JNK (**Fig. S2B** and **S2C**) led to a complete electrophoretic shift of WT Cx43CT.

Next, using the conditions established above, we assessed differences in electrophoretic mobility of WT GST-Cx43CT treated with pure and active ERK, p38, and JNK. Quantification of gel shifts by ImageJ (n=7) indicated that while ERK and JNK-treated GST-Cx43CT proteins undergo a shift to at least a P1 form, p38-treated Cx43 does not display a significant shift as compared to the negative control (**Fig. 2C-E**) or as compared to GST-Cx43CT WT treated with active ERK (**Fig. S2C**). We then utilized the same samples (**Fig. 2C**) in western blotting analyses with phospho-specific antibodies against S255, S262, S279, and S282 (**Fig. 3A**). Antibodies against pS255 are no longer commercially available and were shared by Dr. Paul Lampe's laboratory (Fred Hutch Cancer Center, Seattle, WA). Antibodies against pS279 and pS282 were highly specific, while pS255 antibodies displayed some level of non-specific detection in the control (no MAPK) sample. Unfortunately, pS262 antibodies were the most non-specific, with all four samples (including the negative control) displaying the same level of signal across multiple repeats. We attempted further dilutions of pS262 antibodies to 1:2000 and 1:5000 but were unable to achieve better specificity (data not shown). It is important to note that in these experiments, the highest antibody specificity was achieved when using a less sensitive chemiluminescence reagent

(see Methods). Clear differences in phosphorylation of GST-Cx43CT WT by each MAPK were observed (**Fig. 3A** and **3B**). ERK phosphorylated all three serine sites (255, 279, and 282), while p38 had lower overall levels of Cx43 phosphorylation, toward all sites, and particularly toward S255. JNK, on the other hand, had robust levels of S255 and S279 phosphorylation, with lower levels of S282 phosphorylation. We approached these assays in a qualitative manner; we felt that statistical analyses of these experiments would not be appropriate given the important caveat that there are likely differences in the specificity of each Cx43 phospho-serine antibody.

In addition to phospho-specific antibody experiments, we processed kinase assay samples for analysis using phosphoproteomics. This approach allowed us to strengthen evidence for the phosphorylation sites detected by western blotting, while also potentially identifying Cx43 phosphorylation sites that lack phospho-specific antibodies. Additionally, our use of isobaric mass tags in these experiments facilitated relative quantitation of the amount of phosphorylation of a given site across multiple conditions. We were able to detect several (>15) singly and multi-phosphorylated C-terminal Cx43 peptides using this method (**Table S1**). To increase our confidence in phosphosite assignment, limited validation of mass spectra was performed for the phosphorylation sites characterized with phosphoantibodies **(Figs. S3-S6**). The mass spectrometry results largely mirrored the antibody studies, with JNK and ERK demonstrating substantially more activity for Cx43 at each phosphorylation site than p38 (**Fig. 3C, Table S1**). Together, phospho-specific antibody and mass spectrometry data suggest a preference of JNK for S255 compared to ERK. Phosphoproteomics also provided a clear advantage over use of the S262 antibody, indicating a slight preference for ERK phosphorylation at this site. While both approaches captured the preference of JNK for the S279 phosphorylation site, mass spectrometry data showed this phosphorylation event was strongly associated with JNK activity. Due to the high signal intensity of the singly phosphorylated S279 Cx43 peptide, we were unable to validate and confidently identify peptides with single S282 phosphorylation or the double S279/S282 phosphorylation. However, raw data point to potential preferential ERK phosphorylation at S282 (**Table S1**). Strikingly, our discovery mass spec approach identified a strongly ERK-driven phosphorylation event at T290 (**Fig. 3C**, **Table S1**, **Fig. S6**). This site is particularly intriguing since it does not adhere to the canonical P-X-S/T-P or S/T-P motifs. Overall, the phosphoproteomic data corroborate the phosphoantibody findings while providing a more quantitative assessment of differences among the MAPK conditions.

To better identify the effects of each Cx43 phosphorylation event on electrophoretic mobility changes from P0 to P1 and P2 isoforms, we utilized the full series of our phosphomimetic and phospho-dead mutants at S255, 279, and 282. Given ERK's ability to phosphorylate all three sites (S255, S279, and S282 – **Fig. 3**), we chose this MAPK for the kinase assays with the GST-Cx43CT mutants (**Fig. 4**). Kinase assays of each Cx43CT protein with or without addition of active ERK were analyzed by western blotting analyses against each serine site (255, 262, 279 and 282), as well as by electrophoretic mobility shifts using total Cx43 antibodies (**Fig. 4A**; summarized in **4B**). Total Cx43 western blot indicated that WT, 255A, 279A/282A, and triple A mutants were in the P0 form without the addition of active ERK (summarized in **Fig. 4B**). S255E and double E mutant (no ERK) were observed in the P1 form, while the triple E mutant (no ERK) was in the P2 form. Upon treatment with active ERK, WT Cx43CT exhibited a smear (likely representative of a combination of all three forms (P0, P1, and P2). 255A shifted to P1, while 255E shifted to P2. ERK treatment of S279A/S282A led to both P0 and P1 forms, while the double E mutant exhibited both P1 and P2 forms. Triple A mutant treated with active ERK remained unchanged and stayed in the P0 form, while the triple E mutant stayed at P2. Taken together, these results indicate that a shift to P2 requires phosphorylation at all three sites (255, 279, and 282), and in the absence of S255 phosphorylation or 279 and 282 phosphorylation, Cx43 only shifts Cx43 to the P1 form (**Fig. 4A** and summarized in **4B**).

Assessment of serine phosphorylation through immunodetection with phospho-specific Cx43 antibodies indicated that S255 is phosphorylated in the WT, 279/282 double A and double E mutants, demonstrating a very specific signal from these antibodies. Moreover, the pS255 band was observed in the P2 form, concurring with the results of the gel shifts in the total Cx43 WB (**Fig. 4**). Interestingly, levels of pS255 were higher in the double E mutant than in the double A mutant, even though total Cx43 levels were similar.

A fair level of nonspecific signal for S262 phosphorylation was observed in the 'no ERK' samples for all the mutants and the WT. These results yet again point to the low quality of this antibody. However, a large increase - well above the background signal - was observed in the triple E mutant treated with active ERK as compared to that mutant without ERK. This result demonstrates that S262 phosphorylation by ERK is the most efficient once the other three sites have been phosphorylated (**Fig. 4A**).

Robust levels of S279 phosphorylation were observed in the WT and in both S255 mutants. The WT treated with active ERK exhibited a smear (likely indicative of a shift to both P1 and P2). S255A mutant displayed S279 phosphorylation in the P1 form, while S255E mutant contained both P1 and P2 forms. Indeed, these results reinforce the observation from the total Cx43 signal that S255 phosphorylation is necessary for a shift to P2 when S279 is phosphorylated (**Fig. 4**).

Lastly, the detection of S282 phosphorylation indicated that while WT and S255A displayed a shift to P1, the S255E mutant had a shift to P2, yet again demonstrating that the combination of S255 and at least one more serine (here, S282) is needed for a shift to P2. The double and the triple-A mutants did not display any S282 signal, while some level of the nonspecific signal was observed in both the double and triple E mutants, regardless of the presence of active ERK.

Differences in phosphorylation preferences of MAPKs in MDCK cell lysates

To test Cx43 phosphorylation by MAPKs expressed in two mammalian cell lines, we identified cellular conditions under which we would activate a single MAPK at a time (**Fig. 5A**) and then used these active MAPKs to phosphorylate Cx43 through two approaches: (1) in cells (**Fig. 5B**) or (2) in a hybrid protocol (**Fig. 5C**) with pure GST-Cx43CT. Given our issues with some of the phosphoantibodies against Cx43 sites (such as S255 and S262), the hybrid protocol would allow us to more easily detect Cx43 phosphorylation.

We chose to use Madin Darby Canine Kidney cells (MDCK), a cell line that does not express any endogenous Cx43 but can be transfected with full-length WT Cx43 and phosphomimetic/phospho-dead mutants.[40] In addition, we also assessed levels of endogenous Cx43 phosphorylation in pPAECs (porcine Pulmonary Artery Endothelial Cells) (**Fig. 5B**). In both cell lines, ERK was activated by cell treatments with the epidermal growth factor (EGF), while p38 and JNK signaling pathways were activated by treating cells with anisomycin[41] (**Fig. 5A**). Unfortunately, both JNK and p38 are involved in cellular responses to stress and we were not able to identify activators of only JNK or p38 pathway.[20,22,23] Thus, we utilized inhibitors of p38 (SB202190) and JNK (SP600125) in an attempt to downregulate the kinase activity of each of these MAPKs. We utilized MAPKAPK-2/MK2 (mitogen-activated protein kinase-activated protein kinase 2) phosphorylation as a readout of p38 activity and c-Jun phosphorylation to measure JNK activity.[25,42,43] In addition, it has been observed that even though SB202190 and

SP600125 are competitive inhibitors that prevent downstream phosphorylation of p38 and JNK substrates, respectively, there is evidence that binding of these inhibitors will also affect levels of p38 and JNK phosphorylation by their upstream MAP2Ks (MEK3/6 and MEK4/7, respectively).[44–47] Thus, we also monitored levels of p38 and JNK phosphorylation as partial readouts of these two pathways. To inhibit ERK activity, we utilized a dual MEK1/MEK2 inhibitor (U0126)[44] and measured levels of phosphorylated ERK as a readout of this signaling pathway (**Fig. 5A**).

To facilitate analyses of Cx43 phosphorylation by MAPKs from MDCK and PAEC, we compared the activation status and total protein levels of ERK, JNK and p38 in both cell lines upon treatments with EGF, anisomycin and kinase inhibitors (**Fig. 5D**). This side-by-side assessment revealed that levels of total ERK are higher in PAECs than in MDCKs, while EGF is more effective in activating ERK in MDCKs. Levels of total and active JNK were also higher in PAECs than in MDCKs. JNK activity was detected both through antibodies specific to dual phosphorylated JNK activation loop (active JNK) and by monitoring levels of total c-Jun (**Fig. 5D**). By comparing phospho c-Jun blots with total c-Jun blots (data not shown), we have noted that total cJun antibodies can detect both the phosphorylated c-Jun (slower migrating form - solid triangle in **Fig. 5D**) and unphosphorylated c-Jun (faster migrating form - empty triangle in **Fig. 5D**). JNK inhibitor had a partial inhibitory effect on JNK activity (as judged by both active JNK levels and the phospho c-Jun band in the total c-Jun western blot). Levels of total, and especially, active p38 were higher in the PAECs than in the MDCKs. Treatments with the p38 inhibitor decreased phosphorylation of both the activation loop of p38 (active p38) and its downstream substrate: MK2. Similarly to c-Jun, we compared phospho-MK2 antibodies and total MK2 antibodies (not shown), and observed that both the phosphorylated form and the unphosphorylated MK2 can be efficiently detected with total MK2 antibodies: slower migrating phospho-form (solid triangle) and faster migrating unphosphorylated MK2 (empty triangle, **Fig. 5D**).

