

Connexin46 Modulates Cancer Cell Migration Through a Channel- Independent Mechanism

Williams E. Rosales

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

Leonor D.R. Lii-Troncoso

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

Viviana P. Orellana

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

Rodrigo Alarcón

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

Isidora M. León-Fuentes

Clínica Alemana Universidad del Desarrollo

Sofía T. Paredes

Clínica Alemana Universidad del Desarrollo

Catalina Bejer

Clínica Alemana Universidad del Desarrollo

Javiera Baeza

Universidad de Talca

Wendy Gonzalez

Universidad de Talca

Iván E. Alfaro

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

Mauricio A. Retamal

mretamal@udd.cl

Instituto de Ciencias e Innovación en Medicina (ICIM), Clínica Alemana Universidad del Desarrollo

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Abstract

Cell migration is a central process in cancer progression and metastatic dissemination. While connexins (Cxs) classically mediate cell–cell communication through the formation of gap junction channels and hemichannels, accumulating evidence supports additional channel-independent roles in tumor cell signaling. Cx46 is a member of the Cx family with a highly restricted expression pattern under physiological conditions, thus, in humans it has been reported primarily in the eye lens. Interestingly, Cx46 is aberrantly expressed in several types of cancer cells, where it has been implicated in the acquisition of mesenchymal traits and cancer stem cell–like properties. In this study, we identify a previously unrecognized signaling function of Cx46 in the regulation of cancer cell migration. We show that Cx46 expression suppresses migration in HeLa cells. This anti-migratory effect is mediated by the C-terminal domain of Cx46, as deletion of this region abolishes the inhibitory phenotype. Furthermore, we demonstrate that Cx46 directly interacts with Src kinase, promoting Src localization at the plasma membrane and inducing concomitant changes in the intracellular distribution of focal adhesion kinase (FAK), consistent with altered cell adhesion dynamics. Notably, increased Cx46 expression in MCF-7 a breast cancer cell line and SK-Mel-2 melanoma cells reduces cell migration. Together, these findings uncover a novel Cx46–Src signaling axis that have the potential to induce focal adhesion remodeling and cell motility, providing new mechanistic insight into how Cxs regulate cancer cell migration independently of their canonical channel functions.

Highlights

- Cx46 decrease cell migration in HeLa, MCF-7 and Sk-Mel-2 cell lines.
- The C-terminal domain of Cx46 and Src kinase physical interact in HeLa cells.
- The presence of Cx46 induce the internalization of FAK.

1. Introduction

Cell migration is a fundamental biological process involved in tissue development, wound healing, and immune responses [1]. In cancer, however, dysregulated migration contributes to invasion, metastasis, and poor clinical outcomes [2, 3]. Therefore, understanding the molecular mechanisms that drive or suppress cell migration is essential for identifying new therapeutic targets and improving patient survival [3, 4]. Among the proteins implicated in the regulation of cell migration in both physiological and pathological conditions, are connexins (Cxs) [4, 5], which is a family of transmembrane proteins known to form gap junction channels (GJs), which mediate direct intercellular communication [6]. GJs are formed by the docking of two hemichannels [7]. When present in non-junctional regions of the plasma membrane, hemichannels allow the exchange of ions and signaling molecules, such as ATP and glutamate, between the cytoplasm and the extracellular environment [8]. Moreover, it is well established that Cxs exert important biological functions not only through their canonical-associated channel activity but also via non-canonical, channel-independent mechanisms, most of which involve protein–protein

interactions (PPIs) [9, 10]. The mechanisms described by which Cxs regulate cell migration include hemichannel-mediated release of ATP, cytoskeletal architecture modulation and/or promoting epithelial-to-mesenchymal transition, which are directly implicated in cell movement [11–14].

Src kinase is a non-receptor tyrosine kinase that belongs to the Src family kinases, originally identified as the cellular homolog of the Rous sarcoma virus oncogene. It is localized predominantly at the inner surface of the plasma membrane, where it functions as a central signaling hub [15–17]. Through phosphorylation of multiple substrates, Src regulates a broad range of cellular processes, including proliferation, survival, metabolism changes, adhesion, and migration [18, 19]. In the context of cell migration, Src plays a pivotal role by coordinating cytoskeletal dynamics and focal adhesion turnover [16]. Thus for example, activated Src phosphorylates focal adhesion kinase (FAK) and other adaptor proteins, promoting the disassembly and reassembly of adhesion complexes required for directional movement [16, 20]. It also regulates actin remodeling through downstream pathways such as Rho GTPases and PI3K/AKT, thereby controlling lamellipodia and filopodia formation [21, 22]. Aberrant Src activation has been frequently observed in diverse cancers and is strongly associated with enhanced migratory and invasive capacities, contributing to metastasis [23, 24].

Cx46 in humans it is expressed predominantly in the eye lens [25]. For this reason, it has been traditionally studied in the context of lens physiology and cataract formation [26]. However, over the last decades, an increasing body of evidence has revealed that Cx46 may also act as a tumor-promoting factor [27]. Elevated Cx46 expression has been reported in several types of cancer and has been associated with improved survival of tumor cells under hypoxic conditions [28], and the promotion of cancer stem cell (CSC) properties [27, 29] as well as the expression of endothelial-to-mesenchymal transition (EMT) biomarkers [30]. Despite these associations, the precise molecular mechanisms through which Cx46 enhances cell survival, supports CSC maintenance, and facilitates EMT remain poorly defined. It has been proposed that both GJCs and hemichannels formed by Cx46 could influence intracellular signaling by regulating the exchange of metabolites, ions, and signaling molecules with the extracellular environment [31, 32]. On the other hand, and as other Cxs types [33–35], Cx46 may modulate the activity of oncogenic signaling pathways, such as Src or Akt, thereby modulating cytoskeletal organization, adhesion, and cell fate decisions. A better understanding of these mechanisms could be critical for clarifying the contribution of Cx46 to cancer biology and for evaluating its potential as a therapeutic target.

In this work, we aimed to shed light on the mechanisms by which Cx46 modulates cancer cell migration. To this end, we transfected HeLa cells (which exhibit no- or very low endogenous Cx expression) with Cx46 fused to enhanced green fluorescent protein (EGFP) in its C-terminus (Cx46EGFP), as well as with different mutants: one lacking the C-terminal domain (Δ CTEGFP), one containing only the C-terminal domain (CTEGFP). As a transfection control, we transfected HeLa cells only with EGFP. Our main finding is that the C-terminal domain of Cx46 interacts with Src kinase, interaction that correlates with FAK relocalization and inhibition of cell migration. Notably, in MCF-7 (human breast cancer–derived) and SK-Mel-2 (human melanoma–derived) cells, Cx46 expression levels were negatively correlated with their

migratory capacity. These findings suggest that the impact of Cx46 on cancer cell migration may represent a broader phenomenon and could contribute to the development of future strategies aimed at limiting cancer metastasis.

2. Material and Methods

2.1 Cell culture. HeLa cells were cultured in DMEM, MCF-7 in DMEM/F12 and SkMel-2 in RPMI (Thermo Fisher Scientific, Waltham, MA, USA), in each case culture media was supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was replaced every three days. Prior to all experiments, cells were treated for two weeks with Biomyc (Sartorius, Göttingen, Germany) to eliminate mycoplasma contamination, followed by a one-week recovery period without the antibiotic before experiments were carried out.

2.2 Cell Transfection: The following vectors: pRP[Exp]-Bsd-CMV>EGFP (coding for EGFP as a transfection control), pRP[Exp]-Bsd-CMV>hGJA3/EGFP, (coding for Cx46 plus EGFP attached in its C-terminal; Cx46EGFP), pRP[Exp]-Bsd-CMV>hGJA3(aa223-435)/EGFP (coding for the Cx46 C-terminal plus EGFP; CTEGFP), pRP[Exp]-Bsd-CMV>hGJA3(aa1-223)/EGFP (coding for Cx46 without its C-terminal plus EGFP; DCTEGFP), and pRP[Exp]-Bsd-CMV>EGFP/hGJA3 (coding for Cx46 plus EGFP attached to its N-terminal; EGFP-Cx46) were used to transfect HeLa and MCF-7 cells and were purchased from VectorBuilder (Chicago, IL, USA). A schematic representation of these constructs (Figure 1A) and a representative of their molecular weights in a WB analysis (Figure 1B). Cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer instructions. Stable clones were selected using blasticidin (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 10 µg/ml for 1 week. The Cx46 expression was subsequently evaluated by Western blot analysis.

In the case of SK-Mel-2 cells, they were seeded in 6-well plates and cultured in complete RPMI medium. Upon reaching approximately 50% confluence, the medium was replaced with RPMI without antibiotics. Cells were then transduced overnight with lentiviral particles encoding either shRNA targeting Cx46 (Dallas, TX, USA, #SC-60431-V) or scrambled control shRNA (Dallas, TX, USA, #SC-108080), diluted in culture medium containing polybrene (Dallas, TX, USA, #SC-134220) at a final concentration of 5 µg/mL. Following transduction, the culture medium was replaced with complete RPMI. On the next day, cells were reseeded at a ratio of 1:3 to 1:5 to allow expansion. Five days after transduction, selection was initiated using puromycin (10 µg/mL) to isolate resistant colonies. The Cx46 expression was subsequently evaluated by Western blot analysis.

