CONNEXIN 37 REGULATION OF CELL PROLIFERATION THROUGH BINDING INTERACTIONS WITH CONNEXIN 43 AND 14-3-3σ

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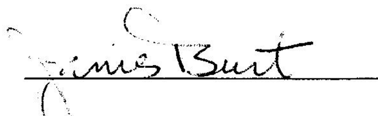

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Abstract

Whereas Cx43 expression has no effect on proliferation of Rin cells, Cx37 exerts a potent growth suppressive effect on these cells. To determine how Cx37 suppresses growth and whether co-expression of Cx43 altered the suppressive effects of Cx37, two cell lines were created. 43tet/37 C4 cells constitutively express Cx43 and the tet-on transcription factor, which supports tetractycline inducible Cx37 expression in these cells. i43/i37 D6 cells constitutively express the tet-on transcription factor, which supports inducible expression of both connexins. The 43tet/37 C4 cells exhibit high Cx43:Cx37 expression ratio, whereas the i43/i37 D6 cells have a low Cx43:Cx37 expression ratio. In both cell lines, Cx37 and Cx43 appear (by reciprocal co-immunoprecipitation experiments) to form heteromeric channels. The proliferative properties of the co-expressing cell lines show that Cx37 growth suppression in Rin cells is relieved in a dose dependent manner by the presence of Cx43. In contrast, we demonstrate that 14-3-3σ may play a role in the growth suppressive mechanism of Cx37 in Rin cells as it is upregulated in cells expressing Cx37 and has the capability of binding to Cx37, presumably at the consensus binding motif in the C-terminus.

Connexin 37 Regulation of Cell Proliferation Through Binding Interactions with Connexin 43 and 14-3-3σ

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Introduction

Gap junctions are intercellular channels, present in most tissues of the body, that allow for passage of ions and communication between cells. These channels are formed by docking at the extracellular surface of two connexons (hemichannels). Each connexon is composed of six connexins; the connexins comprise a gene family with twenty-one members in humans and twenty in mice with nineteen orthologous genes (Sohl et.al 2004).

Connexins have long been recognized as coordinators of tissue function and regulators of growth through three mechanisms. As intercellular channels, gap junctions serve to mediate electrical and chemical signaling between cells. This serves to promote coordinated tissue responses such as contraction/relaxation, cell proliferation and migration. In addition, connexons serve as transmembrane channels, or hemichannels, and modulate activity through paracrine and autocrine methods. This is most often seen in response to injury, ischemia or inflammation. Lastly, connexins interact with multiple cytosolic proteins mainly through the C-terminal cytosolic tail which often contains multiple phosphorylatable sites (Kardami et.al 2007). Through all three functions, intercellular and transmembrane channels, and protein-protein interactions, these proteins coordinate tissue functions. However, their molecular mechanisms of action are incompletely understood.

Nearly all cells express multiple connexins (Sohl et.al 2004). In rat aortic endothelial cells, for example, multiple members of this gene family are expressed. Connexin 37 (Cx37) and connexin 40 (Cx40) dominate but, connexin 43 (Cx43) is also commonly expressed (Gabriels et.al 1998). It has also been shown that Cx43 and Cx37 localize in the zona pellucida of mouse ovarian follicles (Simon et.al 2006) among other tissues. Additionally, it is known that Cx37 and Cx43 are not only expressed within the same tissue types but can also form heteromeric channels, in which each hemichannel (connexon) contains at least two different connexins. Evidence of Cx37-Cx43 heteromeric channels has been shown in mouse neuroblastoma cells (Brink et. al 1997), and junctional plaques in rat myocardium (Larson et. al 2000). It is unclear why multiple connexins might be needed by cells and what the co-expression of connexins contributes to the overall functioning of the cell.

Connexins have been shown in numerous studies to serve as tumor suppressors. Tumorigenesis is often characterized by decreased expression of connexins and increased cell proliferation. However, with the introduction of connexin genes into tumorigenic cell lines, the rate of proliferation is slowed (Kardami et.al 2007). This has been shown with Cx43, Cx32, Cx26 (Kardami et.al 2007) and more recently with Cx37 (Burt et.al 2008). Cx43 is well documented to have a tumor suppressor function in various cell lines including: human osteosarcoma U2OS cells (Zhang et.al 2001), human epithelial cervical cancer HeLa cells (Dang et.al 2003) and human embryonic kidney HEK293 cells (Dang et.al 2006). Interestingly, Cx43 does not have a growth suppressive effect in rat insulinoma (Rin) cells. However, Cx37 is growth suppressive in Rin cells (Burt et.al 2008). Additionally, very little is known about Cx37 and growth suppression in other cell lines. This suggests that there is tissue specificity resulting

in differential growth control based on the cell type, connexin expression and the protein machinery available in the cell. These studies bring into question what unique characteristics may be present when connexins are co-expressed in culture.