We initially attempted to study phosphorylation of full-length Cx43 WT and mutant constructs transiently transfected into MDCK cells that were then treated with EGF (**Fig. 5A**). We were unable to get any specific phosphorylation signal with S255 and S262 antibodies (data not shown) but were successful at detecting S279 and S282 phosphorylation of transfected Cx43 WT and mutants under active ERK conditions (**Fig. 6A** and **6B**). The results of the total Cx43 blot of these transfected samples (**Fig. 6A**) indicate similar electrophoretic mobility shifts as was observed

in our *in vitro* analyses (**Fig. 4**). Namely, upon treatment with the EGF, WT Cx43 underwent a slight shift and ran as a smear, while 255A mutant was observed in the P1 form. Interestingly, the S255E mutant treated with EGF displayed two bands (**Fig. 6C**): one that ran similarly to S255A (P1) and a higher form (P2). Analysis of S279 phosphorylation levels demonstrated that upon treatment with EGF, WT Cx43 had two distinct bands: P1 and P2 (Fig. 6A). The S255A mutant treated with EGF displayed a P1 form. Interestingly, the S255E mutant demonstrated S279 phosphorylation in both P1 and P2 forms. This antibody, yet again, did not pick up any nonspecific signal from the negative controls: double A and double E mutants. Detection of these mutants with the S282 phosphoantibody, on the other hand, was somewhat less specific, showing some background signal in the untransfected lanes. However, both WT and S255A mutants treated with EGF demonstrated a P1 form, while the S255E mutant contained both P1 and P2 forms. Despite difficulties with phosphoCx43 antibodies (S255 and S282), our analyses with total Cx43 (Fig. 6A and B), pS279 and pS282 antibodies (Fig. 6A) concur with our observations *in vitro* (Fig. 4): mimicking and/or actual phosphorylation at all three sites (255, 279 and 282) is required for the full electrophoretic shift to P2.

In addition, we attempted to immunoprecipitate ectopically expressed, full-length Cx43 proteins from these cells in hopes to increase the ability of our phospho-Cx43 antibodies to reliably detect phosphorylation by MAPKs on all three sites (255, 279, and 282). We transfected MDCKs with WT full-length Cx43 C-terminally tagged with GFP and utilized magnetic beads to pull down GFP (Fig. 6D). We pulled down discernable levels of WT Cx43-GFP (probed with total Cx43 antibodies) and detected Cx43 phosphorylation at S255, S279, and S282 in samples treated with EGF. We were unable to observe any signal for S262 (data not shown). Moreover, we failed to immunoprecipitate any WT Cx43-GFP under anisomycin (JNK/p38-activating conditions) – data not shown. We then attempted a different Cx43 pull-down approach with Cx43 antibodies and protein A magnetic beads. However, even though we were able to immunoprecipitate easily detectable levels of total Cx43, the signal from pS279 and pS282 antibodies was low and difficult to interpret due to the high variability in the total immunoprecipitated Cx43 levels (data not shown). Despite these difficulties, we were able to observe phosphorylation by activated ERK on three sites: S255, S279 and S282 on full-length Cx43 ectopically expressed in the MDCKs (**Fig. 6A** and **6D**).

To overcome the unreliability of the phosphoCx43 antibodies in more complex mixtures such as cell lysates and immunoprecipitated samples (**Fig. 6**), we utilized our hybrid kinase assay approach, using purified WT GST-Cx43CT protein immobilized on the glutathione resin, and treated the resin with MDCK lysates containing activated ERK, p38 and JNK (**Fig. 5C**). Lysates of MDCKs treated with various activation/inhibition conditions (with or without starvation, with and without EGF, anisomycin, and upon addition of MAPK pathway inhibitors, **Fig. 5A**) provided robust and specific levels of Cx43 phosphorylation at S279 and S282 (**Fig. 7A**, quantitated in **7B**). Unfortunately, the signals for S255 and S262 phosphorylation were yet again largely nonspecific (based on a high background signal in the untreated triple A mutant used as a negative control). Nevertheless, these analyses allowed us to delineate the effects of the ERK signaling from the contribution of the p38 and JNK pathways. Upon activation of ERK (**Fig. 7C**), high levels of both S279 and S282 were phosphorylated (**Fig. 7A and B**). When both the p38 and JNK signaling pathways were activated and basal levels of ERK activity were eliminated by treatment with the MEK1/2 inhibitor (**Fig. 7C**), strong levels of S279 and S272 phosphorylation continued to persist (**Fig. 7A** and **B**). Moreover, we were able to identify that under full serum conditions, due to more basal ERK activity, detectable levels of Cx43 phosphorylation were present (**Fig. 7A-C**).

Differences in phosphorylation preference of endogenous Cx43 in PAECs

To assess Cx43 phosphorylation in a more native environment, we utilized PAECs, an endothelial cell line that expresses endogenous Cx43. Cx43 protein expression levels have been shown to be robust in these cells and all three electrophoretic forms can be easily observed in whole cell lysates by total Cx43 WB.[40,48] Thus, we utilized the same approach of activation and inhibition of MAPKs in this cell line as in the MDCK cells (**Fig. 5A** and **D**). We hypothesized that the levels of total and phosphorylated Cx43 could be more easily detected in PAEC as compared to the lower levels of transfected Cx43 in MDCK cells (**Fig. 6A** and **6D**).

Indeed, we not only observed strong signals for phosphorylation at S279 and S282 but were also able to separate the three phosphorylation isoforms in all three western blots (pS279, pS282, and total Cx43) (**Fig. 8A**). However, we were not able to detect any specific signal with S255 and S262 antibodies in these samples (data not shown). Despite this limitation, we observed that Cx43 phosphorylated at S279 migrates largely in the P1 and P2 forms, while phosphorylation at S282 is observed mostly in the P0 form (and to a smaller extent, in the P1 form). Total Cx43 antibodies

detected all three forms, with P0 and P1 predominant under ERK activating conditions, with the appearance of the P2 form upon activation of p38 and JNK (**Fig. 8A** and **B**). Comparison of several treatment conditions in these cells also indicated that EGF is not the most ideal activator of the ERK signaling pathway, while cells grown in full serum (FBS) had robust levels of active ERK (**Fig. 8C** and **D**). Highest levels of S279 and 282 phosphorylation were observed under anisomycin treatments (when JNK and p38 are active) and under serum conditions (FBS) when ERK is most active. Taken together, JNK and p38 signaling provides all three isoforms (P0, P1 and P2), while ERK signaling in these cells only produces P0 and P1 isoforms. Despite some antibody difficulties encountered when assessing more complex reactions mixtures (**Figs. 6-8**), our results across multiple phosphorylation sites *in vitro*, in hybrid and fully cellular experiments demonstrate different and consistent Cx43 phosphorylation patterns by each MAPK.

DISCUSSION

Phosphorylation of Cx43 at S255, S262, S279 and S282 is known to mediate GJIC by controlling GJ channel closure, Cx43 ubiquitination and interaction with endocytosis machinery. However, individual contributions of each serine phosphorylation by all three MAPK family members have been difficult to pinpoint.[8,10–13,20,22,23,39] Our study provides a thorough and systematic assessment of substrate specificity by each MAPK toward Cx43, utilizing progressively complex approaches: *in vitro*, in a hybrid mixture of purified and cellular proteins, and directly in mammalian cells. Importantly, our work also establishes that a previously observed (in published mass spectrometry data sets)[49–51] and predicted[52,53] phosphorylation site (T290) is phosphorylated by ERK. (**Figs. 3C**, **S6** and **Table S1**).

The results of our initial kinase assays between purified, active MAPKs (ERK, JNK and p38) and GST-tagged Cx43CT identified substrate specificity differences toward each serine site (**Figs. 2-4**). A combination of three complementary techniques (electrophoretic mobility shifts, western blotting analyses with phospho-specific Cx43 antibodies and LC-MS/MS phosphoproteomics) together with the use of alanine and glutamic acid mutants help simplify data interpretation of more complex cellular experiments (**Figs. 5-8**). Experiments in MDCKs and PAECs help identify cellular conditions necessary for activating and inhibiting individual MAPK signaling pathways (**Fig. 5A**), setting the stage for future Cx43 and GJ functional studies.

Our *in vitro* kinase assay assessed by combination of western blotting and gel shift analyses demonstrate that ERK was able to phosphorylate four serine sites (S255, S279, S282 and S262) – **Fig. 3** and **Fig. 4**. It is important to point out that there was a significant level of non-specific detection with pS255 and even more so, with pS262 phospho-Cx43 antibody (**Fig. 3A** and **Fig. 4A**). These findings stress the importance of using multiple approaches when detecting protein phosphorylation and highlight the utility of phosphosite mutants as controls. For example, while S262 antibody was found to be somewhat nonspecific, our ERK kinase assays with a full panel of Cx43 mutants indicate that S262 phosphorylation is more robust when we mimic phosphorylation at the other three sites (S255E/S279E/S282E triple mutant in **Fig. 4A** as compared to the triple alanine mutant signal).

Analyses of *in vitro* kinase assays between JNK or p38 with pure Cx43CT clearly indicate that each stress-activated MAPK differs in its substrate specificity: JNK prefers S255 and S279, while p38 prefers S279 and S282 (**Fig. 3A** and **3B**, summarized in **Fig. 9A**). Comparison of mobility shift results upon treatments with ERK, p38 or JNK also allowed us to decipher the effects of each phosphorylation event on the appearance of P1 and P2 electrophoretic forms (**Fig. 2C-E** and **Fig. 4**). Namely, we observed that phosphorylation on both S255 and S279 are required for a shift to the P2 form, while phosphorylation at S255 or double phosphorylation at S279/S282 leads to the P1 form. Because we did not have a single S279 or S282 mutant, we cannot definitively conclude that S279 phosphorylation (without S282) is sufficient for the shift from P0 to P1. However, pS282 antibody signal was observed largely in the P0 form, while pS279 signal was seen in the P1 and P2 (**Figs. 4A** and **8A**). These observations were recapitulated through use of glutamic acid mutants as positive controls: S255E and S279E/S282E were in the P1 form, while the triple E mutant was in the P2 form (**Fig. 4A**, summarized in **4B**). Our results are in full agreement with *Sirnes et. al.* who observed S255 phosphorylation in the P2 isoform of cell lysate samples of IAR20 rat liver epithelial cells exposed to the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) treatments.[54] Moreover, our triple alanine mutant lost all ability to undergo an electrophoretic shift, even if S262 was still available for phosphorylation (**Fig. 4**), an observation seen by Warn-*Cramer et. al. 1998* when expressing such a mutant in a mouse Cx43 knock-out (KO) cells treated with EGF.[14]

Our results are in some contrast with the electrophoretic mobility of MAPK site mutants described by *Grosely et. al*.[39] These researchers utilized aspartic acid (D) mutants of some MAPK

sites in two different versions of purified CT of Cx43: a soluble GST-Cx43CT (same as in this study) and TM4-Cx43CT (a membrane-anchored version). The study assessed the effects of mimicking phosphorylation on electrophoretic mobility, changes in secondary structure and backbone dynamics of the CT by circular dichroism (CD) and nuclear magnetic resonance (NMR), respectively. In experiments with the fully soluble mutants, GST-Cx43CT S255D/S279D/S282D mutant migrated in the P1 form, while the S255D/S262D and all single mutants remained in the P0 form. However, the membrane anchored TM4-Cx43CT S255D/S262D mutant was observed in the P1 form, while the TM4-Cx43CT S282D mutant remained in the P0 form. TM4-Cx43CT mutants of other sites were not tested due to difficulties with their expression in *E.coli*.[39] Even though aspartic and glutamic acid substitutions have both been commonly used to mimic phosphorylation in Cx43,[39,40,55,56] it is conceivable that the glutamic acid substitution is able to better recapitulate the effects of serine phosphorylation on Cx43.