2.3 Western blot. Cells (1×10^6 cells) were seeded in a 60mm plate petri, then cultured until 60-70% confluence, then were harvested, and sonicated in 500 µl PBS containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA #1861280). Whole-cell homogenates were

resuspended in NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific, Waltham, MA, USA #NP0008) and separated on NuPAGE 10% BisTris gels (Thermo Fisher Scientific, Waltham, MA, USA #NP0315BOX). Proteins were electrotransferred onto a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA, USA, #PB3310) using a Dry Power Blotter XL transfer system (Thermo Fisher Scientific, Waltham, MA, USA) and blocked with 5% nonfat milk diluted in TBS containing 0.05% Tween-20 (TTBS). The nitrocellulose membrane was then incubated overnight at 4 °C with primary antibodies diluted in 5% nonfat milk in TBST. The following day, membranes were washed at least four times with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies. Finally, chemiluminescence was detected on a blot scanner (iBright FL1500, Thermo Fisher Scientific, Waltham, MA, USA) using the SuperSignal kit (Pierce, Rockford, IL, USA). *Antibodies used:* anti-hCx46 (Santa Cruz Biotechnology, Dallas, TX, USA, #SC-365394), anti-c-Src (Cell Signaling Technology, Danvers, MA, USA, #36D10), anti-c-pSrc-Y416 (Cell Signaling Technology, Danvers, MA, USA, #59548T), anti-c-pSrc-Y530 (Cell Signaling Technology, Danvers, MA, USA, #2105T), anti-FAK (Cell Signaling Technology, Danvers, MA, USA, #3285T), anti-pFAK-Y576/Y577 (Cell Signaling Technology, Danvers, MA, USA, #3281T), and anti-EGFP (Thermo Fisher Scientific, Waltham, MA, USA, #MA5-15256).

2.4 Confocal microscopy. For Immunofluorescence, the cells were seeded for 48 h into 35mm plate petri at a concentration of 1×10^5 cells by plate in complete medium and each plate petri contains 4 coverslips. Then cells were fixed with 4% paraformaldehyde for 10 min at 30°C. Then, cells were washed in PBS for at least three times and permeabilized using PBS supplemented with 0.2% 0.1% Triton X-100 for 10 min at 30°C. After this procedure cells were washed and blocked using goat serum (Thermo Fisher Scientific, Waltham, MA, USA, #16210-064) for 30 min at room temperature. Proteins of interest were detected using the appropriate primary antibodies, which were diluted in goat serum and incubated overnight. The following day, coverslips were washed with PBS and incubated for 60 min at room temperature with Alexa 555–conjugated donkey anti-rabbit (Thermo Fisher Scientific, Waltham, MA, USA, #A31572). After washing off the secondary antibody, coverslips were mounted on microscope slides (1 mm thick) using DAPI Fluoromount G (Electron Microscopy Sciences, Washington, PA, USA). Images acquired and analyzed with a confocal microscope Celldiscoverer 7 with LSM 910 and Airyscan 2, (Carl Zeiss AG, Oberkochen, Germany).

2.5 Co-Immunoprecipitation. Primary antibodies conjugated to magnetic beads were employed following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA #14311D). Briefly, 1 mg of magnetic beads was incubated with 20 µl of primary antibody on a roller at 37 °C for 24 h. The following day, the antibody–magnetic bead complexes were precipitated using a magnet (Cell Signaling Technology, Danvers, MA, USA), the supernatant was discarded, and the pellet was washed three times with the kit's washing buffer and stored in 50 ml of kit's store buffer. Cells grown in 90 mm plastic dishes were harvested and sonicated in 950 µl of cold PBS containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA, #1861280). Then, 50 µl of primary antibody–magnetic bead complexes were added to the cell suspension and incubated overnight at 4 °C under constant agitation to prevent bead aggregation. The next day, the suspension was placed on a magnet, and the collected magnetic beads were washed five times with 1 ml PBS supplemented with 0.01% NP40 and

0.1% Triton X-100 for 10 min at room temperature. After the final wash, the supernatant was discarded, and 100 µl of NuPAGE™ LDS Sample Buffer and heated at 95°C for 3 min to release the proteins bound to the antibody. The presence of the immunoprecipitated proteins was analyzed by Western blot. For detection, a secondary antibody specific for immunoprecipitation was used, as it only recognized primary antibodies in their native state (Abcam, Cambridge, UK #ab131366). As a negative control, immunoprecipitations were performed using a Mouse mAb IgG XP® Isotype Control (Thermo Fisher Scientific, Waltham, MA, USA #02-6100).

2.6 Molecular dynamic studies:

Sequence-based protein-protein interaction (PPI) servers. The sequence-based PPI prediction servers BIPSPi [36] and iFRAG [37] were used. To run the calculations, amino acid sequences of human Cx46 (ID: P12931) and human cSrc (ID: Q9Y6H8) available in the UNIPROT database were used. To identify the amino acids relevant for the PPI, we selected residue pairs that were oriented toward the cytosol, exhibited a solvent-accessible surface area (SASA) greater than 50%—indicating surface exposure—and were predicted by both servers consulted.

Cx46 structural modelling. Homology modeling was performed using MODELLER [38] to generate the full-length structure of human Cx46. The hemichannel model was built using the structure of sheep connexins 46/50 (PDB ID: 7JKC; residues 1–237) and the C-terminal region of rat connexin (PDB ID: 1R5S; residues 239–435) as templates. A total of 20,000 models were generated, and the best model was selected based on the MODELLER score. The selected model was further refined using coarse-grained refinement implemented in the HADDOCK server.

Cx46-cSrc molecular docking. To perform molecular docking calculations, the human Cx46 structural model generated by homology modeling was used. The c-Src structure was predicted using AlphaFold, and the least reliable segments were removed from the model. Amino acids identified through sequence-based protein–protein interaction analyses were used as references for protein–protein docking. Molecular docking and refinement were performed for membrane proteins following the protocol described previously [38]. This protocol consists of four steps: (I) generation of the initial structure from the coarse-grained (CG) model of Cx46 embedded in a pre-equilibrated lipid membrane obtained from the MemProtMD database (<http://memprotmd.bioch.ox.ac.uk/>); (II) replacement of the CG Cx46 structure with an atomistic model, while retaining only the beads representing the phosphate groups of the membrane; (III) molecular docking using LightDock, in which the residue pairs identified in the previous step were used to define the docking grid; and (IV) selection of the best conformations for refinement using a CG-based HADDOCK protocol and scoring. The final model selected corresponds to the protein–protein complex with the best score.

Interaction analysis. Based on the selected model, predictions of the interactions of the Cx46-cSrc complex were made using the PLIP server. The calculations were performed considering the interacting monomer of Cx46 and the cSrc protein.

2.7 Cell migration. The wound-healing assay was performed to evaluate cell migration. For Hela and SkMel-2 cells lines, they were seeded into 24-well plate at a final density of 1.5×10^5 cells by well and these were maintained at 37 °C and 5% CO₂ for 48 h to permit cell adhesion and formation of a confluent monolayer. For MCF7-EGFP and MCF7 Cx46-EGFP cells were also seeded into 24-well plate at a high density of 7.5×10^5 cells by well and cultured by 5 h. The wound-healing assay was performed by creating a linear scratch across the confluent cell monolayer using a sterile 200 µL yellow pipette tip held perpendicular to the plate surface. Detached cells and debris were removed by washing twice with sterile PBS, and fresh complete medium was subsequently added. Images of the wound area were acquired immediately after scratching (time 0) and at defined time intervals until closure was achieved. The wound width and percentage of closure were quantified over time using NIS-element Nikon software. All experimental conditions were conducted in triplicate, and scratches were generated with uniform pressure and orientation to ensure experimental reproducibility.

2.8 Cell doubling time. Approximately 10,000 cells were seeded in a 60 mm Petri dish and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 1× Pen/Strep (Thermo Fisher Scientific, Waltham, MA, USA #15140-122), at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were harvested on day 7. To collect the cells, cultures were washed with 1× PBS, detached with 0.25% trypsin-EDTA (Capricorn Scientific, Pune, Maharashtra, India, #TRY-1B10) for 5 min at 37 °C, and neutralized with fresh complete medium. The cell suspension was centrifuged at 1500 rpm for 5 min at room temperature. The supernatant was discarded, and the cell pellet was resuspended in medium. An aliquot of 10 µL of the cell suspension was mixed with 10 µL of 0.4% Trypan Blue solution, and viable cells were counted using a hemocytometer. At the end of the experiment, the total number of cells obtained on day 7 was used to calculate the cell doubling time using the following formula:

$$Dt = \frac{\ln 2 * Duration(hours)}{\ln\left(\frac{Nt}{N0}\right)}$$

- Dt = Doubling Time
- Nt = Final concentration (day 7)
- N0 = Initial concentration (day zero).

3. Results

3.1 Cx46 expression reduces the migratory capacity of HeLa cells. We previously demonstrated that the expression of Cx46 in MCF-7 promotes several characteristics associated with cancer stem cell (CSC) and epithelial–mesenchymal transition (EMT) phenotypes, including enhanced migratory capacity [30].