The lab of J.Burt at The University of Arizona has found that expression of Cx37 by Rin cells results in suppression of cell proliferation (Burt et.al 2008). It is proposed that this is accomplished in a 14-3-3σ dependent fashion (Fig. 1). 14-3-3σ, aka stratifin, is a protein that is known to play a role in cell cycle regulation in a phosphorylation-dependent manner. Specifically, 14-3-3σ is a negative regulator of the cell cycle when it is expressed at high levels within cells. Its expression is activated by p53 in response to DNA damage. It then acts as a key component of the G2 checkpoint machinery. For example, under normal circumstances the protein complex cdc2-cyclin B1 needs to be translocated to the nucleus in late G2 in order for mitosis to ensue. However, when 14-3-3σ is present it sequesters cdc2-cyclin B1 in the cytoplasm resulting in cell cycle arrest (Chan et.al 1999, Hermeking et.al 1997, Satoh et.al 2006).

Within the Cx37 C-terminus there is a putative consensus site for 14-3-3σ binding. The 14-3-3σ consensus sequence for target proteins is R/K X X pS/pT X P (Uchida et.al 2006). The sequence K S P pS R P appears in the C-terminus of Cx37 with the phosphorylatable Serine at position 321. Data from C-terminus mutations and deletions from the lab of J.Burt (Table 1) are consistent with growth suppressive characteristics when this region of the C-terminus is maintained, allowing 14-3-3σ to bind. Interestingly, there are two consensus sites for 14-3-3σ in the Cx43 C-terminus with the phosphorylatable Serines at 244 and 373. However, S244 is very near where the protein's 4th membrane spanning domain is proposed to exit the membrane and is unlikely to bind 14-3-3σ. Serine 373 is near PKC phosphorylation and ZO1 binding sites, which may alter the ability to bind. Uchida et.al found that there were five 14-3-3σ putative binding sequences in cdc25B, though only three were active. They attributed absence of binding activity to amino acids C-terminal to the 14-3-3σ binding motifs, which alter the efficiency of 14-3-3σ binding (Uchida et.al 2006). Thus, it is likely the environment surrounding the binding motif plays a role in the ability of 14-3-3σ to bind and have an effect. Consequently, this could render the 14-3-3σ binding sites inactive in Cx43 and result in a regulatory mechanism of growth suppression that is uniquely different from Cx37.

The subject of the current study was to evaluate the impact on growth of two Cx37-interacting proteins: 14-3-3σ and Cx43. The goal was two pronged: to explore how Cx37 could promote growth suppression in Rin cells via interactions with 14-3-3σ, and to show how this suppression might be relieved in a regulated fashion via co-expression with Cx43.

Methods

Antibodies and reagents. All general chemicals, unless otherwise noted, were purchased from Sigma Aldrich Chemical. The following antibodies were utilized for immunoblotting and immunoprecipitation: Cx37 antibody was produced by the lab of A.Simon (University of Arizona) (αCx37-18264) (Simon et.al 2006), Cx43 antibody was purchased from Sigma (C6219), and 14-3-3σ (E-11) antibody was purchased from Santa Cruz Biotechnology (sc-166473). Cx37 and Cx43 primary antibodies were detected with anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody from GE Healthcare UK Limited (NA934V). 14-3-3σ antibody was detected with anti-mouse IgG horseradish peroxidase linked secondary antibody

from Promega (W402B). Enhanced chemiluminescence (Super Signal West Dura from Pierce (product #1859025 and 1859024) strategies were used to detect HRP in bands on immunoblots.

Generation of connexin plasmids. Plasmids were created in order to obtain co-expression of Cx37 and Cx43. Two strategies were employed to accomplish this. The first strategy involves a double inducible system for expression of Cx43 and Cx37. Cx43 was inserted into the multiple cloning site of the pTRE2-puro vector for constitutive expression of the puromycin resistance gene but inducible expression of the Cx43 gene. The resulting plasmid is pTRE2-Cx43-puro. Cx37 was inserted into the multiple cloning site of the pTRE2-hygro vector for constitutive expression of the hygromycin resistance gene but inducible expression of the Cx37 gene. The resulting plasmid is pTRE2-Cx37-hygro. Both the pTRE2-Cx43-puro and the pTRE2-Cx37-hygro plasmids were previously created by the J.Burt lab (University of Arizona) (Burt et.al 2008). Both vectors were them simultaneously transfected into iRin cells (described below). This cell line is described as i43/i37.

