Tyk2 phosphorylates Cx43

Regulation of Connexin43 function and expression by Tyrosine kinase 2*

Hanjun Li, Gaelle Spagnol, Li Zheng, Kelly L. Stauch, and Paul L. Sorgen

University of Nebraska Medical Center, Omaha NE 68105 USA

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Abstract

Phosphorylation regulates many aspects of Cx43 function from assembly into functional channels, electrical and metabolic coupling at the plaque, as well as to modulate the interaction with other proteins. Here, we identified and characterized a novel tyrosine kinase that phosphorylates Cx43, Tyrosine kinase 2 (Tyk2). Activation of Tyk2 led to a decrease in Cx43 gap junction communication by increasing the turnover rate of Cx43 from the plasma membrane. Tyk2 directly phosphorylated Cx43 residues Y247 and Y265, and led to indirect phosphorylation on residues S279/282 (MAPK) and S368 (PKC). While this phosphorylation pattern is similar to what has been observed after Src activation, the response caused by Tyk2 occurred when Src was inactive in NRK cells. Knock down of Tyk2 at the permissive temperature (active v-Src) in LA-25 cells decreases Cx43 phosphorylation indicating that although activation of Tyk2 and v-Src leads to phosphorylation of the same Cx43CT residues, they are not identical in level at each site. Additionally, angiotensin II activation of Tyk2 increased the intracellular protein level of Cx43 via STAT3. These findings indicate that like Src, Tyk2 can also inhibit gap junction communication by phosphorylating Cx43.

Introduction

Gap junctions are integral membrane proteins that enable the direct cytoplasmic exchange of ions and low molecular weight metabolites (<1 kDa) between adjacent cells. They provide an intercellular pathway for the propagation and/or amplification of signal transduction cascades triggered by cytokines, growth factors, and other cell signaling molecules involved in growth regulation and development. Gap junctions are formed by the apposition of connexons from adjacent cells, where each connexon is formed by six connexin proteins. Connexins are tetraspanning transmembrane proteins with intracellular N- and C-termini (CT). There are 21 human isoforms and though there is significant sequence homology among connexins, their major divergence occurs in the cytoplasmic domains. Connexin43 (Cx43) is the most ubiquitously expressed connexin isoform and well-studied in terms of structure, function, and regulation (1).

Cx43 channels can be regulated by a variety of molecules and physiological conditions (e.g. Ca2+, pH, and intercellular voltage), including through phosphorylation (2,3). All stages of the Cx43 life cycle (i.e. trafficking, assembly/disassembly, degradation, and channel gating (3)), the regulation of electrical and metabolic coupling, as well as regulation of interactions with other proteins, involve phosphorylation. The Cx43CT is differentially
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Phosphorylated on at least 20 residues and significant progress has been achieved in characterizing the kinases involved (2,4-6). Unfortunately, an understanding of the mechanism(s) by which Cx43 phosphorylation alters channel function is lacking. Several problems contributing to this difficulty include the transient nature of a particular CT phosphorylation state, the ability of many kinases to phosphorylate more than one CT residue, the ability of various kinases to phosphorylate the CT at the same time, and the inability to precisely control which residue(s) are phosphorylated. Strategies employed to overcome these difficulties include the use of phospho-specific CT antibodies (7) and short CT phosphopeptides (8,9). Also well appreciated is the use of Asp (or Glu) substitutions as a mimetic for phosphorylation (10,11).

The negative charge of the phosphate could affect the permeability of ions through the pore, alter the structure of the transmembrane α-helices to influence pore size, or modify the binding affinities of molecular partners involved in Cx43 regulation. Notably, if phosphorylation modifies protein interactions to affect the kinetics of channel assembly/disassembly or degradation, cell-to-cell communication will also be altered. Evidence has emerged that a phosphate can directly block the Cx43CT interaction with ZO-1 (9) and tubulin (8) or enhance the interaction with Nedd4 (12,13). This information is critical because a particular cellular condition can be correlated with a specific Cx43 phosphorylation status to understand which proteins will and will not interact to affect regulation of Cx43. Numerous serine protein kinases have been identified to directly phosphorylate Cx43 (for review see (4)), however tyrosine protein kinases have been limited to the well-studied cases involving c- and v-Src-induced phosphorylation (14-18). In this study, we identified Cx43 as a novel substrate of Tyrosine kinase 2 (Tyk2).

Tyk2, a JAK family member, is a ubiquitously expressed non-receptor tyrosine kinase. Tyk2 associates with the intracellular domains from a wide range of cytokine and growth factor receptors via its N-terminal FERM and SH2-like domains (19-21). This leads to recruitment and phosphorylation of STATs (22), which translocate to the nucleus where they induce transcription of genes involved in diverse biological processes (e.g. cellular proliferation and differentiation, inflammation, and defense against infection (23,24)). Patients with non-functional Tyk2 display a number of immunodeficiency conditions and increased susceptibility to infections caused by defects in IFNα/β, IL-6, IL-10, IL-12 and IL-23 signaling (25). Tyk2 deficient mice display reduced responses to IFNα/β and IL-12 and a deficiency in STAT3 activation (26). The Tyk2/STAT3 axis has also been implicated in the induction of neuronal death in Alzheimer disease (27), as a biomarker for Crohn’s disease (28), and in controlling allergic asthma (29). Marrero et al. (30) first showed that angiotensin II (Ang II) induces Tyk2 activation in rat aortic smooth muscle cells; a finding verified in other studies (31-34). The renin-angiotensin system (RAS) is a key signaling pathway associated with the pathogenesis of cardiovascular disease (35,36). Increased Ang II levels are linked with an increased risk of ventricular arrhythmia, and treatment with angiotensin-converting enzyme (ACE) inhibitors has demonstrated survival benefits in congestive heart failure and myocardial infarction (37-39). A transgenic mouse model of RAS activation by overexpression of ACE restricted to the heart (ACE8/8) has normal ventricular structure; however, it exhibits cardiac oxidative stress, a high incidence of ventricular tachycardia and subsequent sudden cardiac death. With no significant change in Na+ current, these observations were associated with downregulation of Cx43 gap junction intercellular communication (40). Altered
cardiac Cx43 protein level and change in cellular localization were also observed in other models of RAS activation (hypertension, hypertrophy, mechanical stress; (41-44)). We provide evidence of a relationship between Tyk2 activation by Ang II and Tyk2 phosphorylation of Cx43. Tyk2 decreased gap junction stability at the plasma membrane, and through STAT3 activation increased the intracellular protein level of Cx43.