To elucidate the molecular mechanisms underlying these effects, HeLa cells were employed, as they exhibit minimal—if any—Cx expression [39,40], particularly Cx46 [41]. Given the well-established role of the Cx's C-terminal domain as a hub for protein–protein interactions [9,10] and a key regulator of Cx channel function [39,40], we used a Cx46 construct fused to EGFP at its C-terminus (Cx46EGFP) to visualize its expression and subcellular localization. In addition, two Cx46 C-terminal mutants were generated: one lacking the C-terminal domain (Δ CTEGFP) and another containing only the C-terminal domain (CTEGFP). HeLa cells transfected with EGFP alone (EGFP) were used as a transfection control (Figure 1A). Western blot analysis revealed that, when an antibody against the C-terminal of Cx46 was used, a band of approximately 70 kDa was detected in HeLa cells expressing Cx46EGFP, corresponding to the predicted molecular weight of Cx46 fused to EGFP. Additionally, two main bands of approximately 40 and 46 kDa were observed in HeLa cells transfected with CTEGFP. The 40 kDa band likely corresponds to the molecular weight of the C-terminal domain fused to EGFP, whereas the 46 kDa band may represent a phosphorylated form of CTEGFP. As expected, no immunoreactivity was detected in HeLa cells transfected with EGFP or Δ CTEGFP (Figure 1B). When an anti-GFP antibody was used, a band of approximately 24 kDa was detected in HeLa cells expressing EGFP. Bands of the expected molecular weight were also observed in HeLa cells expressing Cx46EGFP and CTEGFP, consistent with those detected using the anti-Cx46 antibody. In the case of Δ CTEGFP, a faint band of approximately 41 kDa was detected, which corresponds to the predicted molecular weight of Cx46 lacking its C-terminal domain fused to EGFP (Figure 1C). Using these four cell types, we assessed the migration rate through a wound-healing assay (Figure 2). We found that HeLa-EGFP cells covered $65.0 \pm 4.2\%$ of the wounded area (% of closure) after 24 hours, whereas, unexpectedly, HeLa-Cx46EGFP cells show only $24 \pm 2.3\%$ of closure (Figure 2A,B). Consistent with the regulatory role of the Cx46 C-terminal domain, HeLa-CTEGFP cells also exhibited reduced migration, although the effect was less pronounced ($45.8 \pm 3.4\%$) than that observed in HeLa-Cx46EGFP cells. In contrast, the migration rate of HeLa- Δ CTEGFP cells did not differ significantly from that of HeLa-EGFP cells ($57.3 \pm 5.0\%$) (Figure 2A,B). One possible explanation for these results is a change in the rate of cell division. Therefore, we determined the cell doubling time for each cell type. We found that expression of both Cx46EGFP (24.1 ± 0.3 h for cell duplication) and CTEGFP (23.1 ± 0.2 h for cell duplication) modestly increased the cell division rate compared with HeLa cells expressing EGFP alone (22.1 ± 0.3 h for cell duplication) (Figure 2C). Increased cell proliferation would be expected to enhance apparent migration in wound-healing assays, as a greater number of cells would more readily repopulate the wounded area. In contrast, we observed a marked reduction in cell migration, suggesting that the inhibitory effect of Cx46 on migration is not driven by changes in cell proliferation and may, in fact, be underestimated.

3.2 Only Cx46EGFP forms functional hemichannels at the plasma membrane, whereas CTEGFP and Δ CTEGFP do not. The opening of Cx hemichannels can influence cell migration [44–46]. Therefore, we wanted to determine whether Cx46 and its C-terminal mutants form functional hemichannels. Despite the significant differences observed between HeLa-EGFP, HeLa-CTEGFP, and HeLa- Δ CTEGFP, under control conditions, the rate of dye uptake in all cell types was very low and likely lacked biological relevance (Figure 3A,B). In contrast, under divalent cation free solution (DCFS), only HeLa cells

expressing Cx46EGFP exhibited a marked increase—more than 20-fold— in the rate of dye uptake compared to the rest of the cell types (Figure 3A,C). These findings demonstrate that only HeLa cells expressing wild-type Cx46 are capable of forming functional hemichannels at the plasma membrane. To further support this conclusion, we analyzed the subcellular localization of these constructs using fluorescence microscopy. In HeLa cells expressing EGFP alone, the fluorescence signal was homogeneously distributed throughout the cytoplasm and nucleus (Figure 3D, EGFP). In contrast, Cx46–EGFP was primarily detected at the cell periphery and in perinuclear compartments, and occasionally within the nucleus as small fluorescent puncta (Figure 3D). Both CTEGFP and Δ CTEGFP exhibited a homogeneous cytoplasmic distribution and were not visibly enriched at the cell edges. This localization pattern further supports the notion that CTEGFP and Δ CTEGFP are unable to form hemichannels at the plasma membrane.

3.3 Intracellular signaling pathways, but not extracellular purinergic signaling, mediates the reduction in cell migration in HeLa cells. As mentioned earlier, hemichannels serve as pathways for the release of signaling molecules such as glutamate, NAD⁺, and ATP, among others [47–49]. In particular, ATP is well recognized as an extracellular signaling molecule involved in cancer cell migration [50–52]. Therefore, we investigated whether extracellular ATP, potentially released through hemichannels, could influence HeLa cell migration (Figure 4A,B). HeLa cells (expressing EGFP or Cx46EGFP) were exposed to ATP at concentrations ranging from 0 to 500 μ M. Notably, none of these concentrations affected cell migration, regardless of Cx46 expression, suggesting that activation of P2X/P2Y receptors is not involved in this process. To further test this, we evaluated the effect of the general P2X receptor inhibitor PPADS (10 μ M) on cell migration. Similar to ATP treatment, PPADS did not produce any detectable change in the migration rate (Figure 4A,B). Then, we test the effect of inhibitors of important proteins in in cancer cell migration such as PI3k, AKT1 and Src [16,53,54]. Inhibition of PI3K (BEZ235, at 1 μ M) caused a marked reduction in cell migration in both HeLa-EGFP and HeLa-Cx46EGFP cells (Figure 4C,D). However, these effects were not statistically different between the two cell types (Figure 4E). In contrast, inhibition of AKT1 (Akt inhibitor VIII, at 1 μ M) did not produce any significant change in migration in either HeLa-EGFP or HeLa-Cx46EGFP cells. Notably, inhibition of Src (SU6656, at 1 μ M) reduced the migration rate, and this effect was more pronounced in HeLa cells expressing Cx46EGFP (Figure 4F), suggesting Cx46 may increase the cell's dependence on Src for migration (e.g., Cx46 could recruit or activate Src, or modulate focal adhesions and the actin cytoskeleton).

3.4 Cx46 promotes Src re-localization via protein–protein interactions. Because our previous results suggested a possible interaction between Cx46 and Src, we examined this PPI using co-immunoprecipitation and high-resolution confocal microscopy. We found that Cx46 co-immunoprecipitated with Src and vice versa, demonstrating a close association between these two proteins (Figure 5A). This observation was further supported by confocal microscopy. In HeLa–EGFP cells, Src was predominantly distributed throughout the cytoplasm and, to a lesser extent, at sites of cell–cell contact. (Figure 5B, EGFP). In contrast, in HeLa-Cx46EGFP cells, Src was predominantly localized at cell–cell contact sites (Figure 5B, Cx46EGFP). To determine whether Src co-localizes with Cx46 at these contact regions, we performed high-resolution confocal co-localization analysis.

Cx46EGFP (green signal) showed strong co-localization with Src (red signal) both in the cytoplasm and, more prominently, at cell–cell interfaces (Figure 5C). These findings suggest that Cx46 and Src can form a functional protein–protein complex. To further explore this interaction, we performed a bioinformatic analysis to predict potential contact regions between Cx46 and Src. A structural model of the human Cx46 hemichannel embedded in a POPC plasma membrane revealed that Cx46 is able of interacting with Src. The predicted interaction site involves a region within the Cx46 C-terminal tail (amino acids between positions 308–335) and residues in Src that are primarily distributed within the kinase domain (SH1 region, aa at positions K319, M383, L445, Q500, E508, E513, Y514), although additional amino acids in the SH3 (aa at position 118) and SH2 (aa at position 155) also appear to contribute (Figure 5D). This bioinformatic analysis is consistent with our previous findings showing that the C-terminal domain alone is sufficient to decrease cell migration, whereas a Cx46 construct lacking the C-terminal domain (Cx46DCT) is unable to do so.

3.5 The presence of Cx46 correlates with Src phosphorylation at sites associated with its

activation. Because Cx46 interacts with Src in HeLa cells, we next evaluated whether this interaction affects Src phosphorylation. We used antibodies that recognize Src phosphorylated at Y419, which is associated with activation [55], and at Y530, which is associated with inhibition of Src [56]. First, we examined total Src levels in HeLa-EGFP and HeLa-Cx46EGFP cells. Src expression in HeLa-Cx46EGFP cells was ~52% of that observed in HeLa-EGFP cells, although this difference was not statistically significant (Figure 6A). In contrast, phosphorylation at Y419 showed an ~213% increase in HeLa-Cx46EGFP cells, despite the reduction in total Src levels (Figure 6A). In contrast, phosphorylation at Y530 was not significantly changed in HeLa-Cx46EGFP cells compared with HeLa-EGFP cells (Figure 6A). Next, we analyzed the potential co-localization of Cx46 with phospho-Y419 and phospho-Y530 Src in HeLa-Cx46EGFP cells (Figure 6B). The phospho-Y419 signal was mainly localized in the cytoplasm and nuclear regions. Only a low degree of co-localization was observed between Cx46 and phospho-Y419 Src, as indicated by the weak orange signal, which was notably lower than the strong co-localization observed between Cx46 and total Src (Figure 5C), where most of the merged signal appeared orange. In contrast, phospho-Y530 Src was distributed in the cytoplasm and along the cell edges and showed a higher degree of co-localization with Cx46 than the phospho-Y419 form. Thus, despite a slight reduction in total Src expression, Cx46 expression appears to favor a more active Src conformation.