The second strategy involves an IRES vector that constitutively drives expression of an mRNA that encodes Cx43 and the tet-on transcription factor rtTA. This plasmid was created by amplifying rtTA out of the pTet-On vector (Clontech, Mountain View, CA) using PCR. The primers used for PCR amplified out the rtTA transcript with the addition of a *NotI* restriction enzyme site in the 5' primer. The rtTA gene was then digested with *NotI*. The pIRES vector was digested with *NotI*, and the rtTA insert was ligated into the vector at multiple cloning site (MCS) B. The rtTA insert contained an *EcoRI* site which would hinder insertion of the next transcript therefore, the *EcoRI* site in the rtTA gene was mutated using the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Subsequently, pIRES-rtTA with *EcoRI* mutation was digested with *EcoRI*. The Cx43 insert was prepared by digesting pcDNA m43 with *EcoRI*. The sample was purified on a gel and extracted with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The m43 *EcoRI* digested insert was ligated into the MCS A of pIRES rtTA with *EcoRI* mutation. The ligation reaction was transformed into Maximum Efficiency DH5α Competent Cells (Invitrogen, Carlsbad, CA) following the manufacturer's instructions; a colony was removed from the Luria Broth (LB) agar plate with ampicillin, and miniprepped using the alkaline lysis method by Maniatis. The sample was sent to sequencing to insure proper orientation of the insert. Upon positive detection of the insert's orientation, the ligation reaction was transformed into Subcloning Efficiency DH5α Competent Cells (Invitrogen, Carlsbad, CA) following the manufacturer's instructions; a colony was removed from the LB agar plate with ampicillin, and maxiprepped with the QIAgen Maxiprep Kit (Qiagen, Valenica, CA). The resulting plasmid is pIRES-Cx43-rtTA-neomycin. The vector was then simultaneously transfected (described below) into Rin1046 cells with the previously described pTRE2-Cx37-hygro vector. This cell line is described as 43tet/37.

Cell culture and expression vectors. Communication deficient rat insulinoma (Rin) cells (Rin1046-38) were obtained from R.Lynch (University of Arizona). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Sacramento, CA) and antibiotics (300 µg/ml penicillin and 500 µg/ml streptomyocin) at 37°C in a humidified, 5% CO_2 incubator. Previously, pTet-On (Clontech, Mountain View, CA) was transfected into Rin cells using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions to produce an inducible Rin cell line (iRin) where inserted genes of interest can be expressed upon induction with doxycyline (dox; an analog of tetracycline). Cells

were cultured in supplemented RPMI as previously described with the addition of 300 μg/ml neomycin (G418; Gibco). Previously created iRin37 cells contain pTRE2-mCx37 and express Cx37 upon dox induction. Cells were cultured in supplemented RPMI as previously described with the addition of 300 μg/ml G418 and 100 μg/ml hygromycin. NIH 3T3 cells were obtained from J.Martinez (University of Arizona Cancer Center). Cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics (300 μg/ml penicillin and 500 μg/ml streptomyocin), at 37°C in a humidified, 5% CO_2 incubator.

Two cells lines were created with the addition of the newly generated connexin containing plasmids described above. The cell line 43tet/37 was created when Rin 1046 cells were simultaneously transfected with pIRES-Cx43-rtTA-neomycin (2 μg) and pTRE2-Cx37-hygro (2 μg) using Lipofectamine according to the manufacturer's instructions. Stably transfected cells were selected with 300 μg/ml G418 and 100 μg/ml hygromycin in RPMI supplemented medium. Cells were screened for constitutive expression of Cx43 and Cx37 expression upon dox (2 μg/ml) induction. Based on expression levels, the 43tet/37 C4 clone was chosen for experimentation. The cell line i43/i37 was created when iRin cells were simultaneously transfected with pTRE2-m43-puromyocin (2 μg) and pTRE2-mCx37-hygromycin (2 μg) using Lipofectamine according to the manufacturer's instructions. Stably transfected cells were selected with 300 μg/ml G418, 100 μg/ml hygromycin and 0.5 μg/ml puromycin in RPMI supplemented medium. Cells were screened for expression of Cx43 and Cx37 upon dox induction. Based on expression levels, the i43/i37 D6 clone was chosen for experimentation.

Immunoblotting. Whole cell protein was prepared for Western Blot analysis. Cells were washed three times with PBS, harvested from their dishes by scraping and then pelleted by centrifugation at 8,000 *g* for 5 minutes. Sample buffer [100 mM Tris, 4% SDS, 10% glycerol, 5 mM NaF, 0.25 mM Na_3VO_4, 2 mM PMSF and 0.02% bromophenol blue with added protease inhibitor cocktail (Roche catalogue# 11836153001); pH 6.8], was added to lyse the cells, and then the solution was sonicated briefly. Centrifugation at 16,000 *g* for 10 minutes was utilized to remove debris. The sample's protein concentration was determined with a BCA assay (Pierce Chemical, Rockford, IL). Samples (15-50 μg of total protein) underwent electrophoresis on 12% Tris HCl gels (Bio-Rad, Hercules, CA). The protein was transferred onto a nitrocellulose membrane for antibody staining. The blot was blocked in 5% non-fat-dry-milk (NFDM) and then placed in the appropriate primary antibody in 1% NFDM. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescnce strategies were used to visualize the blot using the Kodak ID Image Station.