Results

Tyk2 directly interacts and phosphorylates the Cx43CT domain-Src is the only known tyrosine kinase to both directly phosphorylate the Cx43CT domain (pY247 and pY265) and affect GJIC (45,46). However, proteomic discovery-mode mass spectrometry (MS) data identified other tyrosine residues (Y267, Y286, Y301, and Y313) as potential phosphorylation targets (PhosphoSitePlus web site). Unfortunately, the kinases involved were not identified nor was the functional significance of phosphorylation at these sites. In this study, we focused on the JAK family of tyrosine kinases because of the potential direct link between Cx43 and RAS in the pathogenesis of cardiovascular disease (35,36,47,48). An in vitro tyrosine phosphorylation screen performed by Eurofins Scientific (KinaseProfiler) found that Tyk2 phosphorylated purified Cx43CT (236-382; Figure 1A). JAK1 and JAK2, also JAK family members, had significantly less ability to phosphorylate the Cx43CT domain (PhosphoSitePlus web site). Unfortunately, the kinases involved were not identified nor was the functional significance of phosphorylation at these sites. In this study, we focused on the JAK family of tyrosine kinases because of the potential direct link between Cx43 and RAS in the pathogenesis of cardiovascular disease (35,36,47,48). An in vitro tyrosine phosphorylation screen performed by Eurofins Scientific (KinaseProfiler) found that Tyk2 phosphorylated purified Cx43CT (236-382; Figure 1A). JAK1 and JAK2, also JAK family members, had significantly less ability to phosphorylate the Cx43CT domain (JAK3 was not tested because it is not expressed in the heart). To confirm the interaction between Cx43 and Tyk2, purified GST-tagged Cx43CT236-382 was immobilized on glutathione-Sepharose beads and lysate from MDA-MB-231 cells that express Tyk2 (but not Cx43) was used in a pull-down assay (Figure 1B). Tyk2 can be pulled-down by GST-Cx43CT but not GST in MDA-MB-231 cells. Src was used as a positive control.

Identifying the Cx43CT tyrosine residue(s) phosphorylated by Tyk2-Purified Cx43CT236-382 was incubated in vitro with active Tyk2 (Life Technologies) as described in (49,50) and phosphorylation was confirmed using an anti-phospho-tyrosine antibody (Figure 2B). After trypsin digestion, Tandem MS/MS identified phosphorylation at Y247, Y265, Y267, and Y313 (Figure 2A and Table 1). To confirm tyrosine phosphorylation can occur at residues other than Y247 and Y265, the in vitro kinase assay was performed using the Cx43CT236-382(Y247,265F) (2YF) construct and phosphorylation detected with an anti-phospho-tyrosine antibody (Figure 2B). The data indicate that while a majority of Tyk2 phosphorylation occurs on Y247 and Y265 (of note, Y247 and Y265 phospho-specific antibodies will be used in the next sections to further characterize these sites), other tyrosine sites (i.e., Y267 and Y313) can be phosphorylated by Tyk2 in vitro.

Tyk2 phosphorylates Cx43 residues Y247 and Y265 leading to decreased stability of the gap junction plaque in normal rat kidney epithelial (NRK) cells-To determine if Tyk2 phosphorylation of Cx43 occurs in cells, we initially tested in NRK cells if Cx43 (endogenously expressed) and Tyk2 (not expressed; transiently transfected with a constitutively active construct, Tyk2V678F) colocalize (Figure 3). The images suggest little-to-no colocalization in NRK cells that are over-expressing active Tyk2. However, in those same NRK cells, Cx43 is significantly decreased at the plasma membrane. The data suggests over-expressing active Tyk2 is leading to internalization of Cx43 from the plasma membrane.

Next, tyrosine phosphorylation levels were evaluated in the NRK cells using the only available Cx43 tyrosine phospho-specific antibodies pY247 and pY265 (Figure 4). Constitutively active Tyk2 increased both Y247 and Y265 phosphorylation levels as compared to control (non-transfected cells).
Importantly, the presence of active Tyk2 did not cause activation of Src. Since MAPK and PKC were reported to phosphorylate Cx43 to down regulate Cx43 GJIC in response to active Src in different cell lines (51,52), we investigated if Tyk2 also indirectly affects serine phosphorylation. Cx43 serine phosphorylation levels were evaluated using phospho-serine specific antibodies to pS279/282 (MAPK target) and pS368 (PKC target). pS279/282 and pS368 levels were up-regulated in the presence of active Tyk2. Finally, while the amount of Cx43 at the plasma membrane decreased (Figure 3A), active Tyk2 directly or indirectly caused an overall increase in the total amount of Cx43. Numerous studies have shown that differentially phosphorylated Cx43 results in multiple electrophoretic isoforms: a fast migrating isoform (P0), and multiple slower migrating isoforms (P1 and P2) (2,53,54). In particular, the P2 isoform has been found at the stage of gap junctional plaques. Consistently, Western blots (here and next sections) show an increase in the lower migrating P0 and P1 isoforms of Cx43 in the presence of active Tyk2.

Cx43 gap junction channels are localized in detergent-insoluble junctional plaques (55,56). To confirm the increased amount of Cx43 is not leading to an increase in the amount of Cx43 at junctional plaques, we assessed the level of Triton X-100 detergent solubility. The detergent extraction Western blot data show that active Tyk2 in NRK cells decreased the detergent-insoluble fraction of Cx43 (Figure 5A). Quantification of the data revealed that the control insoluble to insoluble + soluble ratio (I/(S+I)) was 67.1±6.0%. However, the number of assembled gap junctions significantly decreased to 19.5±0.9% in the presence of active Tyk2. The decreased amount of Cx43 in junctional plaques was corroborated by a biotinylation assay which detected a significant decrease in the amount of Cx43 at the plasma membrane (Figure 5B).