3.6 Cx46 expression is associated with reduced FAK and pFAK localization at cell edges. The Src–focal adhesion kinase (FAK) signaling axis plays a central role in cell migration and acts as a key regulator of directional motility [57,58]. Dysregulation of Src–FAK signaling has been widely implicated in cancer cell invasion and metastasis [59,60]. We therefore investigated whether changes in Src kinase phosphorylation and localization affect FAK phosphorylation. Western blot analysis revealed no significant changes in total FAK levels or in its phosphorylation at Tyr576/577, residues associated with FAK activation (Figure 7A,B). However, the subcellular localization of activated FAK (pFAK) was markedly altered in the presence of Cx46 (Figure 7C). Thus, in HeLa-EGFP cells, pFAK was localized in the cytoplasm by more predominantly at the cell edges, forming structures resembling filopodia. In contrast, these structures were absent in HeLa-Cx46EGFP cells, where the pFAK signal was mainly cytoplasmic.

3.7 Cx46 expression is associated with reduced cell migration in breast and skin cancer cell lines. Finally, we investigated whether the effect of Cx46 is cell-type dependent or represents a broader phenomenon. For this purpose, we used MCF-7 cells (a human breast cancer cell line), which express low endogenous levels of Cx46 (Figure 8A), that's why we transfected them with EGFP as a control or with Cx46EGFP. Western blot analysis showed that MCF-7-EGFP cells express endogenous Cx46, detected as a faint band at ~46 kDa. In contrast, MCF-7 cells transfected with Cx46EGFP exhibited a stronger ~46 kDa band, along with an additional ~70 kDa band corresponding to the Cx46EGFP fusion protein. Notably, MCF-7 cells, which express lower levels of Cx46, migrated faster (0.63 ± 0.02 wound closure at 24 h) than cells transfected with Cx46EGFP (0.45 ± 0.02 wound closure at 24 h) (Figure 8B). Similarly, we examined the effect of Cx46 on cell migration in a human melanoma cell line (Sk-Mel-2), which endogenously expresses Cx46, in this case these cells were transfected with a shRNA scramble as a control (Figure 8A, shScramble). In these cells, Cx46 was detected as a ~46 kDa band and as higher-molecular-weight forms, likely corresponding to phosphorylated species, as previously reported [61]. Sk-Mel-2 cells transfected with an shRNA targeting Cx46 exhibited an ~70% reduction in Cx46 expression (Figure 8A, shCx46), accompanied by an ~35% increase in cell migration compared to control cells. These results suggest that the presence of Cx46 reduces cell migration independently of cell type.

4. Discussion

In this work we identify Cx46 as a negative regulator of cancer cell migration and demonstrate that this effect is mediated by a channel-independent signaling mechanism involving direct interaction with Src kinase and disruption of focal adhesion signaling. These findings expand the non-canonical functions of Cx46 and reveal a previously unrecognized Cx46–Src–FAK axis that constrains migratory behavior in cancer cells. Expression of Cx46 in HeLa cells produced a robust reduction in migration that depended on the presence of the C-terminal domain. Deletion of the C-terminal domain completely abolished this effect, whereas expression of the C-terminal domain alone partially recapitulated it, supporting the concept that the Cx46 C-terminal domain acts as a signaling scaffold rather than a structural component of intercellular channels. Although Cx46 expression modestly increased proliferation, migration was strongly inhibited, indicating that reduced motility is not secondary to altered cell growth and may in fact be underestimated in wound-healing assays.

Although full-length Cx46 can form functional hemichannels permeable to ATP in both cancer and normal cells [61–63], purinergic signaling did not contribute to migration control in our model. Neither exogenous ATP nor P2X receptor inhibition affected migration, arguing against a role for hemichannel-mediated ATP release. Instead, the isolated Cx46 C-terminal domain was sufficient to suppress migration, despite lacking transmembrane regions and the ability to form hemichannels or gap junction-like structures [41,64]. These findings strongly support a non-canonical, channel-independent role for Cx46 in regulating cell migration. Mechanistically, we identify Src kinase as a key effector of Cx46-dependent migration control, consistent with its established role in migratory signaling [65,66]. Src inhibition reduced migration in all cells but had a significantly greater effect in Cx46-expressing cells, suggesting that Cx46 reshapes Src-dependent signaling networks. In line with this, Cx46 directly

associated with Src and promoted its relocalization to cell–cell contact regions, accompanied by increased phosphorylation at the activating Y419 site despite reduced total Src levels. The interaction of Cx46 with Src residues located at positions 118 and 155 could plausibly modulate Src activity by interfering with its intramolecular regulatory mechanisms. Residue E118 is positioned within the SH3 domain, which plays a central role in maintaining Src in its inactive conformation through interactions with the proline-rich linker region. Binding of Cx46 near this site could destabilize the SH3-mediated autoinhibitory interaction, thereby favoring an open conformation of the kinase. Similarly, residue 155 is located within the SH2 domain, which stabilizes the inactive state through binding to phosphorylated Y530. Association of Cx46 with this region may perturb SH2-dependent intramolecular contacts, reduce its conformational restraint and promote therefore the kinase activation [67–69]. Together, interactions at these sites could shift Src toward an active state, facilitating Y416 autophosphorylation and enhancing downstream signaling. However, the interaction between Cx46 and Src must be investigated in greater detail using molecular dynamics simulations and site-directed mutagenesis to unequivocally determine the precise mechanism underlying this interaction.

Downstream of Src, focal adhesion kinase (FAK) is a central regulator of directional migration [58,60,70]. While total FAK expression and activation remained unchanged, Cx46 altered the subcellular distribution of phosphorylated FAK, leading to loss of pFAK enrichment at the leading edge and filopodia-like structures. This redistribution likely disrupts focal adhesion dynamics and provides a mechanistic basis for impaired migration. Given the broader roles of internalized FAK in cell survival and transcriptional regulation [71], Cx46-mediated modulation of the Src–FAK axis may impact additional cancer-related processes.

Notably, in this study we determined that Cx46 suppressed migration in breast, skin and ovarian cancer cells. At first glance, these findings may appear to contrast with previous reports, including our own [30], in which Cx46 promoted migratory and EMT-associated features in specific cellular contexts. However, this apparent paradox underscores a fundamental principle of Cx biology: Cxs act as context-dependent signaling platforms rather than unidirectional regulators of cancer behavior. Differences in expression levels, interacting partners, subcellular localization, and post-translational modifications are likely to shift Cx46 function between pro- and anti-migratory states. In the present study, Cx46 engages Src and disrupts Src–FAK signaling at focal adhesions, thereby limiting migration. In other cellular environments, alternative interaction networks may dominate, leading to opposite phenotypic outcomes. Such plasticity provides a mechanistic framework to reconcile seemingly contradictory roles of Cxs in cancer progression. While our results support a role for Src in mediating the anti-migratory effects of Cx46, we cannot exclude the involvement of additional signaling partners or pathways that may contribute in a context-dependent manner. Furthermore, although our data indicate a channel-independent mechanism, the relative contribution of Cx46 channel functions under different cellular or microenvironmental conditions remains to be determined.

5. Conclusion

In this work, we demonstrate that Cx46 suppresses cancer cell migration through a hemichannel-independent mechanism involving direct interaction with Src kinase and spatial dysregulation of Src–FAK signaling (Figure 9). These findings highlight the importance of connexin-dependent intracellular signaling in cancer and suggest that targeting connexin–kinase interactions may offer new opportunities to modulate tumor cell motility and metastatic potential.

Declarations

Authorship contribution statement. MAR: Conceptualization, Writing – original draft, Writing – review & editing. MAR: Literature revision. WER, LDRT, VPO, RA, IML-F, STP, CB, IEA: performed experiments and data analysis. JB, WG: performed bioinformatic analysis MAR: Figure design.

Generative AI statement. During the preparation of this work, the authors utilized ChatGPT 4.0 for editing to enhance language clarity. Following its use, the author thoroughly reviewed and edited the content as necessary, taking full responsibility for the accuracy and integrity of the publication.

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Declaration of competing interest. The authors declare that a patent application is currently under review in the United States (Ref. No. 40-1917-US).

Data and materials availability. The datasets generated and/or analyzed during this study are available from the corresponding author upon request.