Co-immunoprecipitation. Cells for co-immunoprecipitation were washed three times with PBS. Subsequently, one ml of non-denaturing lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) NaN_3, 1X NaF/Na_3VO_4, 2 mM PMSF, 1 tablet Roche Mini Complete Protease Inhibitor Cocktail] was added and the cells were harvested by scraping. The cell lysate was homogenized by passage through a 25 gauge needle 5 times followed by a 5 minute incubation period on ice. Samples were centrifuged for 15 minutes at 16,000 *g* at 4°C. The cell lysate was divided into 500 μl aliquots for each sample and placed in a clean 1.5 ml tube. 30 μl of 50% Protein A sepharose beads (Pierce product# 20333) were added to each tube and mixed overnight at 4°C on a rocker to clear the lysate. The samples were centrifuged for 5 minutes at 16,000 *g* at 4°C. Reciprocal immunoprecipitation was performed:

one tube of a given sample received 1 μg of antibody one; and, the other tube of a given sample received 1 μg of antibody two (either Cx37 and Cx43 antibodies or, Cx37 and 14-3-3σ antibodies). Samples were incubated for 1-2 hours at 4°C on a rocker. To each sample-antibody solution, 500 μl of PBS and 30 μl 50% Protein A sepharose beads were added and then mixed overnight at 4°C on a rocker. Samples were centrifuged for 2 seconds at 16,000 *g* at 4°C and the supernatant was removed. The beads were washed via resuspension with 1 ml cold wash buffer [0.1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) NaN_3]. Centrifugation and resuspension with wash buffer was repeated 3 times while keeping the samples on ice. Beads were rinsed with 1 ml cold PBS and centrifuged for 2 seconds at 16,000 *g* at 4°C and the supernatant was removed. 60 μl of 1X sample buffer was added to each sample. The samples were either stored at -20°C or prepared for immunobloting. Each sample was divided in half for reciprocal immunoblotting: one half of the sample was blotted with antibody one; and, the other half of the sample was blotted with antibody two (either Cx37 and Cx43 antibodies or, Cx37 and 14-3-3σ antibodies).

Proliferation. Proliferation assays were performed on cells plated into six-well plates at a density of 3 X 10^4 cells/well for all cell lines except for the RinGVA line which was plated at 1 X 10^4 cells/well. The day of initial cell plating represents day 0. An entire six-well plate is devoted to one day of cell counting with half of the cells receiving doxycyline (dox) treatment while the other half not. Twenty-four hours following plating, dox (2 μg/ml) was added to three of the six wells. Every 48 hours the cells were fed with their respective medium either with or without dox, as appropriate. Every 3 days, for either 21 days or 15 days, the cells in the wells devoted to that day of counting were harvested with use of trypsin. The number of cells in each well was determined via a hemocytometer. The three dox minus and the three dox plus cell counts were averaged respectively and plotted to visualize the proliferative properties of the cell line.

Results

Co-expression of Cx37 and Cx43 in Rin cells. Two methods were employed to create cell lines which express both Cx37 and Cx43. Since Rin cells expressing Cx37 are growth suppressed (Burt et.al 2008), an inducible expression system was used to create Rin cells that can be propagated for many generations without Cx37 expression, but easily induced to express this protein, as needed, experimentally. The first method yielded the cell line i43/i37 D6: iRin cells were simultaneously transfected with pTRE2-Cx43-puro and pTRE2-Cx37-hygro. Each plasmid results in transcription of two mRNAs, one that encodes for Cx gene expression under a promoter activated by the tetracycline responsive transcription factor (tet+), and the other encodes for antibiotic resistance under a constitutive promoter. Thus, expression of each connexin is only present after induction with doxycyline (2 μg/ml) for 24 hours. The second method yielded the cell line 43tet/37 C4: Rin1046 cells were simultaneously transfected with the generated plasmid pIRES-Cx43-rtTA-neomycin (see methods) and pTRE2-Cx37-hygro. The pIRES vector results in the transcription of an mRNA that encodes both Cx43 and a tetracycline sensitive transcription factor and pTRE2-Cx37-hygro vector results in two transcripts encoding Cx37 and the neomycin resistance. With dox induction, tet+ can interact with the pTRE2-Cx37-hygro plasmid and drive expression of Cx37. Hygromycin resistance is encoded under a constitutive promoter.

The two cells lines were tested for their relative expression of each of the two connexin proteins after 24 hours of 2μg/ml dox induction, which was previously shown to produce maximal Cx37 expression in iRin cells (Burt et.al 2008). The co-expressing cell lines' expression of the connexin proteins was compared to standardized amounts of Cx-GST on an immunoblot. Looking at Cx37 relative expression (Fig.2), visualization revealed that i43/i37 D6 cells have greater Cx37 expression than 43tet/37 C4 cells (Fig.2*A*). Using the Cx37-GST standards, the net intensity of the band was plotted against the pmol concentration of the Cx37-GST protein (Fig. 2*B*). The trendline generated from these data was used to calculate the pmol concentration of Cx37 in our samples relative to the amount of total protein loaded. It was found that (Fig. 2*C*) i43/i37 D6 cells express Cx37 in the range of 5.5 X 10^{-04} to 1.0 X 10^{-03} pmol/μg. This variability is likely a result of the bands not being uniform in nature on the blot. Comparatively, the 43tet/37 C4 cells were calculated to express in the range of 6.6 X 10^{-04} to 3.4 X 10^{-04} pmol/μg. However, it is likely that the generated equation has a difficult time extrapolating at lower net intensities given that the trendline does not cross the axis at (0,0). In the end it is significant that the immunoblot detects a clear difference in Cx37 expression levels between the two cell lines with i43/i37 D6 representing high Cx37 expression, and 43tet/37 C4 representing low Cx37 expression. It has been shown by J.Burt (University of Arizona) that the iRin37 cell line expresses Cx37 in a functional range, resulting in growth suppression, of 8.21 X 10^{-03} to 4.23 X 10^{-04} pmol/μg. The i43/i37 D6 cell line falls squarely within this functional range of Cx37 protein expression. And if the 43tet/37 C4 values are accurate, it falls at the lower end of the functional range or just beyond it.