Our mass spectrometry analyses largely supported the findings of the gel shift assays and western blots, while providing the added benefit of identifying previously uncharacterized sites (**Figure 3C, Figs. S3-S6, Table S1**). One notable shortcoming of our efforts was an inability to distinguish single phosphorylated S282 and double phosphorylated S279/S282 peptides from the dominant singly phosphorylated S279. This was due in part to the close proximity of these two serines in the Cx43CT amino acid sequence, a challenge encountered in other mass spectrometry studies of Cx43.[57] Future efforts could attempt to capture these additional phosphorylation events through improved chromatography (longer HPLC gradients, smaller diameter capillary beads), mass spectrometer methods (m/z exclusion lists), or co-elution studies with synthetic heavy-labeled phosphopeptides.

To the best of our knowledge, our work is the first to identify T290 as a MAPK site (**Fig. 3C**, **Fig. S6**, **Table S1**). Even though T290 fully lacks the P-X-S/T-P or S/T-P motifs typically phosphorylated by MAPKs, our *in vitro* mass spectrometry analyses detected T290 phosphorylation in ERK-treated (and to a lesser extent, JNK and p38-treated Cx43CT) (**Fig. 3C**, **Table S1**, **Fig. S6**). T290 phosphorylation has never been reported in any direct peptide sequencing or mass spectrometry analyses of Cx43.[15,57,58] For example, work by Axelsen *et al* evaluated Cx43 phosphorylation sites by mass spectrometry from Cx43 samples isolated from ischemic vs. control rat hearts; the researchers observed phosphorylation at 255 and 262, while phosphorylation at S279, S282 and T290 was not detected.[57] Warn-Cramer and colleagues detected S255, S279 and

S282 phosphorylation in recombinant Cx43CT treated with recombinant active ERK.[15] Cooper *et al* observed phosphorylation at S255 *in vitro* when Cx43 was treated with cdc2 and in cells on peptides containing S255, S279 and S282 residues (although these specific sites were not mapped).[58]

Chen and colleagues utilized an informatics server (NetPhosK 1.0) to examine phosphorylation motifs in Cx43. While T290 was predicted as a phosphorylation site, it was attributed to PKC by NetPhosK (Chen et al).[59] Even though the server has since been updated (NetPhos 3.1), it is still limited to a set number of kinase motif predictions (ATM, CKI, CKII, CaM-II, DNAPK, EGFR, GSK3, INSR, PKA, PKB, PKC, PKG, RSK, Src, cdc2, cdk5 and p38 MAPK).[60,60] We assessed Cx43 motifs through the NetPhos 3.1 server that yet again, predicted PKC and to a lesser extent, cdc2 as kinases for T290 phosphorylation. We also consulted PhosphositePlus[52,53] for any references to proteomic data sets that have detected T290 phosphorylation. Indeed, phosphorylation at T290 has been observed in a few high throughput proteomics (HTP) data sets.[49–51] Importantly, to the best of our knowledge, our findings are the first to attribute T290 phosphorylation specifically to ERK.

Interestingly, a novel T290 mutation was observed in a patient with erythrokeratodermia variabilis et progressiva (EKVP) - a rare congenital skin disorder genetically linked to mutations in connexin genes.[61] Namely, the study described one patient who had a combination of P283L and T290N mutations, while a second (unrelated) patient had only a single mutation (P283L) (**Fig. 9B** - red triangles). While not tested, it is likely that P283L mutant may not become readily phosphorylated on S282 (as it lacks the canonical phosphorylated motif for S282 phosphorylation - **Fig. 1B** and **C**). Both patients exhibited Cx43 mislocalization (intracellular rather than in the plasma membrane) and the patient with the double mutation demonstrated a more severe EKVP phenotype.[61] A more recent Cx43 functional study by the Laird laboratory of single (P283L) and double (P283L/T290N) Cx43 mutants expressed in rat epidermal keratinocytes and HeLa cells concluded that these mutations either extend the residency of Cx43 GJs in the plasma membrane or slow down Cx43 internalization and degradation.[62] These results, together with our identification of T290 as an ERK site, indicate the potential importance of T290 in the regulation of Cx43 internalization and degradation together with the well-characterized effects of S255, S262, S279 and S282 in these cellular processes.[11,12,48] However, the authors did not test phosphorylation at any serine sites in their P283L mutant (such as S282) and were unable to conclude that a

combination of both mutations (P283L/T290N) is the sole reason for yielding a more severe phenotype of EKVP described by Li *et al*.[61,62]

Many excellent *in vitro*, cellular and *in vivo* studies have demonstrated combinations of Cx43 phosphorylation events under a wide array of activating conditions, such as EGF, TPA (12-O-tetradecanoylphorbol-13-acetate), VEGF (vascular endothelial growth factor) and in many physiological contexts, tissues, and organs.[7,11,12,14,23,34,54,63–65] What makes the assessment of all this literature difficult is the activation status of all three MAPK family members and whether sites that were not reported were ever tested. It is thus helpful to have a systematic *in vitro* assessment of phosphorylation events that help with data interpretation of cellular phosphorylation measurements undertaken under the same treatment conditions (this study) where activation level of each MAPK is evaluated and phosphorylation-dead alanine mutants are used as negative controls.

It is, nonetheless, important to emphasize the difficulties we encountered with either non-specific or low phospho-Cx43 signals from phospho-Cx43 western blotting analyses in complex cellular mixtures: (**Fig. 5B** and **Figs. 6-8**). We were, however, able to better interpret these results because of the Cx43 alanine mutants used as negative controls. Additionally, our use of and comparison between two cell lines allowed us to build in complexity from a hybrid approach to a fully cellular study of endogenous Cx43 phosphorylation (**Fig. 7** vs. **Fig. 8**). We felt that it was important to activate or inhibit ERK, JNK and p38 using identical conditions in both cell lines: EGF treatments to activate ERK, MEK1/2 inhibitor to inhibit ERK, anisomycin to activate both JNK and p38, and respective JNK and p38 inhibitors to inhibit JNK or p38 activity. While we were not able to fully inhibit JNK or p38 using their inhibitors, we were able to identify conditions where only ERK, mostly JNK or mostly p38 were activated in both MDCK and PAECs (**Fig. 7B**, **Fig. 8C** and compared in **Fig. 5D**). For example, in the MDCK experiments (**Fig. 7A** and **B**), we were able to separate the phosphorylation events on S279 and S282 that were due to ERK rather than due to the combined JNK + p38 activity vs. JNK alone or p38 alone (**Fig. 7A** and **B**). In PAECs, it was clear that ERK activity alone was not enough to produce the P2 phospho-form, while activity of JNK and p38 in these cells lead to the appearance of the P2 phospho-form. Taken together, comparisons of Cx43 phosphorylation in these two cell lines indicate higher ERK preference for Cx43 in MDCKs and JNK/p38 preference in the PAECs - results that agree with

our observations that stress activated MAPKs (JNK and p38) are more active and abundant in PAECs while ERK activity is more robust in the MDCKs (**Fig. 5D**).

Finally, this work provides a much-needed confirmation that members of the MAPK family and their signaling pathways are not all created equal when it comes to phosphorylation of Cx43 on five phosphorylation sites. Importantly, these residues (S255, S262, S279, S282, and T290) lie within or near binding sites of actin interacting proteins (drebrin), trafficking chaperones (CIP85), other kinases (Src), ubiquitin ligases (NEDD4), and clathrin adapter protein 2 (AP2) - summarized in **Fig. 9B**.[66] It will thus be important to delineate the impacts of each MAPK pathway (ERK, JNK and p38) on all five sites in the fine-tuned regulation of Cx43 protein life cycle and gap junction function in human health and disease.

MATERIALS AND METHODS

Mutagenesis of Cx43

The pGEX-6p2-WTCx43CT plasmid was a kind gift from Paul Sorgen, University of Nebraska.[39] Plasmid containing full-length Cx43 for mammalian expression (Cx43-eGFP-NI) was a kind gift from Matthias Falk, Lehigh University. It contains a double-stop codon between Cx43 and eGFP and was used to generate mutants at S255, S279, and S282.[40] S255A/E and S279/S282A/E Cx43 mutants were generated using the following mutagenesis primers:

S255A**:** 5'-CAC GCC ACC ACT GGC CCA CTG **GCA** CCA TCA AAA GAC TGC GGA TCT-3'

S255E: 5'-CAC GCC ACC ACT GGC CCA CTG **GAA** CCA TCA AAA GAC TGC GGA TCT-3'

S279A/S282A: 5' CC TCA CCA ACG GCT CCA CTC **GCA** CCT ATG **GCA** CCT CCT GGG TAC AAG CTG G 3'

S279E/S282E: 5' CC TCA CCA ACG GCT CCA CTC **GAA** CCT ATG **GAA** CCT CCT GGG TAC AAG CTG G 3'

To make the triple mutant for bacterial expression, S279/282 mutants were used as templates with the S255 mutagenesis primers. All mutagenesis PCR reactions were amplified using PfuUltra II Fusion HS DNA Polymerase (Agilent). The parental (template) DNA was digested with the restriction enzyme DpnI (New England Biolabs). Plasmid DNA was purified

using the QIAquick PCR Purification Kit (Qiagen). The resulting DNA was transformed into DH5*α E. coli* cells (Invitrogen/Thermofisher). DNA was then isolated using the QIAprep Spin Mini-Prep Kit (Qiagen) and sent for sequencing to ensure that the mutagenesis reactions were successful. Cx43 constructs with a stop codon in the eGFP-NI plasmid were purified with QIAprep Midi Prep Kit (Qiagen) suitable for Lipofectamine2000 (Invitrogen/Thermofisher) transfection following the manufacturer's transient transfection protocol.

Protein Expression and Purification of Cx43 WT and Mutants

GST-tagged Cx43CT WT and mutants were expressed in BL21 (DE3) *E.coli* cells. Cx43 expression was induced with 0.75 mM IPTG at optical density ($O.D_{600nm}$) of 0.6-0.8, and the *E.coli* were grown overnight at 30°C. Bacterial cultures were centrifuged at 8,000 x g for 20 minutes and resuspended in lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1mM PMSF) for sonication (3 times for 30 seconds at 70% amplitude). The resulting lysate was centrifuged at 12,000 x g to separate the soluble protein fraction and incubated with glutathione resin (GenScript) for 30 minutes while shaking at room temperature (RT). The beads were washed in wash buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) and centrifuged at 3000 x g after each wash. The beads were then incubated in elution buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 20 mM glutathione) for 15 minutes at RT. The eluent was collected, dialyzed into a storage buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA), and stored at -20ºC. Concentrations of GST-tagged Cx43CT proteins were determined by measuring absorbance at 280 nm.