Interplay between Src and Tyk2 in the regulation of Cx43-Murakami et al. (1998) observed that v-Src transfected cells indirectly caused constitutive activation of Tyk2 and STAT3. Therefore, a modified NRK cell line containing a temperature sensitive v-Src (called LA-25), commonly used in the gap junction field to characterize Cx43 regulation by v-Src (51,57), was used to study the effect of Tyk2 on Cx43 tyrosine phosphorylation levels upon Src activation. v-Src is active in this cell line at the permissive temperature (35°C) and not at the non-permissive temperature (40°C). Of note, temperature alone does not affect gap junction communication in NRK or LA-25 cells (58) and Tyk2 is endogenously expressed in LA-25 cells. To begin with, Western blot data confirmed that active v-Src at 35°C resulted in activation of endogenous Tyk2 (Figure 6A). Immunostaining data then indicated that after 4 hrs of v-Src activation, endogenous Tyk2 colocalizes with Cx43 at the plasma membrane (Figure 6B). After 12 hrs, there is a decrease in the level of Cx43 and Tyk2 colocalization as well as the amount of Cx43 at the plasma membrane. These results are similar to the overexpression of active Tyk2 in the NRK cells (Figure 3). Next, Tyk2 siRNA was transfected into the LA-25 cells (35°C) to differentiate the effect of v-Src and Tyk2 on direct and indirect phosphorylation of Cx43 (Figure 7). Tyk2 siRNA significantly reduced the level of active Tyk2 (>75%). As important, the Tyk2 siRNA does not affect the level of active v-Src. Knockdown of Tyk2 in the presence of active v-Src significantly decreases the level of Y247 and Y265 phosphorylation as compared to control (scramble siRNA). Indirect serine phosphorylation was also affected by the knock-down of Tyk2. While pS279/282 phosphorylation was reduced by ~50%, phosphorylation of pS368 was reduced by almost ~75% in the absence of Tyk2. Finally, the knock down of Tyk2 led to a decrease in the total level of Cx43. Altogether, the data...
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indicate that similarities exist in the residues affected by Src or Tyk2, but differences exist in the level of phosphorylation at each site and the overall amount of protein.

**Tyk2 increases total Cx43 protein level through the STAT3 pathway**—In both the NRK and LA-25 cells, active Tyk2 caused an increase in the total protein level of Cx43. To determine the mechanism for this effect, cycloheximide was used to compare the turnover rate of Cx43 with or without Tyk2 (Figure 8). Western blot analysis of Cx43 from NRK cells revealed that active Tyk2 increased the Cx43 turnover. The data suggest the increased protein level of Cx43 is not a result of direct tyrosine or indirect serine phosphorylation. Next, RT-PCR from cell lysate of NRK cells transfected with active Tyk2 was used to determine if active Tyk2 indirectly affects the level of Cx43 mRNA. Using different numbers of RT-PCR cycles, the data revealed that active Tyk2 caused an increase in the level of Cx43 mRNA (Figure 9A). Actin was used as a loading control. Because Tyk2 activates the STAT signaling pathway, we tested if this is the mechanism leading to the increased mRNA and protein levels. Experiments performed using NRK cells incubated with the STAT1/3/5 inhibitor Nifuroxazide or the STAT3/5 inhibitor SH-4-54 eliminated the effect of Cx43 mRNA. Western blot analysis was used to investigate wild type (WT) Tyk2 transfected NRK cells treated with Ang II (Figure 11). The transfection of WT Tyk2 alone caused a basal level of activation, however the presence of Ang II significantly increased the amount of active Tyk2. The presence of Tyk2 or Ang II alone caused a significant increase in the level of Y247, Y265, S279/282 and S368 phosphorylation. While the phosphorylation level of Y247, S279/282, and S368 was the greatest in the presence of both Tyk2 and Ang II, only S279/282 phosphorylation was statistically significant compared to Tyk2 alone. Interestingly, while the presence of Tyk2 or Ang II alone caused an increase in the level of total Cx43 that was further increased in the company of both, the presence of Tyk2 but not Ang II caused an increase in the lower migrating P0 and P1 isoforms of Cx43.

**Phosphorylation of Cx43 residue Y247 prevents the binding of tubulin**—Previous studies have identified that Cx43 phosphorylation can inhibit the interaction with the ZO-1 PDZ-2 domain (pS373; (9)) or enhance the interaction with the Nedd4 WW2 domain (pS279/282; (12)). Additionally, a Cx43 phospho-peptide (pY247) was not able to interact with purified tubulin in vitro (8). Since pY247 level was significantly increased by Tyk2 in cells (Figures 4 and 11), a biotin-tagged Cx43 pY247 phosphorylated peptide or non-phosphorylated (K234-S255) version was used in a pull down assay to assess the interaction with β-tubulin in NRK cell lysate (Figure 12). The data indicate that Tyk2 phosphorylation of Y247 would inhibit the interaction with β-tubulin.

**Discussion**

Up until now, Src has been the only non-receptor tyrosine kinase to directly phosphorylate Cx43 leading to an inhibition of gap junctional intercellular communication and subsequent gap junction disassembly (59,60).
In this study, we identified another tyrosine kinase, Tyk2, which can directly phosphorylate Cx43 on residues Y247 and Y265, and lead to indirect phosphorylation on residues S368 and S279/282. While this phosphorylation pattern is similar to what has been observed after Src activation (51), importantly, the response caused by Tyk2 (e.g. overexpression of the constitutively active version) occurred when Src was inactive in NRK cells. This indicates that different signaling pathways can lead to the same cellular response to regulate Cx43 gap junctions. Unexpectedly, when v-Src was activated in the LA-25 cells at the permissive temperature, Tyk2 was activated as well. This observation is not unique to the LA-25 cells as constitutive activation of Tyk-2 was observed in a human gallbladder adenocarcinoma cell line transfected with v-Src (61). A significant decrease in the phosphorylation of Cx43 residues Y247, Y265, S279/282, and especially S368 (75% decrease) was observed in the LA-25 cells at the permissive temperature when the expression of Tyk2 was knocked down. The data suggest that although activation of Tyk2 and v-Src lead to phosphorylation of the same Cx43CT residues they are not identical in level at each site. Additionally, the data would suggest that activation of both Tyk2 and v-Src would work together to amplify the phosphorylation response on Cx43. Differences in the level of phosphorylation between Tyk2 and Src can be explained from the knowledge that the Tyk2 SH2 domain does not need a phosphate to interact with its substrate, combined with the absence of the SH3 domain (62).