Looking at Cx43 relative expression (Fig.3), visualization revealed that 43tet/37 C4 cells have greater Cx43 expression than i43/i37 D6 cells (Fig. 3*A*). The same procedure was utilized to establish a trendline using Cx43-GST standardized protein amounts and the resultant net intensity of the band on the immunoblot (Fig. 3*B*). The trendline generated from these data was used to calculate the pmol concentration of Cx43 in our samples relative to the amount of total protein loaded. It was found that (Fig. 3*C*) i43/i37 D6 cells express Cx43 in the range of (-1.8 X 10^{-04}) to 8.0 X 10^{-04} pmol/μg. The negative value is likely due to the trendline's inaccuracies when extrapolating at lower net intensities. Comparatively, the 43tet/37 C4 cells are calculated to express in the range of 9.6 X 10^{-03} to 3.0 X 10^{-03} pmol/μg. Visualization of protein expression and calculated concentrations detect a clear difference in Cx43 expression levels between the two cell lines: 43tet/37 C4 representing high Cx43 expression, and i43/i37 D6 representing low Cx43 expression. Note that this is opposite to the trend seen with Cx37 expression such that, i43/i37 D6 has high levels of Cx37 expression and low levels of Cx43 expression. In contrast, 43tet/37 C4 has high levels of Cx43 expression and low levels of Cx37 expression.

Cx37 and Cx43 heteromerize. It has been shown that Cx37 and Cx43 are not only co-expressed in multiple cell types but that they form heteromeric channels, in which each hemichannel (connexon) contains a combination of each connexin, Cx37 and Cx43. Evidence of Cx37-Cx43 heteromeric channels has been shown in mouse neuroblastoma cells (Brink et. al 1997), and junctional plaques in rat myocardium (Larson et. al 2000). We expected that our co-expressing clones would behave similarly and reveal that the two connexin proteins heteromerize when co-immunoprecipitated. The stably transfected i43/i37 D6 and 43tet/37 C4 clones were dox induced (2 μg/ml) for 24 hours and then the cells were harvested under non-denaturing conditions. Either antibodies to Cx37 or Cx43 were used to immunoprecipitate (IP) the connexin protein out of solution. If Cx37 and Cx43 heteromerize, the use of either connexin antibody to IP should pull

both proteins out of solution since they are bound together in a hemichannel (connexon) configuration. The IP lysates were then subjected to reciprocal immunoblotting (IB) to detect if the two connexin proteins are bound together. When the samples underwent IB with Cx37 (Fig. 4*A*), Cx37 was detected in the Cx37 IP lanes. Additionally, there is a stronger band for the i43/i37 D6 clone than the 43tet/37 C4 clone which is consistent with our findings in the relative expression experiments. However, this blot does not reflect that Cx37 and Cx43 heteromerize since there is nothing present in the Cx43 IP lanes.

When the IP lysates underwent IB with Cx43 (Fig. 4*B*), Cx43 was detected in the Cx43 IP lanes and the Cx37 IP lanes reflecting that Cx37 and Cx43 do heteromerize in our co-expressing subclones. In the i43/i37 D6 clone it is evident that there is a band at approximately 43 kD in each of the IP lanes. For the 43tet/37 C4 clone, Cx43 was detected in the Cx43 IP lane. Looking closely at the sample in the Cx37 IP lane, there may be a faint band present. Co-immunoprecipitation and the formation of heteromeric channels in the 43tet/37 C4 clone should not be ruled out since the amount of Cx37 may be too low to detect with these measurements. The relative expression blot revealed very low Cx37 expression in the 43tet/37 C4 clone. Nonetheless, Cx37 is present and detectable with 37 IP and 37 IB (Fig. 4*A*), although the band is faint.

Cell proliferation in co-expressing cells. To determine whether co-expression of Cx37 with Cx43 relieves Cx37's growth suppressive effect, we compared the proliferative properties of the induced co-expressing i43/i37 D6 and 43tet/37 C4 cell lines with iRin, iRin37 and Rin GVA cells over a 15 or 21-day period. All cell lines were evaluated with doxycyline (dox+) and without doxycycline (dox-). The iRin and RinGVA (Fig. 5 *A* and *B*) cells all grew rapidly until density-dependent slowing of proliferation occurred near the end of the 21-day period. While the iRin37 dox- cells grow similarly to those noted above, their dox+ counterpart, which express Cx37, has significantly slowed proliferation during the 21-day time course. This finding (Burt et.al 2008) suggests that Cx37 is growth suppressive in Rin cells and that Cx43 (present in the Rin GVA cells) is growth permissive in Rin cells.