Protein Expression and Purification of Active MAPKs:

Mitogen-activated protein kinase (MAPK) cascades are not naturally found in prokaryotic systems. Therefore, they lack the necessary upstream kinases needed to phosphorylate MAPK such as ERK. The methods for expressing active MAPKs were performed are described previously by Khokhlatchev *et al*. and by Miller *et al*..[67,68] Plasmids encoding constitutively active, GST-tagged MEK1 (MEK1-pGEX4T1), His-tagged ERK (ERK2-pET28), GST-tagged p38a (p38a-pGEX4T1) and constitutively-active, His-tagged MKK6 (MKK6-pET28) were kind gifts from Benjamin Turk, Yale University, Department of Pharmacology. Plasmids encoding a constitutively active MEKK (MEKK-C-pBR131) and His-tagged JNK2/untagged MEK4 (SAPKa-MKK4-pET15)

were a kind gift from Dr. Melanie Cobb, University of Texas Medical Center. Appropriate plasmid combinations were co-transformed into BL21 (DE3) *E. coli* cells using equal amounts of plasmid DNA in presence of both ampicillin (100 µg/mL) and kanamycin (50 µg/mL): ERK2-pET28 with MEK1-pGEX4T1, p38-pGEX4T1 with MKK6-pET28, and SAPKa-MKK4-pET15 with MEKK-C-pBR131.

ERK2 and MEK1 co-expression was induced with 0.4 mM IPTG at OD_{600} of 0.6-0.8, and proteins were expressed overnight at 18°C. Bacterial cultures were centrifuged at 8,000 x g for 20 minutes and resuspended in 10 mL of lysis buffer (20 mM Tris-Cl, 140 mM NaCl, 10 mM imidazole, 10% glycerol, 0.1mM EDTA, 1mM PMSF), and lysed by sonication. The supernatant was incubated with 2 mL of nickel resin (GenScript) at RT for 20 minutes and washed once with lysis buffer and another three times with wash buffer (20 mM Tris-Cl pH8.8, 10 mM imidazole, 140 mM NaCl, 10% glycerol, 0.1mM EDTA). Following the initial washes, the resin was then centrifuged and ERK was eluted in 20 mM Tris-Cl, pH 8.8, 400 mM imidazole, 10% glycerol, 0.1mM EDTA), and dialyzed overnight into dialysis buffer (20 mM Tris-Cl, pH 7.5, 140 mM NaCl, 10% glycerol, 0.5 mM DTT). His-ERK was further purified using 1 mL glutathione resin (GenScript) to remove any residual GST-tagged MKK1.

Purification of active, His-tagged rat JNK2 has been previously described.[69] GST-tagged p38 expression was induced with 1 mM IPTG at $O.D_{600nm}$ of 0.6-0.8 and the *E.coli* were grown overnight at 30°C. Bacterial cultures (300 mL) were then centrifuged at 8,000 x g for 20 minutes and resuspended in 3 mL of lysis buffer (20 mM Tris-Cl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1mM PMSF) and lysed by sonication. Cell lysates were centrifuged at 14,000 x g for 20 minutes to separate the soluble supernatant from the insoluble pellet. To determine the best amount of time that p38 takes to bind to glutathione beads, the supernatants were incubated with Glutathione beads for 20 minutes and 90 minutes at RT or 12 hours at 4°C). After incubation, samples were washed four times in wash buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol). Following initial washes, 10 mL of elution buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 20 mM glutathione) was added, and samples were rocked at RT for 15 minutes to elute GST-p38. Samples were then dialyzed in storage buffer (20 mM Tris-Cl, pH 7.5, 140mM NaCl, 10% glycerol, 0.5 mM DTT) and kept at -80°C in 1 mL aliquots. The concentration of all purified proteins was determined by measuring absorbance at 280nm.

In Vitro and hybrid kinase assays and SDS-PAGE Mobility Shifts

In vitro phosphorylation assays were carried out at 25°C for 1 hour in 20 mM Tris-Cl, pH 7.6, containing 150 mM NaCl and 10% glycerol, 2 mM ATP and 10 mM $MgCl_2$ (termed Kinase Reaction Buffer). GST-Cx43CT WT or S255, S279, and S282 mutant concentrations were kept at 6 µM. The typical reaction volume was 200 µL. Reactions were started by the addition of active MAPKs at a final concentration of 0.5 µM and were quenched with 4x SDS-PAGE Laemmli sample buffer.[70] Kinase reactions generated for LC-MS/MS analyses were quenched with SDS (0.2% final concentration). Phosphorylation by ERK, p38, and JNK was analyzed via immunoblotting with phospho-specific antibodies against S255, S262, S279, and S282 (see below). For hybrid phosphorylation reactions, 6 µM solutions (500 µL total volume) of pure, GST-Cx43CT (WT or mutants) were added to 200 µL of glutathione beads preequilibrated in 20 mM Tris-Cl, pH 7.6 containing 150 mM NaCl and 10% glycerol (termed Hybrid Assay Buffer). GST-tagged Cx43CT proteins were allowed to bind to the beads for 30 min at RT with gentle rocking. Glutathione beads were washed 3 times in 1 mL of hybrid assay buffer to remove unbound GST-Cx43CT proteins. Lysates (400 µL) of pre-treated MDCK cells (see 'Lysate Preparation' section) were then added to GST-Cx43CT proteins and incubated while gently rocking at RT for 1 hour. To ensure that enough ATP and $MgCl_2$ were present in these hybrid reaction mixtures, ATP and $MgCl_2$ were added to final concentrations of 2 mM and 10 mM, respectively.

Phosphorylation of Cx43 by ERK, p38, and JNK was analyzed by immunoblotting bead samples with phospho-specific antibodies against S255, S262, S279, and S282. The activation status of ERK, p38, and JNK was confirmed by immunodetection of active and total kinase antibodies and of downstream substrates of JNK and p38 in MDCK lysate samples (see below).

SDS-PAGE and Western Blotting Analyses

Protein samples were resolved on a 4-20% acrylamide (ThermoFisher) pre-cast Tris-Glycine gels and stained with Coomassie Brilliant Blue stain or transferred for western blotting analyses onto 0.4 µm nitrocellulose membrane (Bio-Rad) in Tris-Glycine buffer with 20% methanol for 1 hour at 100 V. For the detection of phosphorylated proteins with phospho-specific antibodies, membranes were blocked with 5% BSA in 1x TBS with 0.1% Tween20 (TBS-T) buffer for 1 hour at RT or overnight at 4°C. For the detection of total proteins, membranes were blocked in 5% nonfat milk and TBS-T. Membranes were washed 3x in TBS-T and probed with primary antibodies

in TBS-T for 1 hour at RT or overnight at 4ºC. After three additional washes, membranes were treated with appropriate goat anti-rabbit-HRP or goat anti-mouse-HRP secondary antibodies (Cell Signaling Technology) at 1:5000 dilution in TBS-T for 30 min at RT. After five additional washes, membranes were treated with ECL Clarity (enhanced chemiluminescence) or ECL Clarity Max Reagent (Bio-Rad) for 5 min and detected on a ChemiDoc™ Gel Imaging System (Bio-Rad). The following primary antibodies were purchased from Cell Signaling Technology and used at 1:2000 dilution: total Cx43, active ERK, total ERK, active JNK, total JNK, active p38, total p38, active MAPKAPK-2 (MK-2) – pThr334 and total MK-2. α-tubulin antibodies (1:5000), pS262-Cx43 (1:2000), pS279-Cx43 (1:2000), pS282-Cx43 (1:2000) were from ThermoFisher and histidine-tag antibodies (1:2000) were from R&D Systems. pS255 rabbit polyclonal antibodies were a kind gift from Dr. Paul Lampe (Fred Hutchinson Cancer Center, Seattle, WA), and were used at 1:2000 dilution in TBS-T. When probing for purified proteins, such as *in vitro* phosphorylated Cx43 or activated MAPKs, a less sensitive ECL reagent was used (Bio-Rad – Clarity ECL), while ECL Clarity Max reagent (Bio-Rad) was used to detect signals from mammalian lysate samples and in the immunoprecipitants.

Electrophoretic Mobility and Densitometry Analyses

ImageJ (NIH) was used to analyze Coomassie-stained gels for electrophoretic mobility changes and western blots for changes in Cx43 phosphorylation levels at specific serine sites. For mobility shifts, the pixel area between each peak maximum and the higher MW peak minimum was quantified in Keynote (gray in **Fig.2D**). Seven independent kinase assays were quantitated in this manner.

Kinase Assay Sample Preparation for Mass Spectrometry

SDS was added to kinase assay reactions to a final concentration of 0.2% (v/v). Denatured reactions were then reduced with 10 mM dithiothreitol (DTT) in 50 mM HEPES (pH 8.5) and alkylated with 55 mM iodoacetamide in 50 mM HEPES. Samples were then subjected to single-pot, solid-phase-enhanced sample preparation (sp3) for cleanup and tryptic digestion as follows: samples were incubated with sera-mag speed beads and 50% ethanol for 8 minutes at room temperature, using 10 µg of beads per 1 µg of protein (kinase + substrate). Lysate-bead mix was incubated on a magnetic rack for 2 minutes and the supernatant was discarded. Beads were washed

three times with 200 µL of 80% ethanol. Proteins were digested for 18 to 24 hours on bead with sequencing grade trypsin (Promega) in 50 mM HEPES buffer at 1:50 trypsin to protein ratio. Peptides were collected in the supernatant by incubating beads on a magnetic rack, and peptide concentrations were measured by bicinchoninic acid (BCA) assay (Pierce). 5 µg of each sample was lyophilized and labeled with TMTpro isobaric mass tags.

Phosphoproteomic Analyses of Kinase Reactions

TMT-labeled samples were resuspended in 0.2% trifluoroacetic acid, and pH-adjusted to ≤ 3. Acidified sample was added to Fe-NTA spin columns (ThermoFisher Scientific), and phosphopeptide enrichment was performed as previously described .[71] Eluates were then subjected to LC-MS/MS on the Q-Exactive Plus as follows: 1) Peptides were separated with a 140 min gradient with 70% acetonitrile in 0.2 M acetic acid. 2) The mass spectrometer was operated with a spray voltage of 2.5 kV. 3) Selected ions were HCD fragmented at normalized collision energy 33%. 4) Full MS1 scans were acquired in the m/z range of 350–2000 at a resolution of 70,000, with maximum injection time of 350 ms. The top 15 most intense precursor ions were selected and isolated with an isolation width of 0.4 m/z. 5) MS/MS acquisition was performed at a resolution of 70,000.

Phosphoproteomic Data Analysis

Raw mass spectral data files were processed with Proteome Discoverer version 3.0 (ThermoFisher Scientific) and searched against the human SwissProt database and a custom database containing the rat connexin43 C-terminus using Mascot version 2.8 (Matrix Science). Cysteine carbamidomethylation, TMT-labeled lysine, and TMT-labeled peptide N-termini were set as static modifications. Methionine oxidation and phosphorylation of tyrosine, serine, and threonine were searched as dynamic modifications. Peptide spectrum matches (PSMs) for phosphopeptides were filtered for ion score ≥ 15, search engine rank = 1, precursor isolation interference ≤ 35%, and average TMT intensity ≥ 1000. The Proteome Discoverer PhosphoRS node was utilized for improved phosphorylation site localization and assignment, and PSMs were filtered for Isoform Confidence Probability ≥ 0.7. PSMs with missing values were only retained if values were missing for every replicate of a given condition. A TMT intensity of 1000 was imputed for missing values. All remaining PSMs for Cx43 or MAPK family members with a given modification were summed to generate peptide-level TMT intensities. For select Cx43 phosphorylation sites, spectra were

manually validated to confirm proper site assignment, and TMT intensity values were obtained directly from the appropriate spectra. Mean-normalized abundances for a given phosphopeptide were calculated by dividing each TMT channel intensity value by the average of these values for the peptide.