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Figures

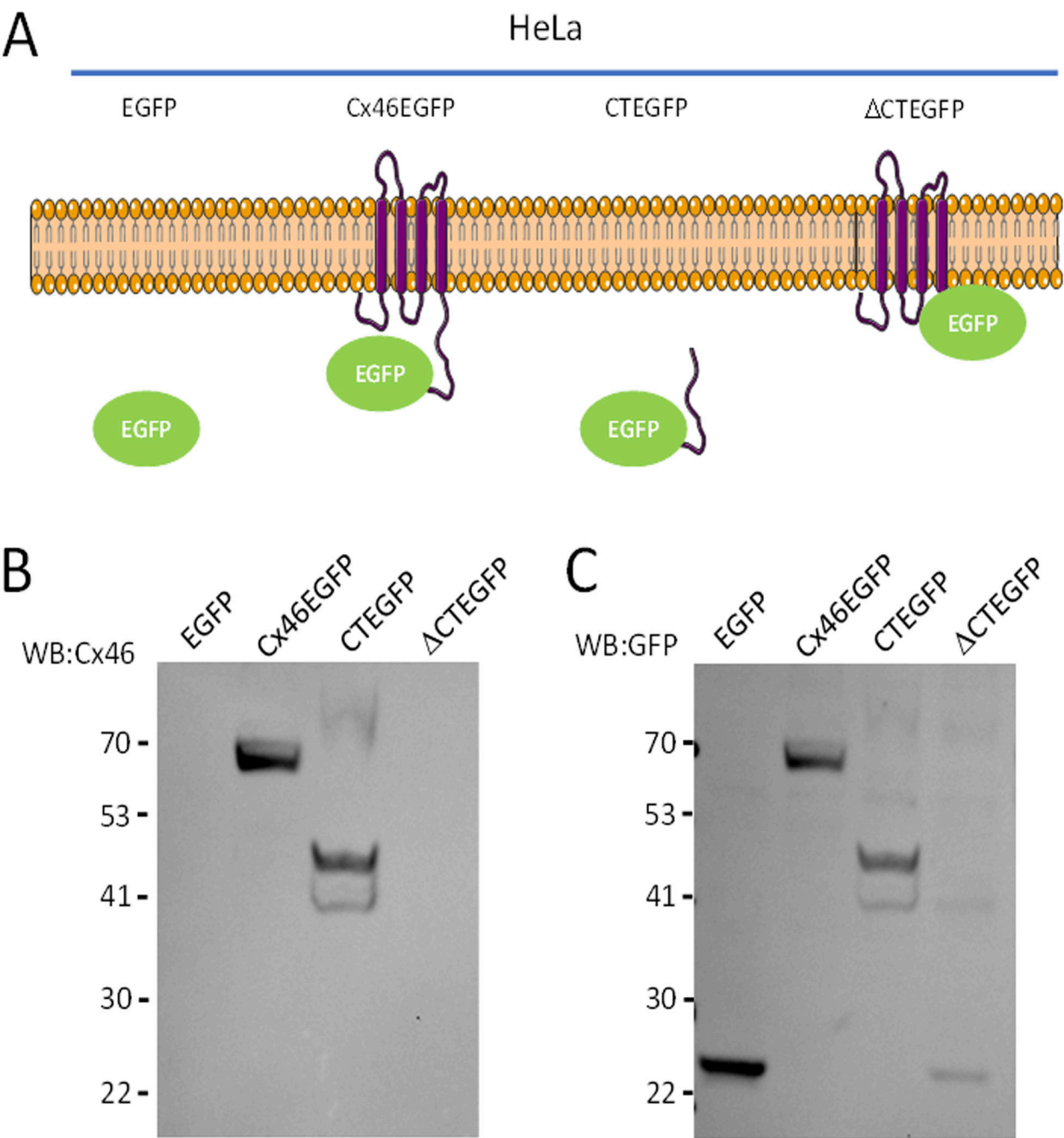


Figure 1

Expression of Cx46 constructs in HeLa cells. (A) Schematic representation of the constructs used in this study: EGFP alone, full-length Cx46 fused to EGFP at its C-terminus (Cx46EGFP), the isolated Cx46 C-terminal domain fused to EGFP (CTEGFP), and a C-terminal-deleted Cx46 mutant fused to EGFP (Δ CTEGFP). (B) Representative Western blot analysis of HeLa cells transfected with EGFP, Cx46EGFP, CTEGFP, or Δ CTEGFP, probed with an anti-Cx46 antibody. Full-length Cx46EGFP is detected as a band at approximately ~70 kDa, whereas CTEGFP is detected as two bands with ~40 and ~48 kDa. As expected, no signal was observed in HeLa cells transfected with Δ CTEGFP, as the antibody recognizes its C-terminal. (C) Representative Western blot analysis of the same samples probed with an anti-GFP antibody, confirming expression of all EGFP-tagged constructs.

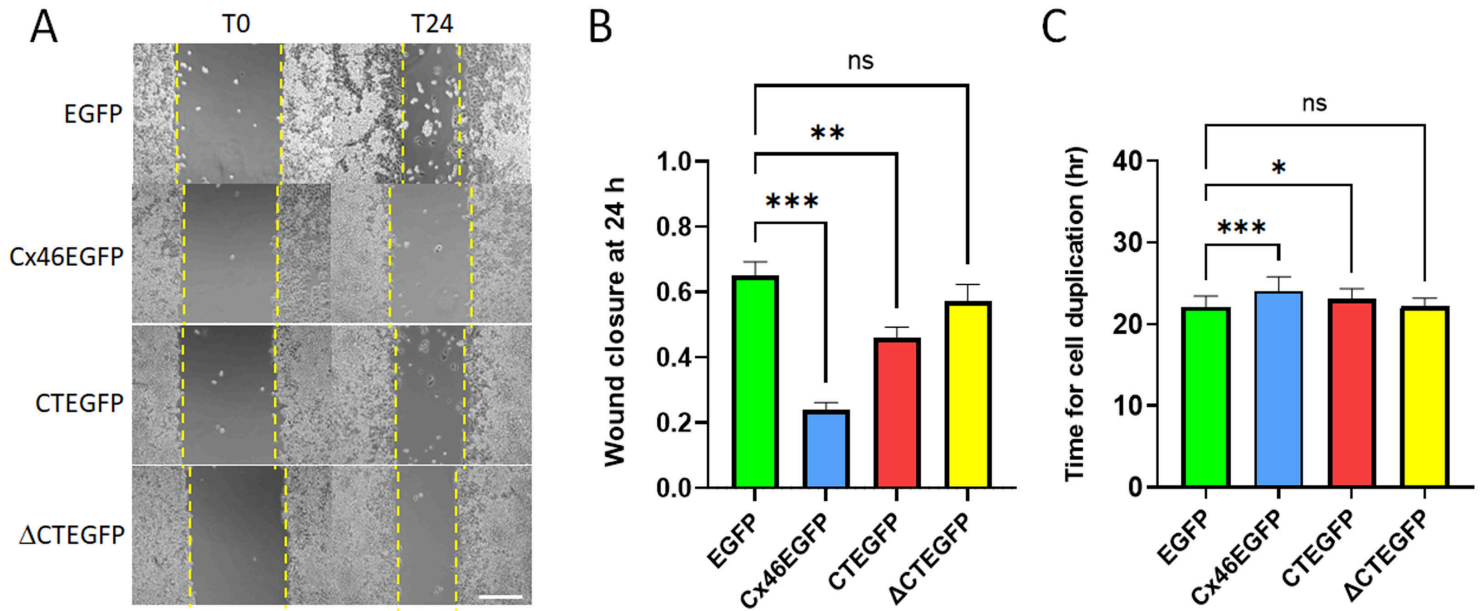


Figure 2

Cx46 suppresses HeLa cell migration through its C-terminal domain. (A) Representative phase-contrast images of wound-healing assays performed in HeLa cells transfected with EGFP, Cx46EGFP, CTEGFP, or Δ CTEGFP at the time of scratching (T0) and after 24 h (T24). Yellow dashed lines indicate wound boundaries. Scale bar: 400 μ m. (B) Quantification of wound closure after 24 h, expressed as the fraction of the initial wound area covered by migrating cells. Expression of full-length Cx46EGFP significantly reduced wound closure compared with EGFP controls, whereas deletion of the C-terminal domain abolished this effect. Expression of the C-terminal domain alone partially reduced migration (n=4). (C) Cell duplication time determined for each condition (n=30). Expression of Cx46EGFP and CTEGFP modestly reduced duplication time compared with EGFP controls, whereas Δ CTEGFP had no significant effect. Data are presented as mean $\pm$ SEM. Statistical significance is indicated (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant).