The co-expressing cell lines i43/i37 D6 (Fig. 5*D*) and 43tet/37 C4 (Fig. 5*C*) show relief of Cx37's growth suppressive effect. 43tet/37 C4 dox- has constitutive expression of Cx43 and no expression of Cx37. It shows rapid proliferation, which exceeds the RinGVA line also expressing Cx43. Upon dox induction (dox+), the cell line expresses Cx37 also. There is a decrease in the total cell count at day 12 while maintaining a similar trend in proliferation. This suggests that Cx37 still has a growth suppressive effect; however, Cx43 appears to compensate and continues to allow for growth but at a decreased rate. i43/i37 D6 dox- does not express any connexins. It shows steady proliferation in a near linear fashion. However, upon dox induction (dox+), the cell line expresses both Cx37 and Cx43. Growth is maintained similar to the dox-state through day 9, but at days 12 and 15 the growth is suppressed. Again this suggests that Cx43 relieves the Rin cells of the pronounced growth suppression seen in the iRin37 cells; however, Cx37 still plays a role in the proliferative properties of the cells.

Presence of 14-3-3σ. It is hypothesized that 14-3-3σ binding to the C-terminus of Cx37 is what results in the growth suppressive properties of Cx37 in Rin cells. To determine if this hypothesis is probable, we first needed to determine the presence of 14-3-3σ in the cell lines of interest. Based on our communications with J.Martinez (University of Arizona Cancer Center), the NIH 3T3 cell line is known to express 14-3-3σ. This cell line serves as a positive control to compare

the expression of 14-3-3σ with the other cell lines utilized in the J.Burt lab (University of Arizona). Initially immunoblotting was performed with the NIH 3T3 cell line and the iRin37 dox+ cell line using the 14-3-3σ (E-11) antibody purchased from Santa Cruz Biotechnology, Inc. (Fig. 6*A*). The manufacturer states that the antibody will detect a band at 30kD for the sigma isoform of 14-3-3. However, both the control lane (NIH 3T3) and the experimental lane (iRin37 dox+) show a multiple banding pattern. The most pronounced band appears at 50kD and not 30kD. Subsequently, we requested the manufacturer's control for the antibody, A431. Upon immunoblotting with the 14-3-3σ (E-11) antibody, there appears to be a pronounced band at 30kD and another band at 40kD. Interestingly however, when an immunoblot was run on another Rin cell line not containing Cx37, iRin, to determine if the expression of 14-3-3σ is a product of dox induction, the lanes appear blank and there is no band detection. Thus, they do not have significant expression of 14-3-3σ. The results do not appear to be due to dox induction since there is no difference in the iRin dox-/dox+ lanes. These blots do not rule out that the NIH 3T3 and the iRin37 dox+ cells express 14-3-3σ. Instead, it leads us to believe that there are modifications to the protein within the cell such as phosphorylation or bonding with other proteins resulting in the multiple banding patterns.

Binding of 14-3-3σ to Cx37 C-terminus. 14-3-3σ is likely present in iRin37 dox+ cells. The next step was to determine if the 14-3-3σ protein binds (to the consensus binding motif in the C-terminus of) Cx37. Reciprocal co-immunoprecipitation was done as previously described using antibodies for Cx37 and 14-3-3σ (E-11). Using reciprocal co-immunoprecipitation (Fig. 6*B*) we were not able to detect that 14-3-3σ is bound to Cx37. However, 14-3-3σ was detected in iRin37 dox+ cells with 14-3-3σ IP and 14-3-3σ IB. Interestingly, in multiple trials this band consistently appears just below 30 kD. Ultimately, this confirms that 14-3-3σ is present in iRin37 dox+ cells despite the multiple banding seen in immunoblots previously. Additionally, we were able to detect Cx37 in the iRin37 dox+ cells once again confirming that the cells do express Cx37 upon dox induction.

In a collaborative effort with the J.Martinez lab (University of Arizona Cancer Center), we were able to show that 14-3-3σ is capable of binding to (the consensus binding motif in the C-terminus of) Cx37 using a GST pull-down assay (Fig. 6*C*). The GST pull-down assay is an in-vitro way to determine protein-protein interactions. This is similar to co-immunoprecipitation but differs in that a bait protein (14-3-3σ) is used instead of an antibody. The bait protein will capture and pull-down any protein-binding partners in the solution. Protein complexes contained within the elution can then be detected with immunoblotting. We sent the J.Martinez lab lysate harvested from the iRin37 dox+ cells and they performed the GST pull-down assay. 14-3-3σ GST (the bait) was used to capture protein-binding partners (prey) in the lysate. The elution was then immunoblotted with Cx37 antibody. This shows that 14-3-3σ can bind to Cx37. The next step is to show that they do bind in Rin cells, and under what conditions this occurs.