Table S1 has a detailed list of abundances and the amino acid sequence of phosphopeptides from the mass spec run. The raw mass spectrometry data and associated tables have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD042575. Data can be accessed for manuscript review with the following credentials:

Username: reviewer_pxd042575@ebi.ac.uk
Password: xmCvof9f

Mammalian Cell Culture, Cell Treatments and Lysate Preparation

Porcine Pulmonary Artery Endothelial (pPAEC) cells were a kind gift of Dr. Matthias Falk, Lehigh University. Madin Darby Canine Kidney cells, strain II (MDCK-II)[72] was purchased from Sigma Aldrich. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin at 37ºC and 5% CO_2. pPAECs express endogenous levels of Cx43, while MDCKII cells do not express any Cx43 but can be transfected with its cDNA.[40,48] Lipofectamine 2000 (Invitrogen/ThermoFisher) was used for transient, full-length Cx43 WT and mutant transfections into MDCKII cells, following the manufacturer's protocol. For 30 mm dishes (or 6-well plates), 2.5 µg of plasmid DNA and 5 µL of lipofectamine were used. Epidermal growth factor (EGF) was purchased from PeproTech and 100 µg/mL stocks were stored in 5% sucrose at -80ºC. Anisomycin, JNK inhibitor (SP600125), p38 inhibitor (SB202190), and MEK1/MEK2 inhibitor (U0126) were purchased from Tocris and stored in DMSO (dimethyl sulfoxide) at -20ºC. Prior to any cell treatments, MDCK or pPAEC were starved for 2 hours in FBS-free DMEM media. Inhibitors (SP600125, SB202190, or U0126) were added following starvation and cells were treated for 1 hour at 20 µM of each inhibitor in FBS-free DMEM. JNK and p38 activation was triggered with or without inhibitors for 1 hour using 1 µg/mL anisomycin in FBS-free DMEM. DMSO was used as vehicle control in samples lacking any anisomycin and MAPK inhibitors.

ERK was activated with 20 ng/mL EGF in DMEM by treating cells for 5 min. All treatment experiments were carried out at 37ºC and 5% CO_2 and halted by removal of treatment media and the addition of 2x Laemmli SDS sample buffer and boiling for 5 minutes or by rinsing the cells with ice cold 1x PBS, and lysing them in pre-chilled lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% NP-40, 1 mM β-glycerophosphate, and 1 mM PMSF. Cell culture dishes with lysis buffer were rocked on ice for 25 minutes, and scraped cell lysates were transferred to pre-chilled 2 mL Eppendorf tubes and centrifuged at 11,000 x g for 5 minutes. The clarified soluble lysate fraction was then used for Cx43 immunoprecipitations or in kinase assays with GST-Cx43CT proteins immobilized on the glutathione beads (as described in the '*In Vitro and Hybrid Kinase Assays'* section above).

Cx43 and Cx43-GFP Immunoprecipitation from MDCK

MDCK cells transfected with full-length Cx43 cDNA or with Cx43WT-GFP were lysed as described above in 300 µL of Lysis Buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% NP-40, 1 mM β-glycerophosphate, and 1 mM PMSF) per 3 cm dish. 30 µL of magnetic protein A-beads (BioRad) preincubated with 1 µL total Cx43 antibodies (Cell Signaling Technologies, Cat. # 3512, to immunoprecipitate full-length Cx43) or GFP-trap magnetic agarose (Chromotek, to precipitate GFP-tagged Cx43) were rocked with 200 µL of clarified cell lysate for 1 hour at RT. Beads were washed in 500 µL of lysis buffer three times and eluted in 80 µL of 2X Laemmli SDS sample buffer and boiled for 5 minutes. Samples were then used in western blotting analyses with phosphoserine or total Cx43 antibodies.

Statistical analyses

Coomassie gel and western blot signals were quantified using ImageJ software (NIH). Quantifications were carried out on experiments that had at least three independent replicates. GraphPad Prism 9 was used for all bar graphs and statistical analyses. All data are represented as mean ± SEM (standard error of the mean), and significance was tested using one-way or two-way ANOVA, as indicated in the figure captions.

Abbreviations

AP-2 (adapter protein 2), Cx43 (connexin 43), CDK (cyclin-dependent kinase), cdc (cell-division cycle, gene that encodes CDC1), CK (casein kinase), EGF (epidermal growth factor), ERK (extracelluar signal-regulated kinase), GJ (gap junction), GJIC (gap junction intercellular communication), GST (glutathione S-transferase), JNK (c-Jun N-terminal kinase), MAPK (mitogen-activated protein kinase), MDCK (Madin Darby canine kidney), MEK/MKK (MAP kinase kinase), MEKK (MEK kinase/MAP kinase kinase kinase), pPAEC (porcine pulmonary artery endothelial cells), MAPKAPK-2/MK2 (mitogen-activated protein kinase-activated protein kinase 2), PKA (protein kinase A), PKC (protein kinase C), TPA (12-O-tetradecanoylphorbol-13-acetate), VEGF (vascular endothelial growth factor).

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Supporting information

This article contains supporting information.

Data availability

All data supporting the results described are in the manuscript and in the supporting information.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

A.T. - conceptualization of all biochemical and cellular experiments except LC-MS/MS analyses, funding acquisition, project administration, manuscript writing and editing, training/supervision of L.L., L.P., R.T., V.V, S.A. and M.C. - (all undergraduate researchers at Moravian University).

F.W. - manuscript editing and supervising J.C.-P. **F.W.** and **J. C.-P.** LC-MS/MS conceptualization. **J. C.-P.** - LC-MS/MS data acquisition, manuscript writing and editing. **L.L.** - cellular and hybrid studies on Cx43 phosphorylation, data analyses and manuscript editing. **L.P.** - identification of cellular conditions for MAPK activation and inhibition. **R.T.** - *in vitro* kinase assays with all MAPKs. **V.V.** - generation and characterization of active p38. **S.A.** - generation and characterization of active JNK. **M.C.** - generation of active ERK and development of the initial ERK/Cx43 assays.

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Figures and Legends:

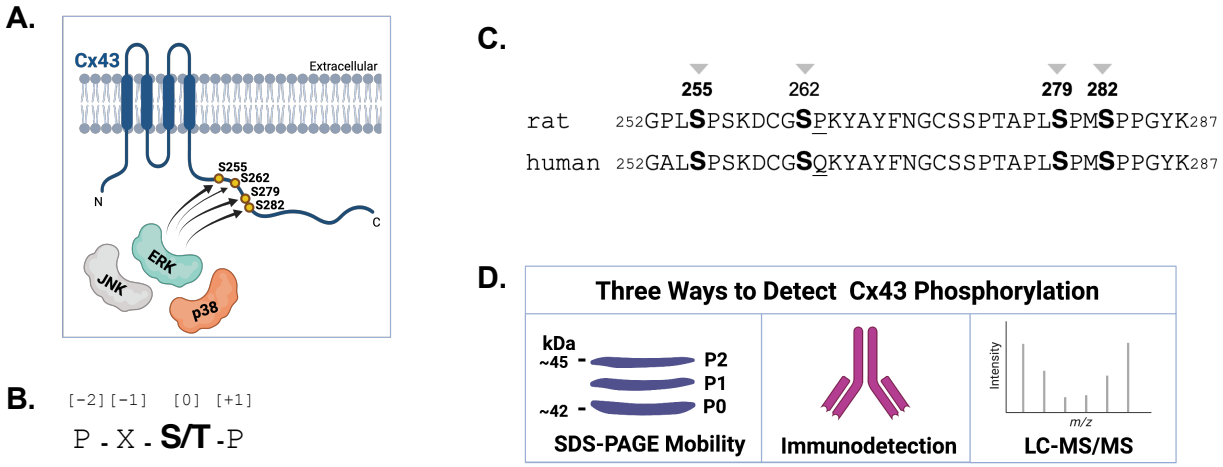

Figure 1: A. Overall question of this study: Do ERK, JNK, and p38 MAPKs display differential phosphorylation preference toward serine residues (255, 262, 279, and 282) on the Cx43 CT? **B.** Phosphorylation motif for MAPK substrates, where S/T is the phosphoacceptor residue at position 0, and there is a preference for a proline residue at +1 and -2 positions. X – any amino acid. **C.** Human vs. rat amino acid sequence alignment within the Cx43CT that harbors the relevant serine residues (shown in bold). While S279 and S282 fully conform to the MAPK motif in both human and rat connexin 43 sequences, S255 in the human sequence lacks a proline at the -2 position in humans. S262, while followed by a proline at +1 in the rat Cx43, it lacks a proline in the human sequence (underlined). Cx43 used in this study is from a rat gene unless otherwise specified. Sequence alignment was generated using Clustal Omega Multiple Sequence Alignment tool. **D.** Cx43 phosphorylation was monitored by three techniques: electrophoretic mobility shifts on an SDS-PAGE from an unphosphorylated, P0 form to the phosphorylated P1 and P2 forms (see text for detail), immunodetection with phosphospecific Cx43 antibodies (to identify Cx43 phosphorylated at S255, S262, S279, or S282), and through use of liquid chromatography and tandem mass spectrometry (LC-MS/MS) of trypsin-digested Cx43 protein samples. Figures A and D were generated in BioRender.com.