A consistent observation from the immunofluorescence experiments was that prolonged activation of Tyk2 led to a near complete removal of Cx43 from the plasma membrane. Brikci-Nigassa et al. (8) showed that a Cx43CT phosphopeptide (pY247) does not interact with purified tubulin. We used a biotinylated Cx43CT control (K234-S255) and phosphopeptide (pY247) in a pull down assay using NRK cell lysate to show that phosphorylation inhibits the Cx43CT/β-tubulin interaction. Increased levels of pY247 by Tyk2 (or Src) would block the Cx43 interaction with tubulin (8). At the gap junction plaque, this may be a mechanism in the disassembly process; at the trans-Golgi network, this may re-route trafficking to the plasma membrane (e.g., lateral membrane vs. intercalated disc) or inhibit trafficking to the plasma membrane, leading to increased intracellular proteasomal and/or lysosomal degradation (63). Other mechanisms that would contribute to the lack of Cx43 at the plasma membrane include phosphorylation of residues S279/282 (MAPK) and S368 (PKC). Phosphorylation of S279/282 increases the binding affinity by two-fold for the WW2 domain from the ubiquitin ligase Nedd4 leading to Cx43 gap junction degradation (12,13,64). Activation of PKC can halt the assembly of new gap junctions and phosphorylation on S368 has been implicated in affecting gating and/or disassembly (65-67). Of note, although not investigated in this study, Src activation also leads to phosphorylation of Cx43 on S373 by Akt (68). The importance of phosphorylation on this site is that the interaction with ZO-1 is inhibited, transitioning Cx43 from the non-junctional plasma membrane into the gap junction plaque, and then through the degradation pathway(s). Less clear is the functional role of phosphorylation on Y265. One possibility is that the Src SH3 domain interaction with Cx43 residues A276-P280 positions the Src kinase domain to phosphorylate Y265, forming a SH2 binding domain (45). The next step would be for the Src SH2 binding domain to interact with pY265 and place the Src kinase domain within distance to phosphorylate Y247. This potential mechanism would explain why pY265 appears here and in other studies to label a larger population of plaques than pY247 (51). Another function of Y265 is to form part of a tyrosine-based sorting motif (YXXΦ, where Y is tyrosine, X is any amino acid, and Φ is a
bulky hydrophobic amino acid) which enables an interaction with the µ subunit of the heterotrimeric adaptor protein complex AP-2 to mediate clathrin-dependent internalization of Cx43. However, the ability of this tyrosine residue to bind AP2 is negatively influenced by tyrosine phosphorylation. For example, phosphorylation of GluN2B residue Y1472 within the YEKL tyrosine-based sorting signal motif by Fyn inhibits AP2 binding and internalization of GluN2B-containing NMDA-type glutamate receptors (69). Similarly, phosphorylation of GluN2A by Src inhibits endocytosis of GluN2A-containing NMDA-type glutamate receptors (70,71).

One difference observed in our (and others) studies performed in vitro as compared to in vivo is the effect of Ang II on Cx43 protein level. We identified that activation of Tyk2 by Ang II in vitro causes an increase in Cx43 protein level via the STAT3 pathway. Other in vitro examples of an increase in Cx43 protein level include exposing freshly isolated aortas of wild-type mice to Ang II for 4 hrs and the aorta smooth muscle cell line A7r5 to Ang II from 2 hrs to 48 hrs (maximum expression was observed at 4 hrs and was maintained to the last time point of 48 hrs) (72,73). Conversely, chronic exposure of Ang II in animal models shows a decrease in the protein level of Cx43. This was seen in the atria and ventricles of the transgenic mouse model of cardiac-restricted overexpression of ACE (ACE8/8) described above (40) and in the double transgenic rat which harbors the human renin and angiotensinogen genes (dTGR) (74). While the length of Ang II exposure (acute vs. chronic) and experimental conditions (in vitro and in vivo) are important factors when interpreting the protein level of Cx43, the combined data can be explained from the study of Kostin et al. (75). Cx43 expression was increased in the compensated hypertrophic stage from the left ventricles of pressure-overloaded human hearts with valvular aortic stenosis, but decreased and heterogeneously distributed throughout the ventricles in the decompensated stage.

We identified that the effect Tyk2 elicited on the Cx43 protein level in NRK cells was mediated through activation of STAT3. However, this increase did not correlate with increased gap junction intercellular communication as Cx43 was not localized to the plasma membrane. Evidence that STAT3 directly increases the transcriptional activity of Cx43 was provided by Ozog et al. (76). Three putative ciliary neurotrophic factor-response elements (binding sites for STAT3 dimers that contain base sequences TTCCN3-5AA) were identified in the promoter region of the mouse Cx43 gene (located at regions −1510, −1179, and −893) as essential for enhanced Cx43 expression. Interestingly, STAT3 activation also leads to down-regulation of E-cadherin (77). One mechanism for the decreased gap junction assembly may be explained by the observation that expression of E-cadherin is necessary to enable the assembly of Cx43 into gap junctions (78).

Conclusion-Phosphorylation of Cx43 at different sites controls gap junction assembly, size, gating, and turnover. Our understanding of these mechanisms has been aided by the identification of the kinases involved and the exact location of their phosphorylation on the Cx43CT domain. Through the use of MS and phosho-specific antibodies, patterns of phosphorylation have been correlated with different cellular conditions (51,79). For example, Solan and Lampe (6) put forth the notion that differential coordination of kinase activation and Cx43 phosphorylation controls the specific routes of disassembly. Their model describes the interplay between CK1, PKA, Akt, MAPK, PKC, and Src; each of which phosphorylates a distinct set of Cx43 residues. Here we identified that more than one kinase can directly phosphorylate the same Cx43CT residues. In addition, Tyk2 and Src alone caused a differential level of phosphorylation at each residue (directly or indirectly), with the
maximum level detected when both were active. These data suggest an additional layer of complexity that may need to be considered when trying to describe the full impact of phosphorylation on Cx43 regulation. Future studies need to address if Tyk2 and Src always work together to decrease Cx43 gap junction communication and if not, what are the cellular conditions where they work independently and does this differentially affect gap junction communication?

**Experimental Procedures**

**Cell culture**—Normal rat kidney (NRK) and LA-25 cells (NRK cells containing temperature sensitive v-Src) were generous gifts from Dr. Paul Lampe (Fred Hutchinson Cancer Research Center). The MDA-MB-231 cell line was from Dr. Melissa Teoh-Fitzgerald (University of Nebraska Medical Center). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific Inc.) and antibiotics in an atmosphere of humidified 5% CO2.