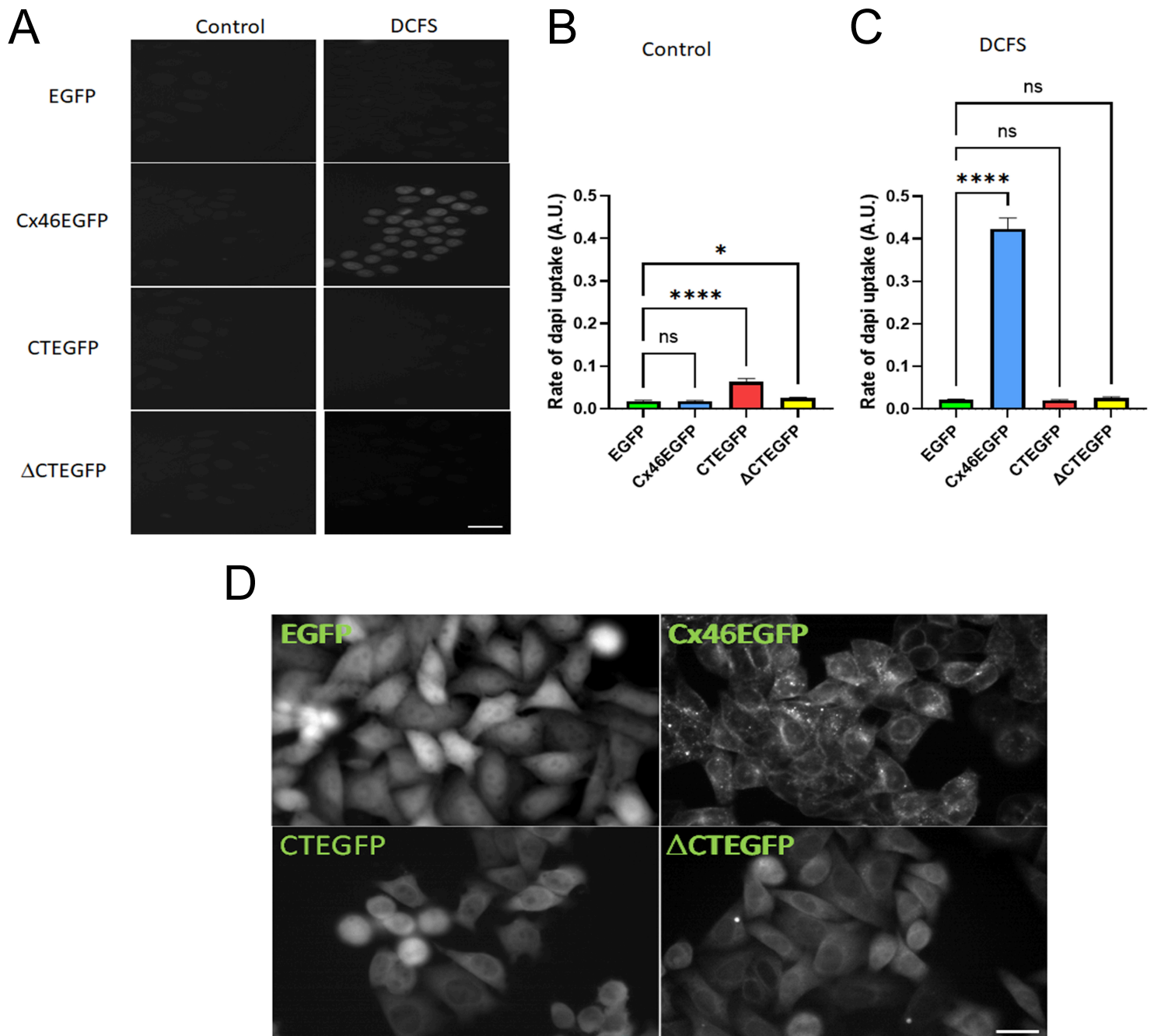


Figure 3

Only full-length Cx46 forms functional hemichannels at the plasma membrane. (A) Representative fluorescence images showing DAPI uptake in HeLa cells expressing EGFP, Cx46EGFP, CTEGFP, or Δ CTEGFP under control conditions (normal extracellular divalent cations) or divalent cation-free solution (DCFS). Robust nuclear DAPI uptake is observed only in Cx46EGFP-expressing cells under DCFS conditions. Scale bar: 10 μ m. (B) Quantification of the rate of DAPI uptake under control conditions (n=6, at least 16 cell analyzed per experiment). All cell types exhibit minimal dye uptake, with no biologically relevant differences between groups. (C) Quantification of the rate of DAPI uptake under DCFS conditions. A marked increase in dye uptake is observed exclusively in HeLa cells expressing full-length Cx46EGFP, whereas CTEGFP and Δ CTEGFP do not differ from EGFP controls (n=6, at least 16 cell

analyzed per experiment). (D) Representative fluorescence microscopy images showing the subcellular distribution of EGFP, Cx46EGFP, CTEGFP, and ΔCTEGFP in HeLa cells. Cx46EGFP localizes to the cell periphery and perinuclear regions, whereas CTEGFP and ΔCTEGFP display a predominantly cytoplasmic distribution. Scale bar: 10 μm. Data are presented as mean ± SEM. Statistical significance is indicated (*p < 0.05; ****p < 0.0001; ns, not significant).

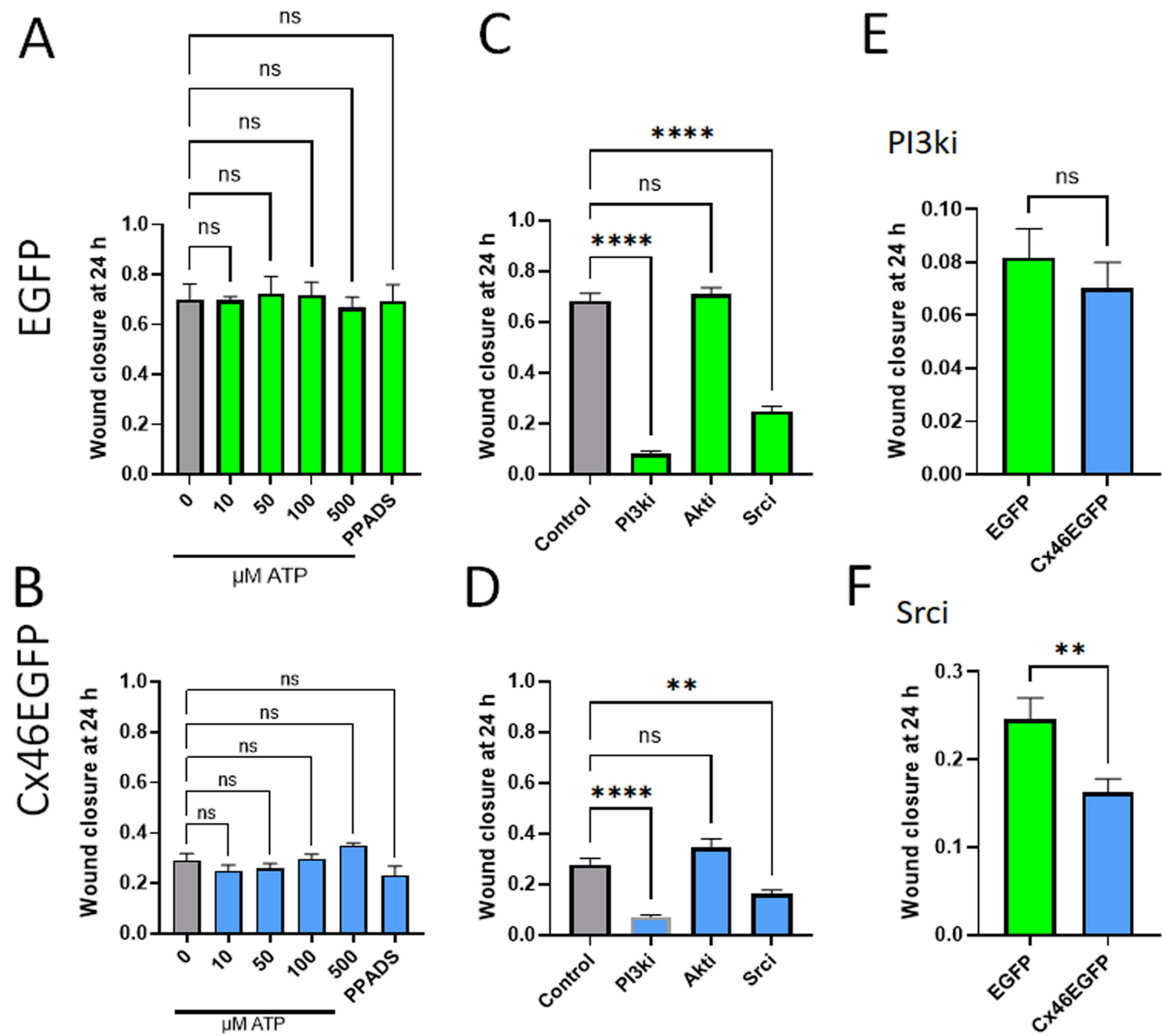


Figure 4

Cx46-mediated inhibition of cell migration involves Src-dependent signaling.

(A,B) Wound closure at 24 h in HeLa cells expressing EGFP (A) or Cx46EGFP (B) following treatment with increasing concentrations of extracellular ATP (0–500 μM) or the purinergic receptor antagonist PPADS (n=4). No significant differences were observed across ATP concentrations or PPADS treatment within each condition. (C,D) Effects of pharmacological inhibition of PI3K (PI3Ki), Akt (Akti), or Src (Srci) on

wound closure at 24 h in EGFP- (C) or Cx46EGFP-expressing (D) cells (n=6). PI3K inhibition significantly reduced wound closure in both conditions, whereas Src inhibition preferentially attenuated migration in Cx46-expressing cells. (E,F) Direct comparison of wound closure at 24 h between EGFP and Cx46EGFP cells following PI3K inhibition (E) or Src inhibition (F). Data are presented as mean $\pm$ SEM. Statistical significance is indicated as ns, not significant; **p < 0.01; ****p < 0.0001.