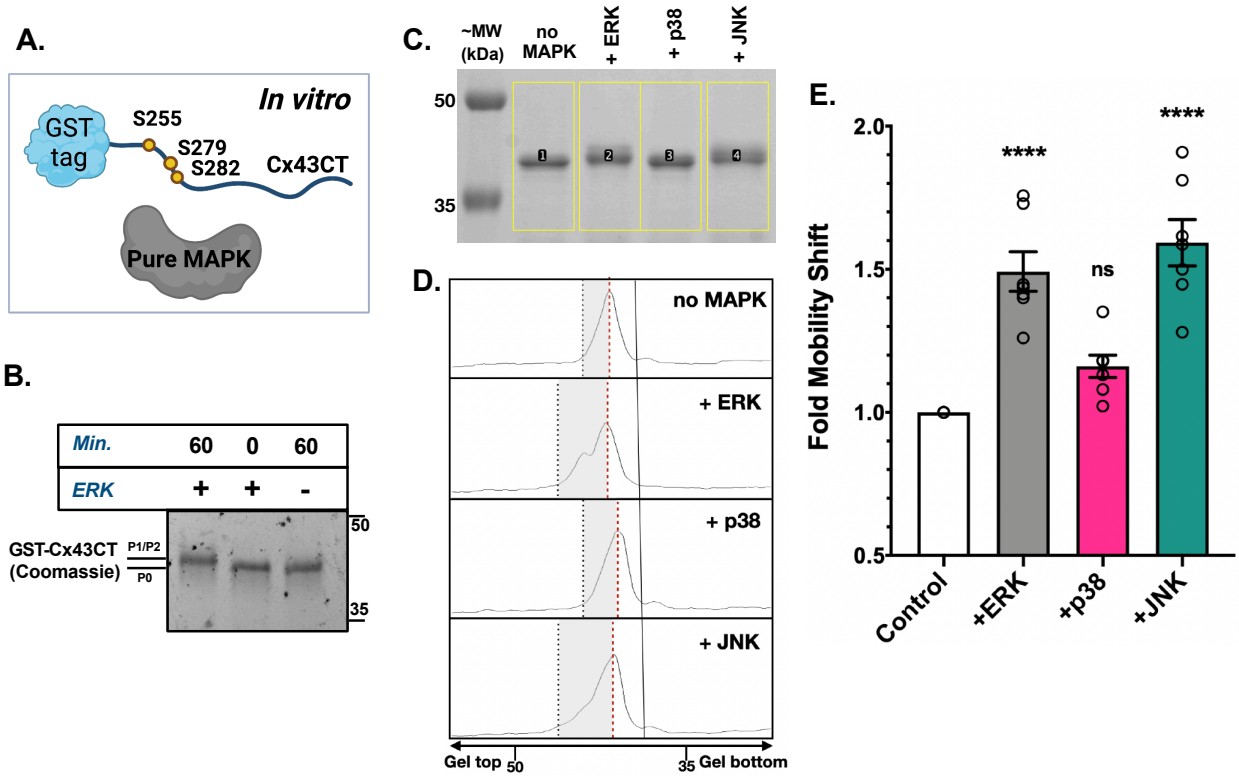

Figure 2: A. *In vitro* approach to studying Cx43 phosphorylation by MAPKs through use of recombinantly-produced and purified GST-tagged Cx43CT protein (6 µM) treated with purified and active MAPKs (His-ERK2, GST-p38a, and His-JNK2 at 0.5 µM each). **B.** Electrophoretic mobility shifts of GST-Cx43CT (WT) treated with active ERK2 (as described in A) and separated on a Tris-Glycine gel (containing 4-20% acrylamide). Appearance of a slower-migrating, phosphorylated form (either P1 or P2) is apparent in the GST-Cx43CT protein treated with active ERK for 1 hr., while both untreated (negative control) Cx43 or treated at t=0 migrated as the unphosphorylated, P0 phospho-form. **C.** A representative SDS-PAGE image of kinase reactions between GST-Cx43CT (WT, 6 µM) and each active MAPK (0.5 µM). GST-Cx43CT kinase reaction sample lacking active MAPK is included for comparison. Yellow boxes and lane numbers indicate areas selected in the ImageJ software used to visualize and quantitate Cx43 mobility shifts. **D.** Representative image of pixel intensity peaks obtained from (C) in ImageJ. Peak maxima are indicated with a dashed red line, while the left-hand side of each peak (from the maximum to the minimum are highlighted in gray rectangles). The width of each gray rectangle (indicative of change in Cx43CT mobility) was measured in Keynote. **E.** Statistical analyses of GST-Cx43CT mobility shifts. Data represent seven biological replicates (n=7) per kinase reaction and statistically significant differences were determined by ordinary one-way ANOVA: $p < 0.0001$ (****), ns-not significant. Error bars are shown in SEM (standard error of the mean). Figure A was generated in BioRender.com.

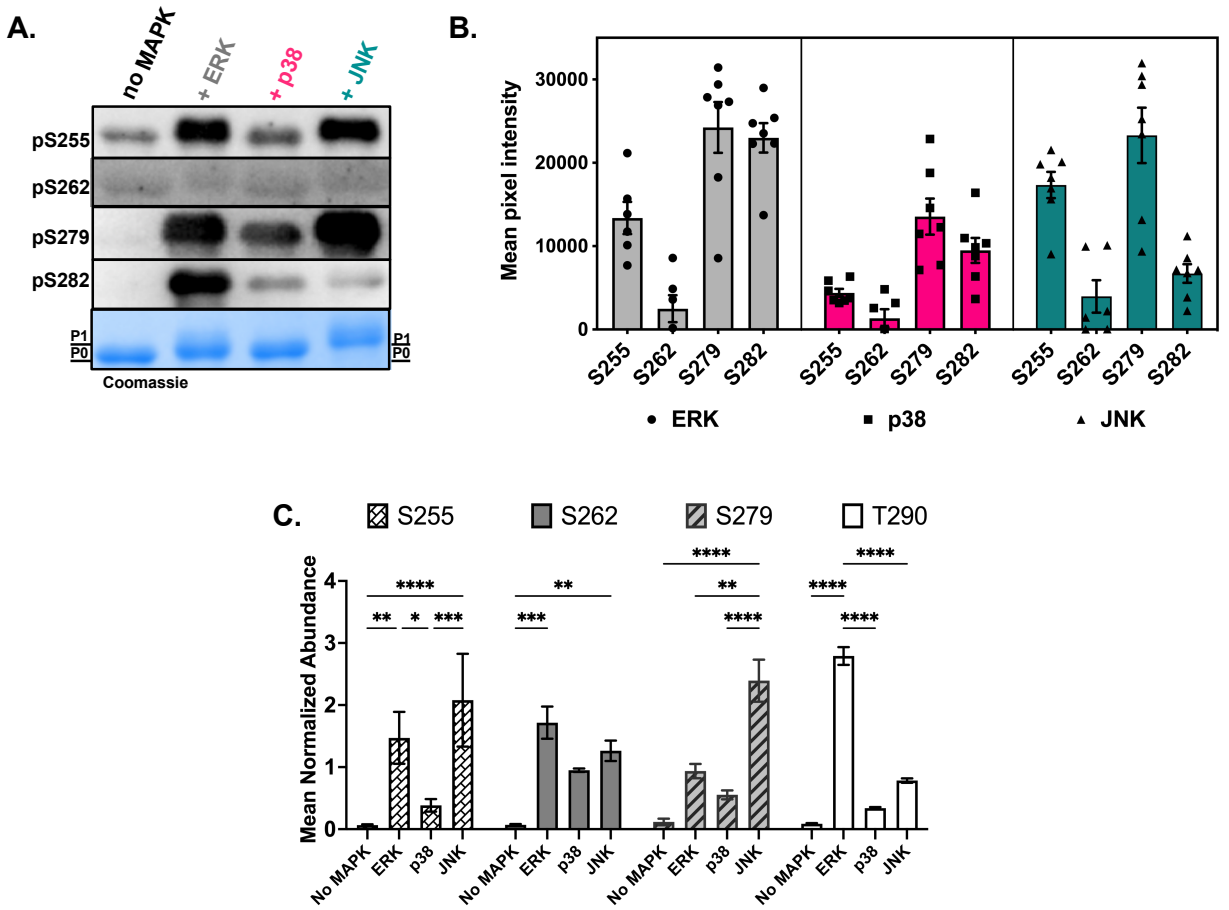

Figure 3: A. Western blotting analyses with phosphoserine-specific Cx43 antibodies on GST-Cx43CT (WT) kinase assay samples with each indicated active MAPK (same reaction conditions as in Fig. 2). Total Cx43 levels and mobility shifts were assessed with Coomassie staining. The reaction without active kinase is included as a negative control. Representative example is shown. **B.** Quantification of mean pixel intensities in ImageJ of phosphoserine Cx43 signal from kinase assays in (A). Data represent seven independent replicates (n=7), error bars are shown in SEM (standard error of the mean). Antibody background signal from the 'no MAPK' control was subtracted from each phosphoserine site signal. Because different phosphoserine antibodies were used, statistical significance was not assessed. **C.** Phosphoproteomics was used to measure the phosphorylation of GST-Cx43CT after incubation with active forms of the indicated MAPKs. Bars represent the relative abundance of phosphopeptides containing the indicated Cx43 phosphorylation sites. Values are normalized to the average abundance measured for GST-Cx43 across each sample condition. Data represent three replicates (n=3) per condition and statistically significant differences were determined by two-way ANOVA Tukey multiple comparisons test: $p < 0.05$(*), $p < 0.005$ (**), $p < 0.0005$ (***), and $p < 0.0001$ (****). Error bars are shown in SEM (standard error of the mean).

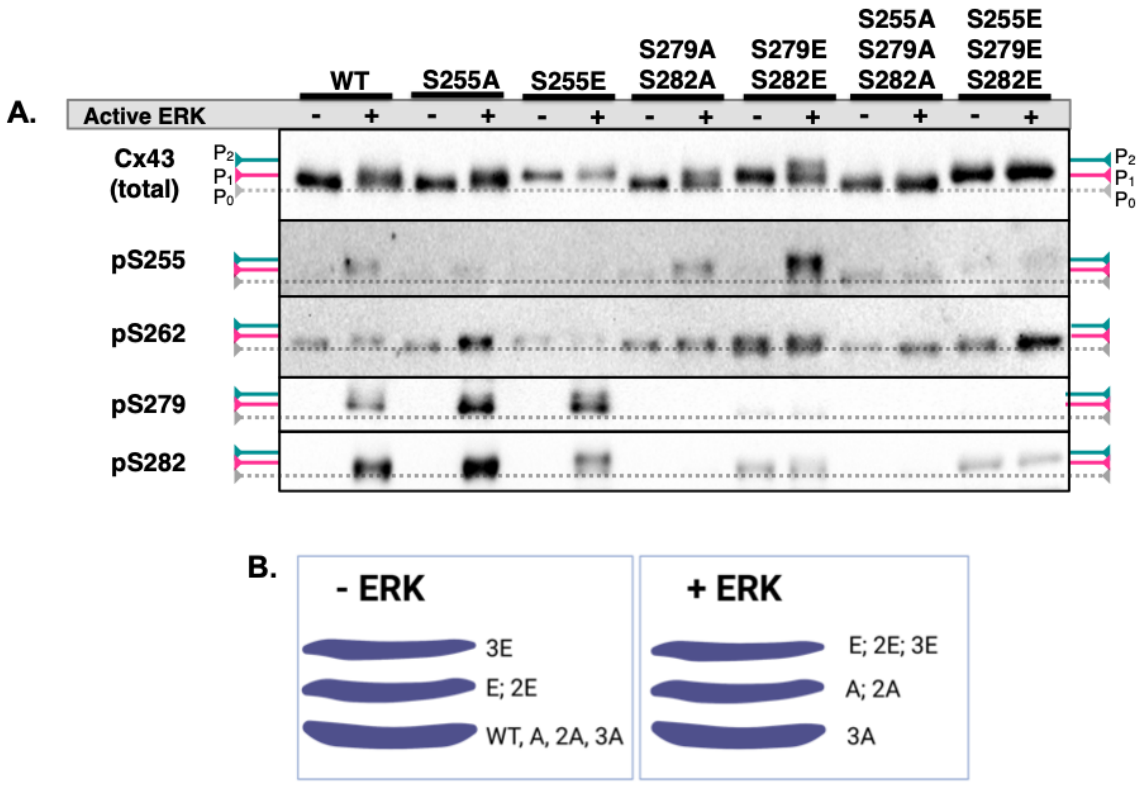

Figure 4: A. Electrophoretic mobility shift and western blotting analyses (total Cx43 and phosphoserine Cx43) of kinase assays between GST-Cx43CT (WT, phosphodead and phosphomimetic mutants) and active ERK (same reaction conditions as in Figs. 2-3). Positions of the unphosphorylated (P0) and phosphorylated (P1 and P2) are indicated. **B.** Summary of results from (A) under two conditions: without and with active ERK. When active ERK was not included, unphosphorylated WT and all alanine mutants were detected in the P0 form. S255E and S279E/S282E were detected in the P1 form, and the triple E mutant was in the P2 form. Upon treatment with active ERK, triple A mutant remained at P0, S255A and S279A/S282A shifted to P1, while S255E, S279E/S282E shifted to P2. The triple E mutant was detected in the P2 form, similarly to the 'no ERK' conditions. Figure B was generated in BioRender.com.