**Antibody and immunostaining**—The following antibodies were used in this study: Cx43 monoclonal antibodies against amino acids 360-382 (Cx43CT1 and Cx43IF1, described in (80,81)); Cx43 polyclonal antibody (C6219, Sigma-aldrich); rabbit anti-Cx43 phosphorylated Y247, rabbit anti-phosphorylated Y265, and rabbit anti-phosphorylated S279/282 (described in (51)) (all generous gifts from Dr. Paul Lampe, Fred Hutchinson Cancer Research Center); rabbit anti-Cx43 phosphorylated Y247, rabbit anti-phosphorylated Y265, and rabbit anti-phosphorylated S279/282 (described in (51)) (all generous gifts from Dr. Paul Lampe, Fred Hutchinson Cancer Research Center); rabbit anti-Cx43 phosphorylated S368 (Millipore); non-specific phospho-tyrosine antibody (P-Tyr-100, Cell Signaling); anti-Tyk2 (Santa Cruz), anti-active Tyk2 (phosphorylated 1054/1055); anti-tubulin β (Sigma); and antibodies against total and active Src (phosphorylated Y416, Millipore), STAT antibodies sampler kit (9939, Cell Signalling).

Cells were immunostained as described previously (82). Briefly, cells grown on cover slips to ~60% confluence were fixed with 3.7% para-formaldehyde for 10 min. Cells were blocked for 30 min at room temperature (RT) by MPS buffer (1x Phosphate-buffered saline (PBS), 1% Goat Serum) containing 0.2% Triton X-100 for permeabilization. Then, cells were immunostained with appropriate primary antibodies at RT for 1 h, followed by several PBS-0.5% Tween washes. Secondary antibodies (Alexa 594-conjugated goat anti-rabbit antibody and/or Alexa 488-conjugated goat anti-mouse antibody) were applied for 1 hr at RT. Images of immunostained cells were acquired with a Zeiss 510 Meta Confocal Laser Scanning Microscope using a 63×1.4 numerical aperture objective with appropriate filters. Colocalization was quantified using the Manders method in Image J (83).

**In vitro kinase assay**—Rat Cx43CT236-382 was expressed and purified as described previously (84). An in vitro kinase screen was performed by Eurofins Scientific using the purified protein. The catalytic domain of JAK1 (866-1154), JAK2 (808-1132), and Tyk2 (875-1187) were used to phosphorylate the Cx43CT. A positive control was used for each kinase (JAK1, GEEPLYWSFPK; JAK2, KTFCGTPYEAPEVRPRILSEEQEMF RDFDYADWC; Tyk2, GGMEIYFEFGKGGKK). Controls and Cx43CT were incubated with the tyrosine kinase and ATP [γ-33P] for 40 min at 30°C and then transferred to P30 Filtermat for substrate capture. Assays were performed in duplicate and acid blanks were used as a negative control. The level of phosphorylation for each sample was determined according to Merck Millipore’s radiometric assay.

**Mass spectrometry**—Purified rat Cx43CT236-382 (12.5 nmol) was incubated with 1.5 µg Tyk2 (amino acids 833-1187, ThermoFisher Scientific) and 500 µM ATP at 30°C for 15 hrs. In the control group, the volume of Tyk2 was substituted by reaction buffer (25 mM HEPES
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[pH 7.5], 10 mM MgCl₂, 0.5 mM EGTA, and 0.005% Brij-35). The reaction was stopped on ice, and 1 nmol of protein was ran on a SDS-PAGE gel and Western blotted using a phospho-tyrosine antibody. 10 nmol of protein was run on a SDS-PAGE gel and stained with Coomassie. The Cx43CT band was cut and sent to the Beth Israel Deaconess Medical Center Mass Spectrometry Facility for analysis (85).

**Glutathione S-transferase (GST) pull-down assay** - The GST pull-down assay was modified from (13). Briefly, purified GST-fusion Cx43CT and GST control proteins were bound to glutathione-Sepharose beads in buffer containing 25 mM Tris-HCl [pH=7.4], 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100 and Complete protease inhibitor (Roche). 4 mg of MDA-MB-231 or LA-25 cell lysate protein (in cell lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, PhosSTOP (Roche), and Complete protease inhibitor) were incubated with GST-fusion Cx43CT or the GST control for 6 hrs at 4°C on a rotating wheel. Beads were washed 5x with cell lysis buffer and bound proteins were eluted with SDS-PAGE sample buffer, and analyzed by Western blot.

**Co-immunoprecipitation (IP)** - NRK or LA-25 cells were lysed in complete lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Na-deoxycholate, 0.5% Triton-X 100, 5 mM NaF, and Complete protease inhibitor), maintained on ice for 30 min, precleared with protein G beads for 30 min at 4°C, and then spun at 12,000 rpm for 15 min. Total protein was assayed using the bicinchoninic acid (BCA) protein assay kit (Pierce). 2 mg lysate was incubated with 2 μg of Cx43 polyclonal or rabbit IgG (4 h at 4°C) and then incubated with 100 μl of protein G sepharose (GE Healthcare) (overnight at 4°C). The sepharose was washed 4x with cold lysis buffer and the co-IP product was analyzed by SDS-PAGE and Western blot.

When detecting tyrosine phosphorylation levels, 5 mM Na₃VO₄ was added in the blocking and primary antibody buffer in order to minimize the loss of phosphorylation. Western blot data were scanned and quantified using ImageJ software as described in (86).

**Tyk2 siRNA treatment** - Tyk2 siRNA (Invitrogen) was used to knockdown Tyk2 expression in LA-25 cells. Oligonucleotide from the non-target pool (Dharmacon) was used as negative control. Lipofectmine RNAi MAX (Invitrogen) was used to carry the oligonucleotide according to manufacturer’s protocol. Cells were treated with Tyk2 siRNA for 36 hrs and then incubated at 35°C for another 12 hrs. Protein levels were detected by Western blot.