One hypothesis is the 14-3-3σ is upregulated in response to Cx37 presence such that they may regulate the actions of one another. Continuing to collaborate with the J.Martinez lab (University of Arizona Cancer Center), we were able to show that 14-3-3σ is upregulated in iRin37 dox+ cells (expressing Cx37) compared to iRin37 dox- cells (not expressing Cx37) (Fig. 6*D*). NIH 3T3 cells were used as a control for 14-3-3σ expression. The β-actin immunoblot shows that this is not a factor of variation in total protein concentration within the sample because similar amounts of protein were loaded into each lane.

Discussion

The growth-suppressive properties of connexins have long been studied. These studies often focused on Cx43, Cx32 and Cx26 growth suppression in various tissues (Kardami et.al 2007). Recently, it has been shown that Cx37 also has growth suppressive properties in Rin cells (Burt et.al 2008). These observations suggest that tissue specificity plays a role in the connexin's proliferative effect in cells. This is likely a factor of the cell type, the machinery present within the cell and the expression level of connexins. It is known that connexins are often co-expressed in cells and some are able to form heteromeric channels (Sohl et.al 2004, Gabriels et.al 1998, Simon et.al 2006, Brink et.al 1997, Larson et.al 2000). Even so, different connexins within the same tissues respond to growth stimuli differently. Gabriels et.al. reported in their 1998 paper that Cx43 and Cx37 were oppositely regulated (Cx37 was down regulated and Cx43 was upregulated) when exposed to a growth stimulus. Through experimentation in the lab of J.Burt (University of Arizona), Cx43 expressing Rin cells are proliferative but, Cx37 expressing Rin cells are growth suppressed. This further suggests counter regulation of these connexins. In our present study we hypothesized that the proliferative properties of Cx43 would relieve the growth suppressive properties of Cx37 when co-expressed in Rin cells.

The first task was to create a cell line that allowed for co-expression of these two connexins which show opposite proliferative properties. Two strategies were used to do this. The lab of J.Burt already utilized an inducible system for Cx37 expression such that a cell line can be maintained for multiple generations without succumbing to the growth suppressive effects of Cx37 yet, Cx37 can be induced to express experimentally. The first of the strategies utilized this inducible Cx37 system but allowed for constitutive expression of Cx43- 43tet/37 C4. This system was useful in allowing us to look at the effects of Cx43 with or without Cx37 expression within the same cell line. We found that the 43tet/37 C4 cell line has high expression of Cx43 and low expression of Cx37. The second cell line introduced here represented the opposite expression profile, i43/i37 D6 has high expression of Cx37 and low expression of Cx43. This cell line requires doxycycline induction of both connexins for expression. This provided a way to look at cell growth without connexin control, and with dual connexin control.

Our data indicate that it is likely that Cx37 and Cx43 heteromize in Rin cells. This is significant because the formation of Cx37-Cx43 heteromeric hemichannels (connexons) or gap junctions may result in unique properties that are not the expected sum of Cx37 and Cx43 properties. For example, Rin37 dox+ cells are profoundly growth suppressed, but we hypothesized that co-expression with Cx43 would relieve much of that effect. We found that Cx37 continues to play a role in the proliferative properties of the co-expressing Rin cells, but, the growth suppression is not as pronounced when co-expressed with Cx43. This suggests that Cx43 is able to compensate for or counteract some of the Cx37 effects and moderately relieve the growth suppression. Our results suggest that the extent of this relief may be attributed to the relative expression of each connexin in the cell lines. The 43tet/37 C4 cell line, with high Cx43 expression, showed less growth suppression than the i43/i37 D6 cell line, with high Cx37 expression. Also, there appears to be altered growth patterns at day 12 in both of the co-expressing cell lines. Further experiments may look at cell cycle time and cell turnover for the co-expressing cell line to explain this key time point.

Cx43 and Cx37 have been shown to have different phosphorylation patterns following activation by growth factor signaling. This makes it highly probable that they are interacting with different binding partners to carry out their proliferative or growth suppressive effects. One of

the binding partners of Cx37 is likely 14-3-3σ, a phosphorylation-dependent negative cell cycle regulator. Within the Cx37 C-terminus there is a consensus site for 14-3-3σ binding. The sequence K S P pS R P appears in the C-terminus of Cx37 with the phosphorylatable Serine at 321. Interestingly, there are also two consensus sites for 14-3-3σ in the Cx43 C-terminus with the phosphorylatable Serines at 244 and 373. However, it is hypothesized that these sites are inactive. S244 is very near where the protein is proposed to exit the membrane and is unlikely to bind 14-3-3σ. The other site, Serine 373, may reveal the importance of differential phosphorylation and other binding partners. This site is near a known PKC site at S368, which could alter phosphorylation patterns and potentially the ability for 14-3-3σ to bind. There is also a ZO1 site nearby, which is a cell-adhesion plaque protein that upon binding regulates transcription factors or growth factor signaling (Kardami et.al 2007). Thus, complexes of Cx43 with PKC or ZO-1 may alter the ability of 14-3-3σ to bind. Moreover, Uchida et.al found that there were five 14-3-3σ binding sequences in cdc25B, a phosphatase involved in cell-cycle regulation. Only three of the five binding sites in cdc25B were active, which they attributed to amino acids C-terminal to the 14-3-3σ binding motif that alter the efficiency of 14-3-3σ binding (Uchida et.al 2006). It is likely that the environment surrounding the binding motif in connexins plays a significant role in the ability for 14-3-3σ to bind. This may be a result of phosphorylation patterns or steric hindrance due to the proximity to the membrane or other bound proteins. Consequently, this could render the 14-3-3σ binding sites inactive in Cx43 and result in a regulatory mechanism of growth suppression that is uniquely different from Cx37.