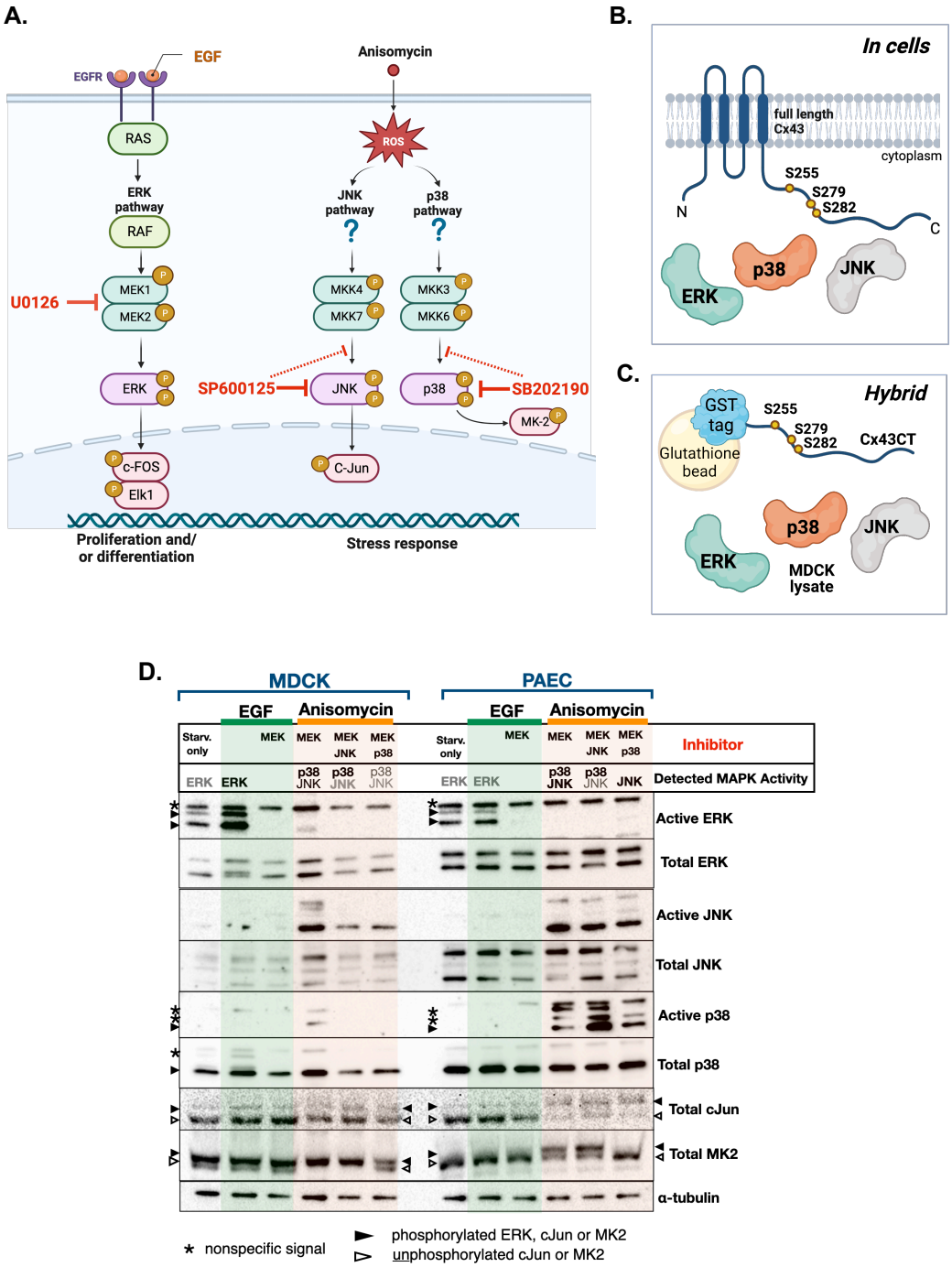

Figure 5: A. MAPK signaling pathways, their activators, and inhibitors relevant to this study. ERK signaling pathway is activated through the action of the EGF receptor (EGFR), Ras GTPase, MAP3K, Raf and MAP2Ks, MEK1 and MEK2. To inhibit ERK in cells, an inhibitor of MEK1 and MEK2 was used (U0126). Anisomycin was utilized as an activator for both JNK and p38 stress pathways, through activation of MEK4/7 and MEK3/6, respectively. To inhibit JNK or p38, SP600125 and SB202190 were used, respectively. These inhibitors are known to target JNK and p38 directly, preventing phosphorylation of downstream targets. However, there is a smaller effect on JNK and p38 phosphorylation by their respective MEKs. Changes in JNK and p38 activity were tested by monitoring phosphorylation of their targets (c-Jun and MK2) and levels of phosphorylated JNK and p38. **B.** Cellular approach to studying full length Cx43

phosphorylation by activated MAPKs. **C.** Hybrid approach in assessing phosphorylation of the immobilized, GST-Cx43CT (on glutathione beads) by activated MAPKs from mammalian cell lysates. **D.** Comparison of MAPK activation and inhibition in two mammalian cell lines relevant to this study: MDCK and PAECs. Cell samples activated with EGF are indicated in green, while those activated with Anisomycin are in orange (see materials and methods for detail). Levels of each MAPK and their activation were accessed using active and total antibodies. Activation/inhibition of JNK and p38 pathway was also assessed with total c-Jun and total MK2 antibodies, respectively. When phosphorylated, c-Jun and MK2 run slower on an SDS-PAGE (solid triangle). Detected MAPK activity for each condition is summarized through use of darker, bolded font (higher activity) vs. lighted, thinner font to indicate lower/basal activity. Figures A, B, and C were generated in BioRender.com.

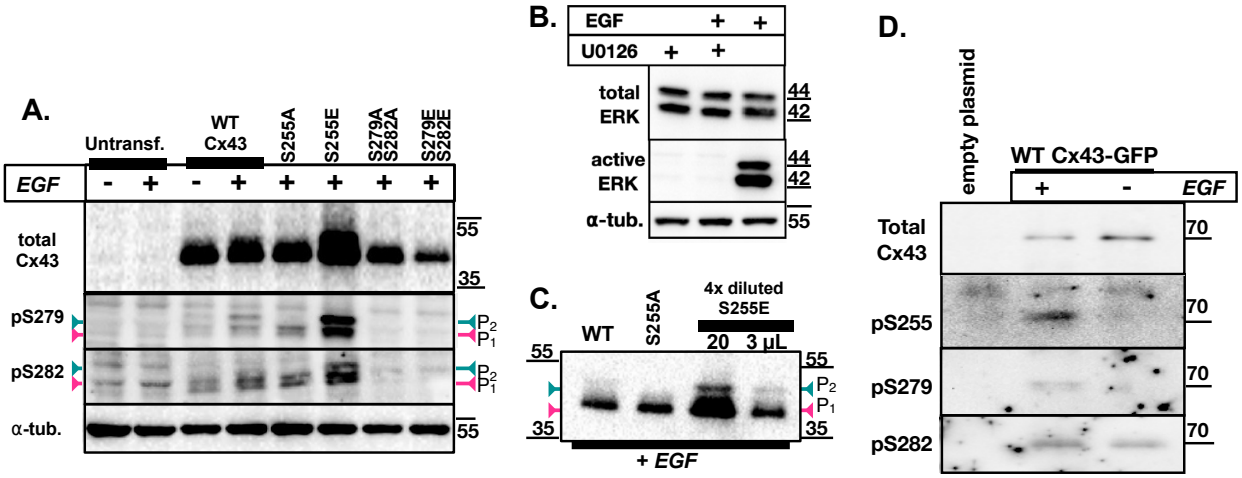

Figure 6: Phosphorylation of full length ectopically expressed Cx43 in MDCK cells. A. Western blotting analyses with total and phosphoserine (pS279 and pS282) Cx43 antibodies of full-length, ectopically expressed Cx43 WT and mutants in MDCK cells upon activation of the ERK signaling pathway with EGF. The signal from α-tubulin was used as a loading control. Higher electrophoretic forms are indicated (P1/P2). **B.** Activation status of ERK in cell lysates (used in A.) upon treatment with EGF with or without addition of the MEK1/2 inhibitor (U0126). **C.** Samples from A (+EGF) re-blotted for total Cx43 once the S255E mutant was diluted down four-fold to see higher electrophoretic bands (P1/P2). **D.** Immunoprecipitated WT Cx43-GFP from transfected MDCK cells treated with EGF and detected with total Cx43, pS255, pS279 and pS282 antibodies.