**Cycloheximide treatment and Cx43 degradation** - Cells equally seeded on 60-mm dishes were grown for 24 hrs to 70-80% confluency before transfection. 24 hrs post-transfection, cells were treated with 100 μg/ml cycloheximide (Cell Signaling) for 1, 1.5, 2, 2.5, 3, 4 hrs to inhibit protein synthesis. Cells were then scraped off and lysed for Cx43 immunoblotting. β-actin was also blotted for quantification.

**Triton-X 100 solubility** - The Triton X-100 solubility assay was modified from the method described in (87). NRK cells grown in 10-cm dishes were rinsed 3x with PBS and scraped into 1 ml lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 100 mM NaCl, PhosSTOP, and Complete protease inhibitor). Then, cells were sonicated for 10 s on ice. Protein estimation was determined using the BCA method. 450 μl cell lysate samples were added to 50 μl of 10% SDS, which was saved as total protein, or to 10% Triton X-100 (final concentration of 1%) and incubated at 4°C for 30 min. Lysates were then separated into cytosolic (supernatant, soluble) and membrane (pellet, insoluble) fractions by centrifugation at 100,000 g for 1 h at 4°C. The pellets were dissolved in 500 μl dissolving buffer (70 mM Tris-HCl [pH 6.8], 8 M urea, 2.5% SDS, 0.1 M DTT, 5 mM NaF, 5 mM Na₃VO₄, and Complete protease
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inhibitor). Equal volume of total lysate, Triton X-100 soluble and insoluble portions, were loaded on a 10% SDS-PAGE and immunoblotted with the Cx43 polyclonal antibody.

Surface biotinylation assay-Surface biotinylation assay followed the method described in (88). Briefly, cells were seeded in 60-mm dishes in replicate and grown to 70-80% confluence. 24 hrs after transfection, cells were washed once with PBS and then 5 ml of ice cold DMEM plus HEPES buffer (pH = 7.4, final concentration 30 mM) was added for 10 min at 4°C. Cells were washed twice with cold PBS-plus (PBS with 1 mM CaCl2 and 1 mM MgCl2). The biotinylation reaction was performed at 4°C using freshly prepared EZ-LinkSulfo-NHS-SS Biotin reagent (Pierce) at 0.5 mg/ml in PBS-plus for 30 min. The reaction was terminated by adding PBS-plus containing 20 mM glycine. Cells were lysed in lysis buffer (25 mM Tris-HCl pH 7.5, 0.05% SDS, 20 mM Glycine, and Complete protease inhibitor) with homogenization by passing through a 25G needle. 500 µg of total protein were incubated with 100 µl of streptavidin-agarose beads (Pierce) on a rotator overnight at 4°C. Beads were then washed 6x and the streptavidin-bound biotinylated proteins were eluted and resolved by SDS-PAGE followed by Western blotting.

Biotin peptide pull down assay-Biotin-tagged phosphorylated (Y247) and non-phosphorylated Cx43CT234-255 peptides (LifeTein) were bound to streptavidin-agarose resin (GenScript) in PBS. NRK cells were lysed in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 0.25% Triton X-100, 2 mM PMSF, 10 mM EDTA, 50 mM NaF, 5 mM Na3VO4, and PhosSTOP and Complete tablets, pH 7.4) and total protein concentration was assessed using the BCA protein assay. A total of 2.5 mg of protein lysate was incubated with each of the resin-bound peptide, overnight at 4°C. Beads were washed 3x with PBS before analyzing the pull-down products by SDS-PAGE and Western blot.

Semi-quantitative reverse transcription (RT)-PCR-Semiquantitative RT-PCR was modified from the method described in (89). Briefly, NRK cells grown in 35-mm dishes were transfected with Tyk2V678F and pretreated with the inhibitor Nifuroxazide (50 µM, Millipore) or SH-4-54 (5 µM, Millipore). RNA extractions were carried out with the RNeasy mini kit (Quiagen), according to the manufacturer’s protocol. RT was performed with ProtoScript cDNA synthesis kit (New England Biolab). The following primers were used: Cx43 5’-AGCCCTCAAAGGAGTTCCAGTGCTCA-3’ and 5’-AGAGCAGCTGACAGCCACA-3’; β-actin 5’-CACCCGCGAGTACAACCTTCTT-3’ and 5’-CCCCATACCCACCACCCACCCAC-3’. Cx43 yielded an amplification product of 160 bp and β-actin of 207 bp. DreamTaq green PCR master mix (Thermo Fisher Scientific) was used for PCR amplification. To find out the exponential phase of amplification, different PCR cycles from 16 to 30 were tested. Each set of reaction included a no-sample negative control.

Statistical analysis-All data were analyzed by GraphPad Prism 5.0 and presented as the standard error of the mean (±S.E.M). Paired t-test was used to compare differences between the experimental group and control or the two parallel experimental groups. P<0.05 was considered to be statistically significant.

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

Author contributions
HL conducted most of the experiments, analyzed the results, and wrote parts of the manuscript. GS and LZ performed the biotin peptide pull down and cycloheximide assays, respectively. GS and KS helped HL analyze the mass spectrometry data and edit the manuscript. PLS conceived the idea for the project, analyzed the results, and wrote parts of the manuscript.
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Figure Legend

Figure 1: Phosphorylation of the Cx43CT domain by JAK tyrosine kinases. (A) An in vitro kinase assay was performed using the catalytic domain of Jak1, Jak2, and Tyk2 to phosphorylate purified Cx43CT. The amount of Cx43CT phosphorylation was compared with a positive control peptide for each kinase (100% signal) (**P<0.01). (B) Purified GST (26 kDa) or GST-Cx43CT (42 kDa) bound on glutathione-agarose beads were incubated with or without MDA-MB-231 cell lysate (top) and the pull-downed product was analyzed by Western blot using an anti-Tyk2 antibody. GST-Cx43CT pull down of Src from LA-25 cell lysate was used as positive control (bottom).

Figure 2: Identification of the Cx43CT tyrosine residues phosphorylated by Tyk2. (A) Sequence of the Cx43CT domain. Highlighted (bold and underlined) are the tyrosine residues identified from mass spectrometry to be phosphorylated from an in vitro kinase assay using the Tyk2 catalytic domain to phosphorylate the Cx43CT. (B) The same in vitro kinase assay in (A) was performed using wild type Cx43CT_236-382 or a Y247,265F (2YF) mutant as substrate and phosphorylation was detected by Western blot using a general anti-phospho-tyrosine antibody. The control (Ctrl) group did not contain Tyk2. The phospho-tyrosine level was quantified using ImageJ software (n=3, **P<0.01).