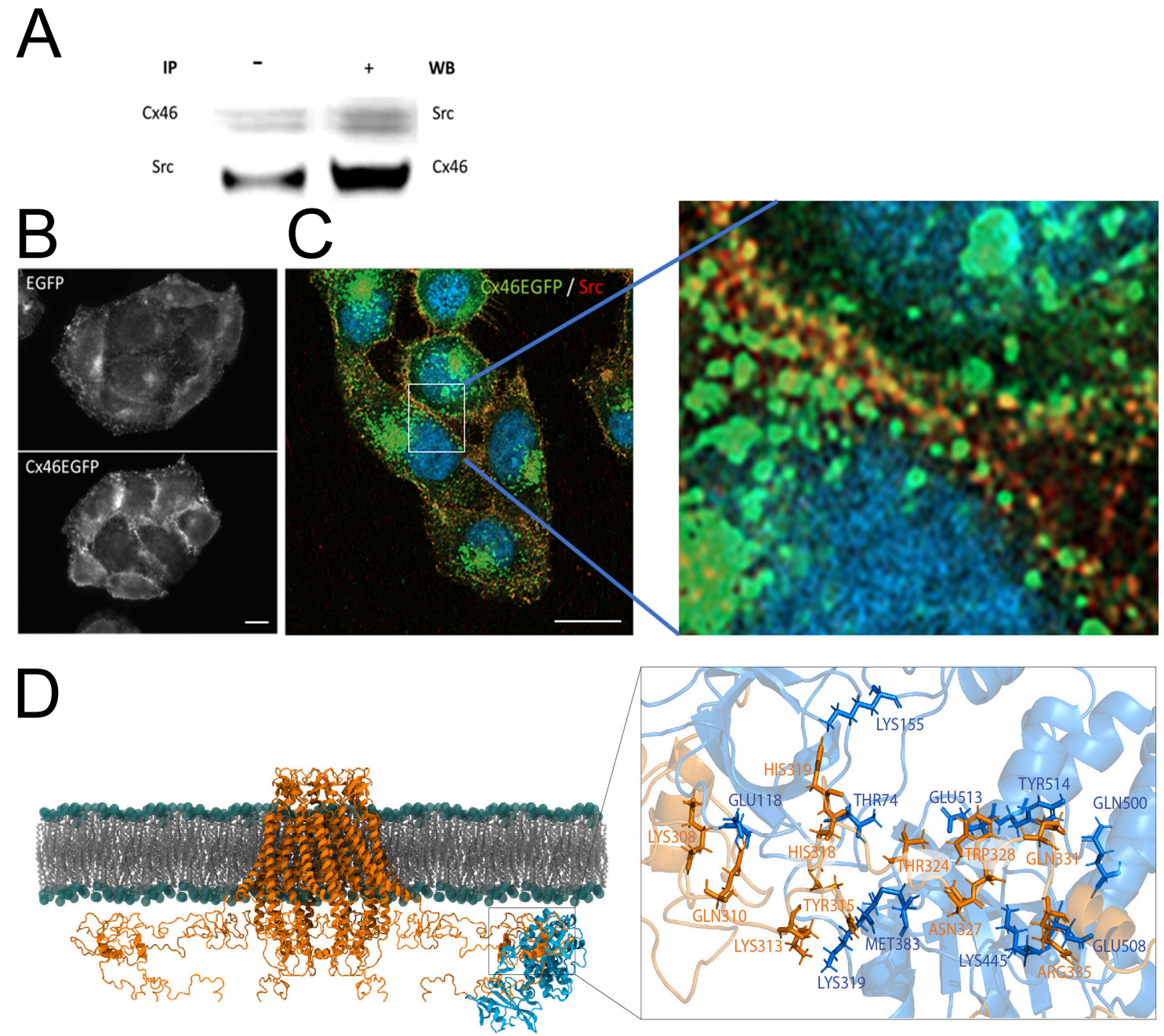


Figure 5

Cx46 physically associates with Src kinase and colocalizes at the plasma membrane.(A) Co-immunoprecipitation analysis showing the association between Cx46 and Src. Cell lysates were subjected to immunoprecipitation (IP) with anti-Cx46 antibodies (+) or without antibody (-), followed by Western blot (WB) detection of Src and Cx46, indicating a specific interaction between both proteins. (B) Representative confocal images of HeLa cells expressing EGFP or Cx46EGFP, showing distinct

membrane-associated localization of Cx46EGFP compared with EGFP control. (C) Confocal microscopy of HeLa cells expressing Cx46EGFP (green) and immunostained for Src (red); nuclei are labeled with DAPI (blue) (Scale bar: 5 μ m). Insets show magnified regions highlighting colocalization of Cx46 and Src at the cell periphery. (D) Molecular docking illustrating the proposed interaction between the C-terminal domain of Cx46 hemichannels and Src kinase at the plasma membrane.

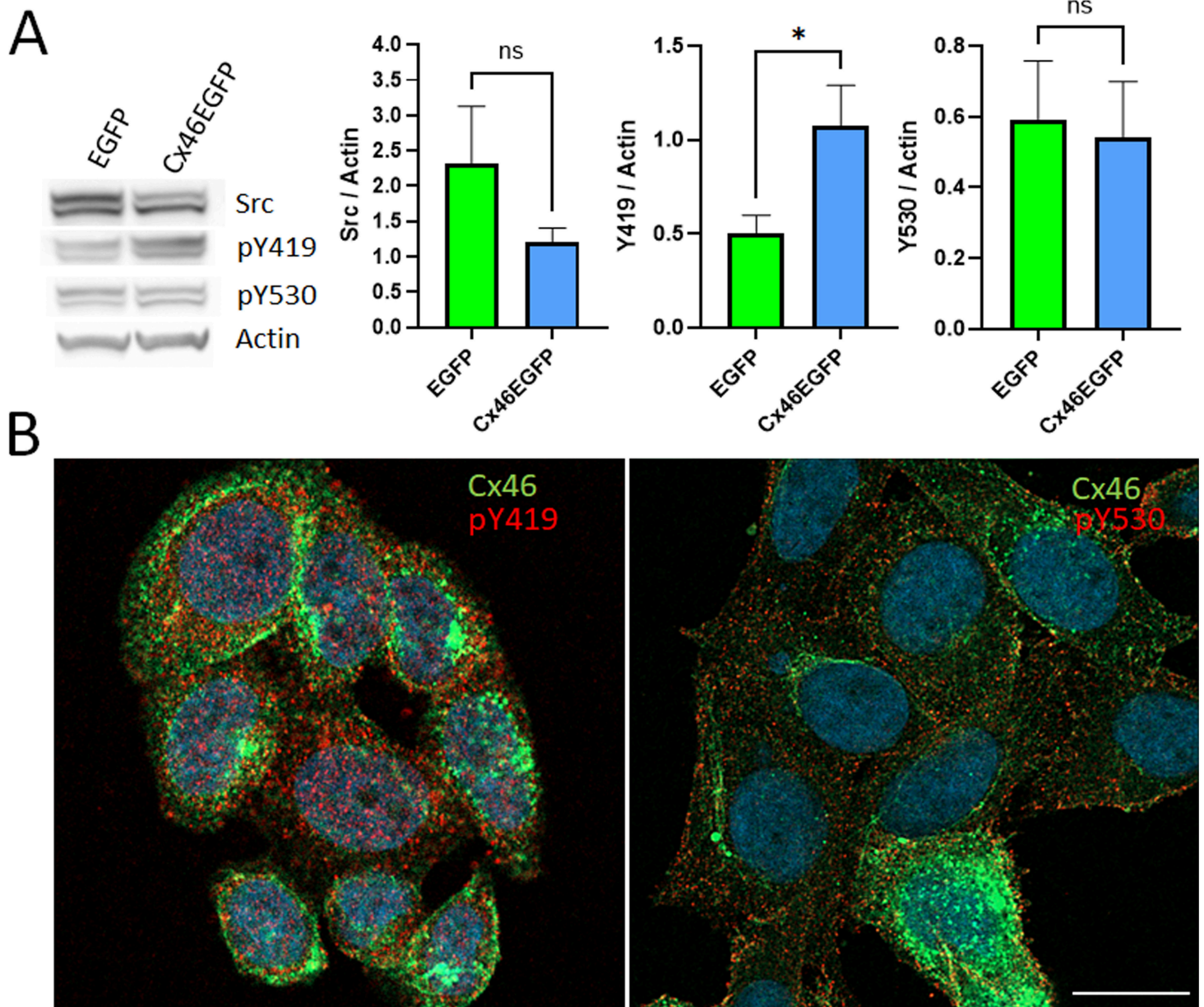


Figure 6

Expression of Cx46 modulates Src phosphorylation status without altering total Src levels.

(A) Representative immunoblots showing total Src, Src phosphorylated at Tyr416 (pY419), Src phosphorylated at Tyr530 (pY530), and actin in cells expressing EGFP (control) or Cx46-EGFP. Densitometric quantification of Src, pY419, and pY530 normalized to actin is shown on the right. Expression of Cx46-EGFP significantly increased Src phosphorylation at Tyr419, while total Src levels and phosphorylation at Tyr530 were not significantly altered. Data are presented as mean $\pm$ SEM. * $p < 0.05$;

ns, not significant. (B) Representative confocal microscopy images showing the intracellular distribution of Cx46 (green) and phosphorylated Src at Tyr419 (left panel, red) or Tyr530 (right panel, red). Nuclei are stained in blue. Cx46 shows partial colocalization with active Src (pY419), whereas no obvious enrichment is observed with pY530. Scale bar, 5 μ m.

