Supplementary Figure Legends

**Figure S1.** Endogenous Cx26 and retrovirally expressed Cx32 are differentially assembled into gap junctions in BxPC3 and Capan-1 cells. **A.** Cells were immunostained for Cx26 and Cx32. Note that in BxPC3 both Cxs are assembled into gap junctions whereas in Capan-1 cells, they are not. **B.** Western blot analysis of lysates from BxPC3 and Capan-1 cells and of lysates from BxPC3 and Capan-1 cells retrovirally transduced with Cx32 (Retro-Cx32). Note that all Cxs are expressed abundantly in both cell types. **C.** Western blot analysis of Cx32 and Cx26 in Total (T), TX100-soluble (S), and TX100-insoluble (I) fractions of cell lysates in BxPC3 and Capan-1 cells. Note that a major fraction of both Cxs is TX100-insoluble in BxPC3 cells but the Cxs remain soluble in Capan-1 cells. The blots were also stripped and re-probed for β-actin.

**Figure S2.** **A.** Colocalization of Cx43 with various markers. A. Colocalization of Cx43 with the secretory markers GM130 and caveolin 2 and with the endocytic marker caveolin 1 (Cav1). BxPC3 and Capan-1 cells were immunostained for Cx43 (red) and various markers (green). **B.** Cx43 (red) extensively co-localizes with Lamp1 (green). For A and B, enlarged images of the boxed regions are shown on the right. **C.** Co-localization of Cx43 with various endocytic and secretory markers was quantitated by calculating the Pearson’s Coefficient (see Materials and Methods). The highest Pearson’s Coefficient (0.8) was obtained in cell images of cells immunostained with monoclonal and polyclonal antibodies targeting Cx26 using monoclonal and polyclonal antibodies followed by detection with Alexa-594- and Alexa-488-conjugated secondary antibodies (Cx-R/G). Note that significant co-localization with Cx43 is measured for only Lamp1 (0.4). The Pearson Coefficients graphed are averaged from 10 separate images from 2 separate experiments. CLT=clathrin.

**Figure S3.** **Cx43 is internalized by the clathrin-mediated pathway in a Rab5 dependent manner.** **A.** Cx43 co-localizes with Lamp1 but not with caveolin 1 (Cav1) in single cells. Cells were immunostained for Cx43 (green) and either for Lamp1 or caveolin 1 (red). Note discernible co-localization of Cx43 with Lamp1 as shown in the enlarged images of the boxed region on the right. **B.** HEK293T cells were transfected with plasmids encoding either GFP or GFP-μ2. Cells were lysed and
immunoprecipitated with a mouse anti-GFP antibody (BD # JL-8). Both input (IN) and pull down (PULL) proteins were separated by SDS-PAGE and analyzed by western blotting. For input 30 µg and for pull-down immunoprecipitates from 700 µg of total protein from cell lysates was loaded in each lane. The membrane in the top panels was probed with a rabbit-anti-GFP antibody. The bottom two panels show a low and high exposure of the membrane probed with anti-Cx43 antibody (Sigma #C6219). C. Cells were transfected with GFP-tagged Rab5-WT, dominant active Rab5 (Rab5-DA) or dominant negative Rab5 (Rab5-DN) and immunostained for Cx43. Note the disappearance of Cx43 puncta (red) in cells expressing Rab5-DN (green). D. Intracellular puncta were quantified in BxPC3 cells expressing Rab5-WT and Rab5-DN. Puncta from 15-20 cells were counted from two separate experiments. Blue and orange represent puncta from transfected and control cells, respectively.