Figure 3: Effect of Tyk2 on the cellular localization of Cx43. (A) Cellular localization of endogenous Cx43 and constitutively active Tyk2 (Tyk2_V678F) in NRK cells detected by immunofluorescence (green, Cx43; blue, DAPI-stained DNA; red, active Tyk2). Scale bar is 20 μm. (B) Quantification of Cx43 expression level at the plasma membrane. Cx43 pixel intensity of 204 cell pairs containing p-Tyk2 was normalized to the Cx43 pixel intensity of 204 cell pairs without p-Tyk2 (Ctrl) by ImageJ software. Cell pairs with p-Tyk2 or without p-Tyk2 were from the same images. The data are representative of three independent experiments (***P<0.001).

Figure 4: Phosphorylation of Cx43 by Tyk2 in NRK cells. Western blot of active Tyk2 (p-Tyk2), total Tyk2, active Src (p-Src), total Src, Cx43 pY247, pY265, pS279/282, pS368 and total Cx43 from NRK cells without (Ctrl) or with transfection of Tyk2_V678F. The Cx43 mobility shifts (P0, P1 and P2) are labeled. Relative protein levels were quantified by analyzing scanned blots using ImageJ software, with normalization of protein expression to the control lane (value set arbitrarily as 100%). The data are representative of three independent experiments (*P<0.05, ***P<0.001).
Figure 5: Effect of Tyk2 on the plasma membrane localization of Cx43 in NRK cells. (A) Triton X-100 solubility assay: Equal amounts of total protein fraction (T), Triton X-100 soluble fraction (S) and the insoluble fraction (I) were run on SDS-PAGE and blotted with anti-Cx43 antibody. Protein levels were quantified to determine the insoluble to insoluble + soluble ratio \([I/(I+S)]\) (*\(P<0.05\)). (B) Biotinylation assay: NRK cells with or without Tyk2\textsubscript{V678F} transfection were cell-surface biotinylated. Biotinylated proteins were pulled down by immobilization on streptavidin agarose beads, and immunoblotted for Cx43. Protein levels of biotinylated Cx43 were quantified (*\(P<0.05\)). Input shows active Tyk2 and Cx43 protein in the cell lysate.

Figure 6: Colocalization of endogenous Tyk2 and Cx43 in LA-25 cells. (A) Western blot of active Src (p-Src), total Src, active Tyk2 (p-Tyk2), total Tyk2, and Cx43 in LA-25 cells at 40°C and 35°C. (B) Cellular localization of endogenous Cx43 and active Tyk2 in LA-25 cells at 40°C or 35°C was visualized by using immunofluorescence (green, Cx43; blue, DAPI-stained DNA; red, Tyk2). Scale bars is 20 µm. Colocalization of Cx43 and p-Tyk2 was analyzed based on 12 images from three independent experiments. Manders method was used to measure green signal (Cx43) coincident with red signal (active Tyk2) over the total intensity of green signal (**\(P<0.01\)).

Figure 7: Effect of Tyk2 knockdown on the phosphorylation level of Cx43 in LA-25 cells. Western blot of active Tyk2 (p-Tyk2), total Tyk2, active Src (p-Src), total Src, Cx43 pY247, pY265, pS279/282, pS368 and total Cx43 from LA-25 cells at 40°C or 35°C treated with scrambled or Tyk2 siRNA for 12 h. Relative protein levels were quantified using ImageJ software, and normalized to the expression level in the scramble-RNA-treated sample at 35 °C (*\(P<0.05\), **\(P<0.01\)).

Figure 8: Effect of Tyk2 on the turnover rate of Cx43 in NRK cells. Control (A) or transfected (B) NRK cells with the constitutively active Tyk2 (Tyk2\textsubscript{V678F}) construct were treated with 100 µg/ml cycloheximide for different durations prior to lysis. Total Cx43 protein was immunoblotted. (C) Protein level of three independent experiments quantified using ImageJ software and normalized to protein level at 0 hr.

Figure 9: Effect of STAT3 activation by Tyk2 on Cx43 mRNA level. (A) RT-PCR shows Cx43 mRNA level with or without transfection of active Tyk2 (Tyk2\textsubscript{V678F}). An appropriate number of cycles was determined by testing different number of cycles (16, 21, 24, 27, 30) for Cx43 amplification (top panel). Cx43 intensity increased up to 24 cycles where a plateau was reached, thus 24 cycles was used to run
Tyk2 phosphorylates Cx43

semi-quantification RT-PCR. Cx43 and β-actin were amplified for 24 cycles and ran on 2% agarose gel (right panel). (B) NRK cells with or without active Tyk2 (Tyk2V678F) transfection for 8 hrs were treated with the STAT3/5 inhibitor SH-4-54 (5 µM) or STAT1/3/5 inhibitor Nifuroxazide (50 µM) for another 16 hrs. Cx43 and β-actin were amplified for 24 cycles and ran on 2% agarose gel. Cx43 and β-actin mRNA levels were quantified by densitometry from three independent experiments (*P<0.05, **P<0.01).

Figure 10: Effect of STAT3 activation by Tyk2 on Cx43 protein level. (A) Western blot of active Tyk2 (p-Tyk2), active STAT3 (p-STAT3), total STAT3, and Cx43 in NRK cells transfected with or without active Tyk2 (Tyk2V678F) and treated with and without SH-4-54 or Nifuroxazide. Cx43 protein level was quantified using ImageJ software (*P<0.05, **P<0.01). (B) Western blot of STAT1, STAT3, and STAT5 from NRK cell lysate. Cell extracts from HeLa cells treated with IFN-α (100ng/ml) for 5 min (9173, Cell Signalling) were used as positive control for blotting STAT1 and STAT5. Jurkat cell lysate was used as positive control for blotting STAT3.

Figure 11: Effect of angiotensin II (Ang II) activation of Tyk2 on the phosphorylation level of Cx43. Western blot of active Tyk2 (p-Tyk2), total Tyk2, active Src (p-Src), total Src, Cx43 pY265, pY247, pS279/282, pS368, total Cx43 and the Angiotensin II type 1 receptor (AT1R) from NRK cells with or without transfected wild-type (WT) Tyk2 and treated with or without Ang II (10^-7 M). Protein phosphorylation level were quantified using ImageJ software (*P<0.05).