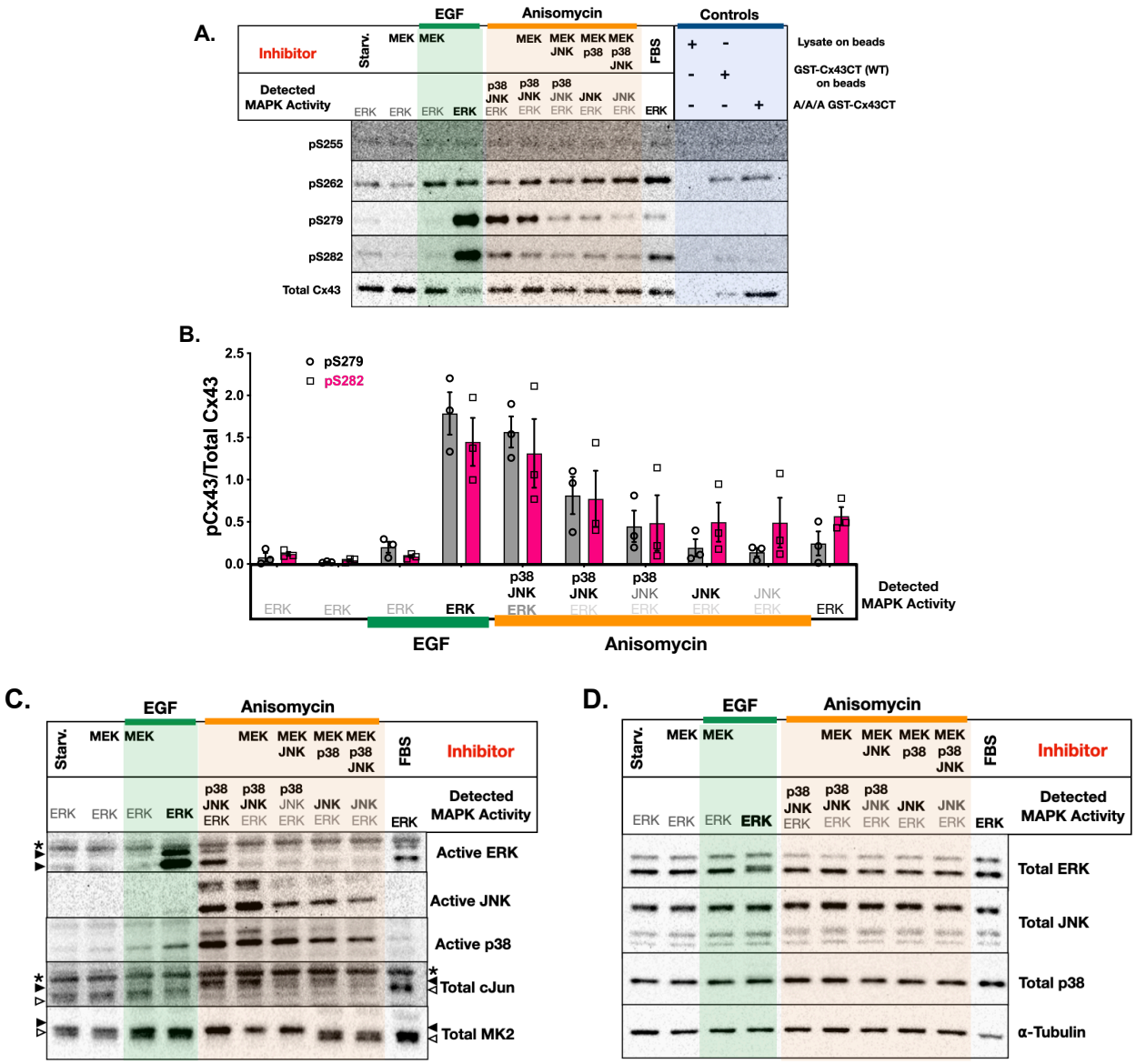

Figure 7: Hybrid kinase assays between purified and immobilized GST-Cx43CT proteins and MAPKs from MDCK lysates. Representative hybrid experiment is shown. **A.** Levels of total and phosphorylated (pS255, pS262, pS279, and pS282) Cx43 proteins on glutathione beads post treatment with activated or inhibited MAPKs. EGF treatments are marked in green, anisomycin treatments are in orange. Presence of inhibitors is indicated. 'Starv.' – treatment with lysate from cells treated with FBS-free starvation media; 'FBS' – treatment with lysate from cells grown in full media. Negative controls are in blue: cell lysate on glutathione beads, GST-Cx43WT on beads, or 2 µM GST-Cx43CT triple A mutant. Detected MAPK activity for each condition is summarized through use of darker, bolded font (higher activity) vs. lighted, thinner font to indicate lower/basal activity. **B.** Quantification of pS279 and pS282 signal from (A.) normalized to the total Cx43 signal. All conditions are in the same order as in (A.) Data are from three independent cell treatments and hybrid kinase assays (n=3). Error bars are in SEM (standard error of the mean). Statistical analyses were not performed due to use of three different antibodies in western blotting analyses. **C**. Western blots of active ERK, JNK, p38 and phosphorylated downstream targets of JNK (c-Jun) and p38 (MK2) in MDCK lysates used in the hybrid experiment. When phosphorylated, total

c-Jun and MK2 levels shift to a slower electrophoretic mobility (solid triangle). Unphosphorylated c-Jun and MK2 remain in the faster migrating form (open triangle). Non-specific signal is indicated with a star (*). **D.** Western blots of total MAPKs and alpha tubulin loading control in MDCK lysates used in the hybrid experiment.

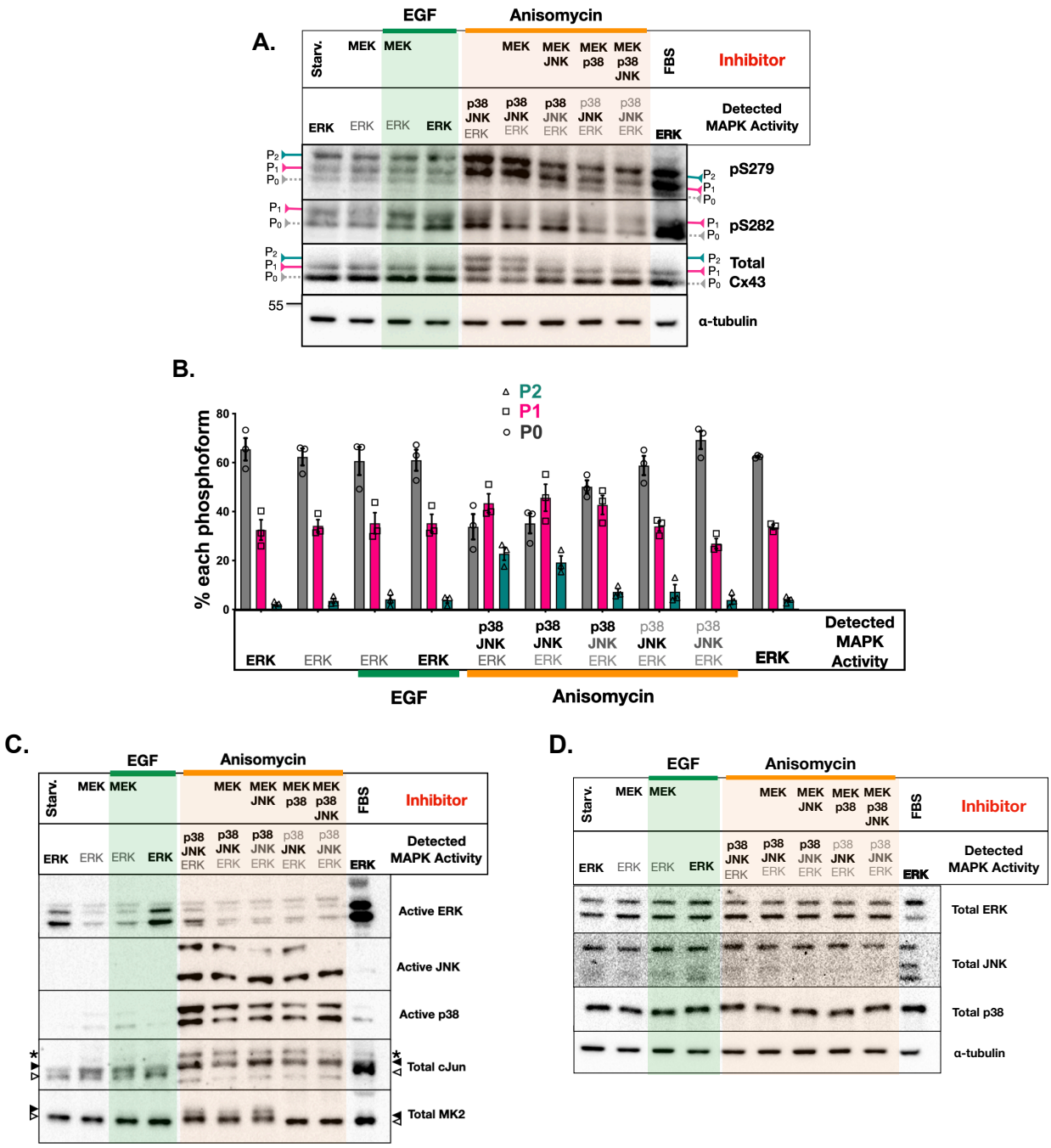

Figure 8: Phosphorylation of endogenous Cx43 in PAECs (representative experiment). **A.** Detection of phosphorylated (pS279 and pS282) and total Cx43 in PAEC lysates upon activation/inhibition of MAPKs. EGF treatments are marked in green, anisomycin treatments are in orange. Presence of inhibitors is indicated. 'Starv.' – lysates from cells treated with FBS-free starvation media; 'FBS' –lysate from cells grown in full media. Electrophoretic forms (P0, P1, and P2) are indicated. Detected MAPK activity for each condition is summarized through use of darker, bolded font (higher activity) vs. lighted, thinner font to indicate lower/basal activity. Western blot against α tubulin is included as a loading control. **B.** Quantification of the total Cx43 signal and the individual electrophoretic forms (P0, P1, and P2) within. Data are an average of three independent cellular experiments (n=3). Error bars are in SEM (standard error

of the mean). **C**. Western blots of active ERK, JNK, p38 and phosphorylated downstream targets of JNK (c-Jun) and p38 (MK2) in PAEC lysates shown in (A.) and quantified in (B.). When phosphorylated, total c-Jun and MK2 levels shift to a slower electrophoretic mobility (solid triangle). Unphosphorylated c-Jun and MK2 remain in the faster migrating form (open triangle). Non-specific signal is indicated with a star (*). **D.** Western blots of total MAPKs and α tubulin loading control (same as in A.) in PAEC lysates in the hybrid experiment in A-C.

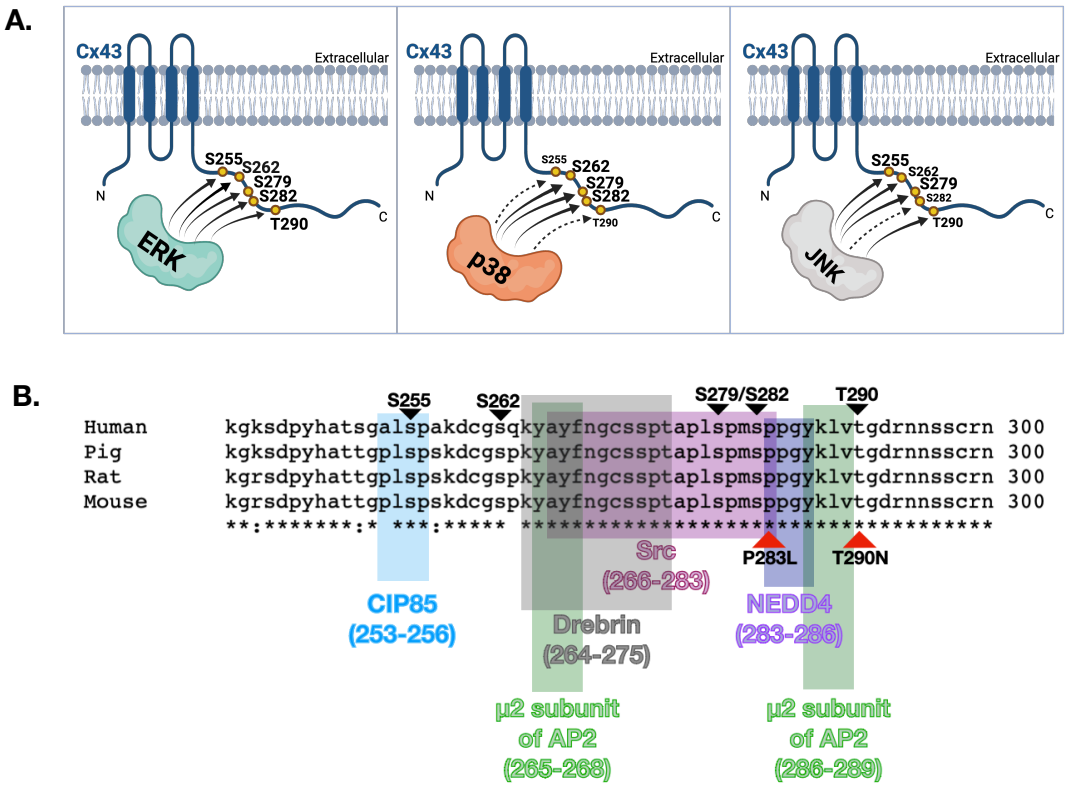

Figure 9: A. Summary of preference by ERK, p38 and JNK toward five phosphorylation sites on the CT of Cx43 (S255, S262, S279, S282, and T290). Dashed arrows and smaller font of site labels indicate lower phosphorylation preference. Figure generated in BioRender.com. **B.** Sequence alignment (generated using Clustal Omega Multiple Sequence Alignment tool) between human, porcine, rat, and mouse Cx43CT sequence (amino acids 241-300). S255, S262, S279, S282, and T250 are indicated with black triangles. Mutants observed in EKVP patients (P283L and T290N) are in red triangles (Ref. 61). Known binding sites of CIP85, Src, NEDD4, AP2, and Drebrin are highlighted (Ref. 66).