**Figure S4. Knock-down of Cx43 in BxPC3 and Capan-1 cells with shRNA.** AB. Lysates from Capan-1 and BxPC3 cells, expressing a shRNA targeting Cx43 (Sh-Cx43) or a scrambled shRNA (Sh-Scr), were immunoblotted for Cx43. Note the robust knock down of Cx43. β-actin was used as a loading control. B. Cx43 knock-down has no off-target effects. The expression of seven junction- or cytoskeleton-associated proteins in Capan-1 expressing Sh-Cx43 and Sh-Scr was analyzed by Western blotting. Sh-Cx43 did not knock down any of the indicated proteins except Cx43. C & D. Parental Capan-1 cells, knock-down cells and knock-down cells expressing one of the Cx43 mutants were microinjected with Alexa 594 (C) or scrape-loaded (D) with rhodamine-conjugated dextran (R-DEX) and Lucifer Yellow (LY). Note that only cells expressing the sorting motif mutant YA-VD-Cx43 show cell-cell transfer of fluorescent tracers. The microinjected cells are indicated by the white arrows.

**Figure S5.** A. LNCaP cells stably expressing WT-Cx43 or the indicated mutants were immunostained for Cx43. Note that YA/VD-Cx43 forms large gap junctions whereas the mutants S279A and S279D form smaller gap junctions and the cells contain many intracellular puncta. B & C. Trafficking and the kinetics of degradation of WT-Cx43 and various mutants. CP43KD cells stably expressing knocked-in WT-Cx43 (WT), YA/VD-Cx43 (YA/VD) or the indicated serine mutants were
cell-surface biotinylated. Biotinylated proteins were pulled down by immobilized streptavidin (Pull), and immunoblotted for Cx43 (B). Note that knocked-in WT-Cx43 and all mutants are biotinylated.  

C. CP43KD cells expressing knocked-in WT-Cx43 or its mutants were biotinylated at 4°C and the kinetics of degradation were determined by incubating cells for various times at 37°C. Note that WT-Cx43 and the mutants are degraded with similar kinetics. Biotinylation of Desmoglein-2 (Dsg-2), a desmosome-associated protein, was used as a control. For the input, 10 µg of total protein was used and probed for Cx43 or Dsg-2.

**Figure S6. A. Subcellular fate of Knocked-in WT-Cx43 and its mutants in CP43KD cells.** A. CP43KD cells stably expressing knocked-in WT-Cx43 (WT), YA/VD-Cx43 (YA/VD), S279A or S279D were seeded at single cell density (see Materials and Methods) and immunostained for Cx43 (red) and clathrin or Lamp1 (green). Note that WT-Cx43 and S279D co-localize robustly with clathrin whereas YA/VD-Cx43 and S279A do not discernibly co-localize. Note also that WT-Cx43 and all mutants co-localize extensively with Lamp1. B. Gap junctions composed of knocked-in YA/VD-Cx43 (YA/VD) in CP43KD cells are internalized into large vesicular puncta. CP43KD cells stably expressing YA/VD-Cx43 were immunostained for Cx43 (red) and caveolin 1 (Cav1) or Lamp1 (green). Note relatively large and varied size of Cx43-containing intracellular puncta. Partial co-localization of intracellular Cx43 puncta with Lamp1 was observed. Note also the lack of co-localization of YA/VD-Cx43 with either Cav1 or clathrin.

**Figure S7. Inhibition of MAP Kinase activity enhances gap junction assembly in LNCaP cells.** A. Cx43-expressing LNCaP cells were treated with 5 or 10 µM U0126 for 30min. Cells were fixed and immunostained for Cx43 (green) and E-cadherin (red). Note that U0126 treatment increases the number as well as immunostaining intensity of Cx43-gap-junction puncta. B. Cells treated with 10 µM U0126 (TREATED) or 0.1 % DMSO (CNT) for the indicated time (in minutes) were lysed and probed with polyclonal antibody to phospho-ERK. Note inhibition of ERK activity. C. The average number of Cx43 gap junction puncta from images of Control (blue bars) and U0126-treated (red bars) cells were counted from 3 random microscopic fields captured with a 63x oil objective. All cell-cell interfaces of
approximately 20 µm or more were counted using the measure tool of Volocity. *=p<0.0001 as determined by the Student’s t-test. Bar = 20 µM in A. Inhibition of MAP Kinase activity has no effect on the assembly of Cx43 into gap junctions in BxPC3 and Capan-1 cells (data not shown).