Figure 12: Effect of Cx43 Y247 phosphorylation on the interaction with β-tubulin. Western blot of β-tubulin from a biotin pull-down assay using a biotinylated-phospho-peptide Cx43CT234-255 (pY247) or non-phospho-peptide (Y247nop) bound to streptavidin beads and incubated with NRK cell lysate. Biotin bound to streptavidin agarose beads was used as negative control. Protein level were quantified using ImageJ and normalized by input (1:20 loaded compared to pull-down groups) from three independent experiments.
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Table 1. Phospho-tyrosine containing peptides identified from mass spectrometry of the Cx43CT domain phosphorylated *in vitro* by Tyk2.

| Peptide sequence | Start-end | Average Mascot Delta Ion Score | *Number of phospho-peptides* | Actual peptide mass | Calculated +1H peptide mass | Phosphorylation site localization |
|------------------|-----------|-------------------------------|-------------------------------|---------------------|-----------------------------|---------------------------------|
| GRSDPyHATTGPLSPSK | 242-258   | 15.35                         | 4                             | 1849.84             | 1850.84                     | Y247                            |
| SDPyHATTGPLSPSK  | 244-258   | 18.5                          | 6                             | 1636.72             | 1637.72                     | Y247                            |
| GRSDPyHATTGPLSPSKDc(Carbamidomethyl)GSPK | 242-264 | 9.37                          | 2                             | 2667.04             | 2668.05                      | Y247                            |
| QASEQNWANySAEQNR | 304-319   | 13.7                          | 2                             | 1974.79             | 1975.79                      | Y313                            |
| yAYFNGc(Carbamidomethyl)SSPTAPLSPm(Oxidation)SPPGYK | 265-287 | 7.14                          | 1                             | 2588.07             | 2588.09                      | Y265                            |
| yAyFNGc(Carbamidomethyl)SSPTAPLSPm(Oxidation)SPPGYK | 265-287 | 13.8                          | 1                             | 2667.05             | 2668.05                      | Y265, Y267                       |

* Peptides with Mascot Delta Ion Score >7 were used to obtain the average Mascot Delta Ion Score according to (90).
Tyk2 phosphorylates Cx43

Figure 1

A

% of Control Signal

**

(\%)

B

- cell lysate  + cell lysate

MDA-MB-231

(kDa)

Tyk2 150
GST 100

Src 72
GST 37

LA-25

GST 37

-GST-Cx43CT

-GST

-GST-Cx43CT

-GST
Figure 2

A

\[ \text{Tyk2 phosphorylates Cx43} \]

B

|       | Ctrl       | +Tyk2     |
|-------|------------|-----------|
| WT    | 75-        | 50-       |
| 2YF   | 37-        | 25-       |
|       | 20-        | 15-       |

**pTyk2**

|       | pCx43CT    |
|-------|------------|
| 2YF   | = Cx43CT   |

\[ \text{WT} \quad \text{2YF} \]

\[ \text{Y}_{247,265}^{247,265F} \]

\[ \text{E}_{236}^{247} \text{HATTGPLPSKDCGSPK}_{265}^{267} \text{FNGCSSPTAPLS} \]

\[ \text{PPG}_{266}^{266} \text{KLVTGDRNNSSCRNY}_{301}^{301} \text{NKQASEQN} \]

\[ \text{W}_{311}^{311} \text{SAEQNRMQAGSTISNSHA} \]

\[ \text{QPFDPDDNQNAKKVAAGHELQPLAI} \text{VDQRPS} \]

\[ \text{RASSRASSRPRPD} \text{DEL}_1^{382} \]
Figure 3

A

Cx43  pTyk2  Merge

B

![Bar chart showing Cx43 pixel intensity comparison between Ctrl and pTyk2 conditions.](chart.png)
Tyk2 phosphorylates Cx43
Figure 5

Tyk2 phosphorylates Cx43

A

B
Figure 6

Tyk2 phosphorylates Cx43
Figure 7

Tyk2 phosphorylates Cx43
Figure 8

A

|        | 0  | 1.5 | 2   | 2.5 | 3   | 4   |
|--------|----|-----|-----|-----|-----|-----|
| Ctrl   |    |     |     |     |     |     |
| Cx43   |    |     |     |     |     |     |
| β-actin|    |     |     |     |     |     |

B

|        | 0  | 1.5 | 2   | 2.5 | 3   | 4   |
|--------|----|-----|-----|-----|-----|-----|
| +Tyk2_cys73F |    |     |     |     |     |     |
| Cx43   |    |     |     |     |     |     |
| β-actin|    |     |     |     |     |     |

C

|     | 0  | 1.5 | 2   | 2.5 | 3   | 4   | 6   | 8   | 10  |
|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ctrl|    |     |     |     |     |     |     |     |     |
| Tyk2_cys73F |    |     |     |     |     |     |     |     |     |
Figure 9

Tyk2 phosphorylates Cx43
Figure 10

A

| STAT inhibitor | TYK2 | SH-4-54 | Nifuroxazide |
|---------------|------|---------|--------------|
| Tyk2<sub>Y705</sub> | -    | -       | -            |
| p-Tyk2        | -    | +       | +            |
| p-STAT3 (Y705) | -    | +       | +            |
| t-STAT3       | -    | +       | +            |
| Cx43          | -    | +       | +            |
| β-actin       | -    | +       | +            |

B

| Cell extracts | NRK (kDa) | Jurkat (kDa) | Stat5 control |
|---------------|----------|--------------|---------------|
| STAT1         | -90      | -70          | -90           |
| β-actin       | -50      | -50          | -50           |
| STAT3         | -70      | -70          | -70           |
| β-actin       | -50      | -50          | -50           |
| Stat5 control | NRK (kDa)| NRK (kDa)    | NRK (kDa)     |
| STAT5         | -90      | -70          | -90           |
| β-actin       | -50      | -50          | -50           |
| β-actin       | -37      | -37          | -37           |
Tyk2 phosphorylates Cx43
Tyk2 phosphorylates Cx43

Figure 12
Regulation of Connexin43 function and expression by Tyrosine kinase 2
Hanjun Li, Gaelle Spagnol, Li Zheng, Kelly L. Stauch and Paul L. Sorgen

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