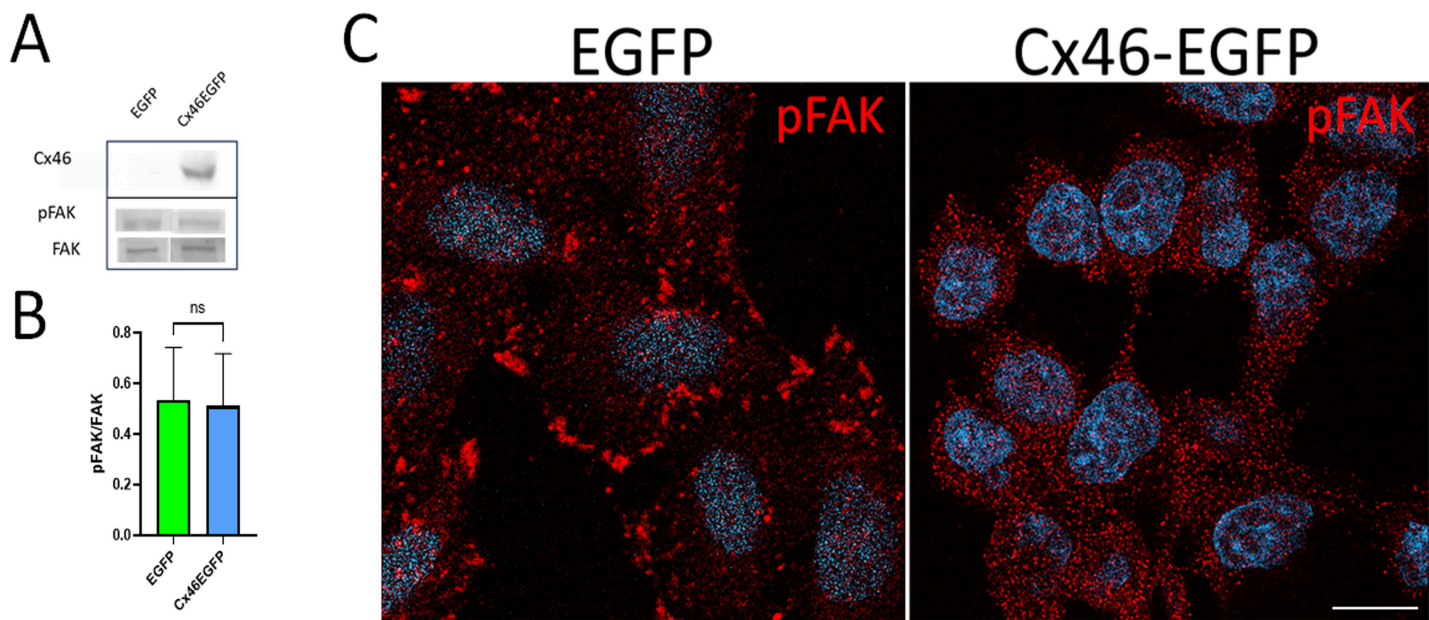


Figure 7

Cx46 expression does not alter total FAK levels or global FAK phosphorylation.(A) Representative immunoblots showing Cx46, phosphorylated FAK (pFAK), and total FAK in cells expressing EGFP or Cx46-EGFP. (B) Densitometric analysis of pFAK normalized to total FAK reveals no significant differences between EGFP and Cx46-EGFP–expressing cells. Data are presented as mean $\pm$ SEM; ns, not significant. (C) Representative confocal microscopy images showing the distribution of phosphorylated FAK (pFAK, red) in EGFP- and Cx46-EGFP–expressing cells (green). Nuclei are shown in blue. Differences in pFAK subcellular distribution are observed upon Cx46 expression. Scale bar, 5 μ m.

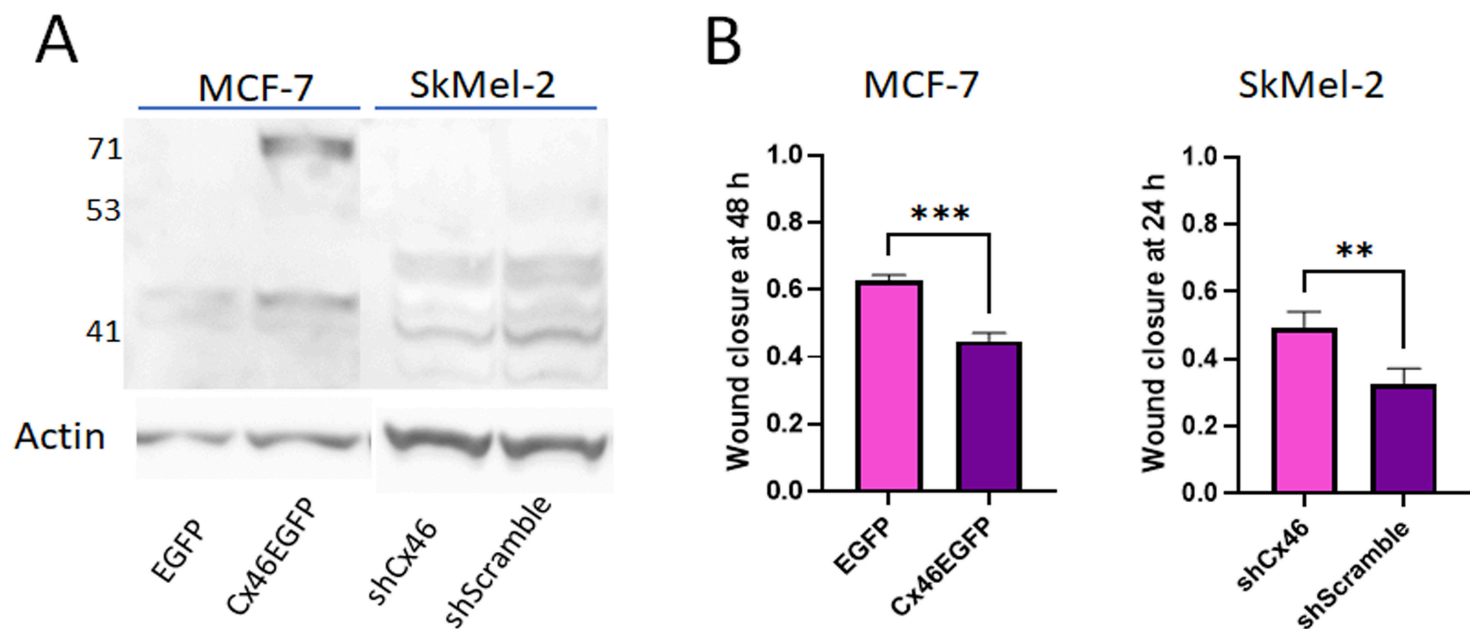


Figure 8

Cx46 expression impairs cell migratory capacity. (A) Representative immunoblots showing Cx46 expression in MCF-7 cells transfected with EGFP or Cx46-EGFP, and in SkMel-2 cells expressing shRNA targeting Cx46 (shCx46) or a scrambled control (shScramble). Actin was used as a loading control. (B) Quantification of wound-healing assays showing wound closure in MCF-7 cells at 48 h after scratch and in SkMel-2 cells at 24 h after scratch. Cx46 overexpression significantly reduced wound closure in MCF-7 cells, while Cx46 silencing significantly decreased wound closure in SkMel-2 cells compared with their respective controls. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

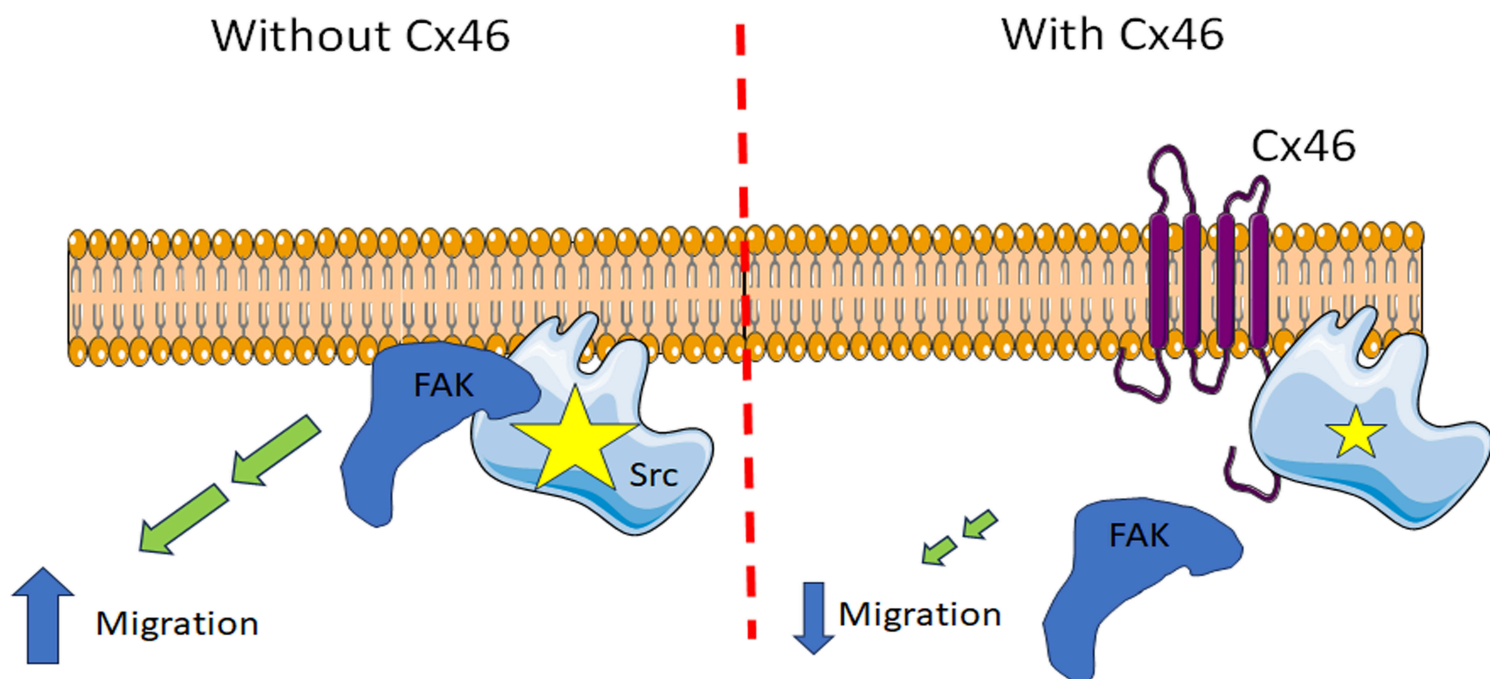


Figure 9

Proposed model for Cx46-dependent regulation of Src signaling and cell migration.

Schematic representation of Src–FAK signaling in the absence (left) or presence (right) of Cx46. In cells lacking Cx46, Src associates with FAK at the plasma membrane, promoting signaling pathways that support cell migration. In contrast, Cx46 expression alters Src localization and/or availability at the membrane, leading to reduced Src–FAK functional coupling and decreased migratory capacity.