It is hypothesized that the growth suppressive properties of Cx37 work through a 14-3-3σ dependent mechanism (Fig. 1) and that the consensus binding motifs in Cx43 are inactive. This hypothesis is supported by the proliferative properties of Cx37-mutants from the J.Burt lab (Table 1). Oftentimes, the Cx37-mutant expressing cells are growth suppressed when the 14-3-3σ consensus binding motif is maintained in the C-terminus. It is postulated that 14-3-3σ binding to the consensus binding motif on the C-terminus of Cx37 may aid in activating 14-3-3σ such that it binds to and sequesters in the cytoplasm cell-cycle regulatory proteins necessary in the nucleus for cell cycle progression, and thereby resulting in cell cycle arrest. The binding of cdc2-cyclin B1 has been previously described as being sequestered in the cytoplasm by 14-3-3σ (Chen et.al 1999). This arrests the cells at the G2/M boundary (Satoh et.al 2006). Other cell-cycle regulatory complexes containing the appropriate binding motif for 14-3-3σ are probable players as well. For example, the 14-3-3σ-cdc25C complex doesn't allow for dephosphorylation of cdc2 thereby preventing mitosis (Hermeking et.al 1997). Interestingly, Cx37 growth suppression is accomplished by arresting the cell cycle at the G1/S boundary (Burt et.al 2008). This does not rule out the role of 14-3-3σ because it has many potential binding partners; it may suggest that Cx37 activation of 14-3-3σ supports interaction of this protein with currently unknown cell-cycle regulators participating in the G1/S transition. This is an area that needs further study to gain an enhanced understanding of what occurring.

Our data suggest that 14-3-3σ is able to bind to Cx37 (Fig. 6*C*). Whether or not this binding occurs within the Rin cells is still uncertain as there was no reciprocal binding shown by co-immunoprecipitation (Fig. 6*B*). Nevertheless, interaction of these proteins should not be ruled out since 14-3-3σ binding to target proteins depends on phosphorylation of those proteins. Typically, only a small percentage of the total amount of a given protein expressed by a cell is in a specific phosphorylation state, thus interaction between 14-3-3σ and Cx37 could be occurring but not detected. The conditions within the cells at the time of their harvest may not have been appropriate for the necessary phosphorylation pattern to be present. Another possibility is that

14-3-3σ binds transiently during its activation and as a result, at any given moment the number of complexes present is too small to be detected. All of these scenarios, and others, are probable situations and thus, more research is needed. One experimental strategy for increasing the chance of observing 14-3-3σ/Cx37 complexes would be to induce differential phosphorylation of Cx37 in the Rin cells, perhaps by introducing a constitutively active form of a kinase that targets the consensus site, and then assess 14-3-3σ binding by co-IP strategies. This could also be done with Cx43 to determine if Cx43 is growth permissive in Rin cells because it requires a specific (and different) internal or external environment in order to become growth suppressive, which is typically unavailable in our cultured cells. In the end, it is promising that 14-3-3σ is able to bind Cx37 in a GST pull-down assay based on the affinity of the proteins for one another. This offers some insight as to what may be occurring in the Cx37 expressing Rin cells that results in their growth suppressed state and provides additional areas for future study.

In summary, we demonstrated two methods to obtain Cx37-Cx43 co-expressing cell lines. These cells lines showed reciprocal expression levels of each of the connexin proteins. We provided evidence that Cx37-Cx43 in these new cell lines likely form heteromeric channels. In co-expressing cell lines, we showed that the growth suppressive effect of Cx37 is moderately compensated for or counteracted by Cx43 expression. In addition, we demonstrated that 14-3-3σ may be part of the underlying mechanism of Cx37-mediated growth suppression in Rin cells as it is upregulated in these cells following induction of Cx37 expression and has the ability to bind to Cx37. In the end, there remain many unanswered questions. More experiments with the Cx37-Cx43 co-expressing cell lines need to be done in order to obtain a clearer picture of how Cx37 suppresses growth and how Cx43 co-expression might attenuate the growth suppressive effect of Cx37. Also, work needs to be continued with 14-3-3σ to explain the multiple banding patterns seen in immunoblots and to determine the next step in the signaling cascade.

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Figures

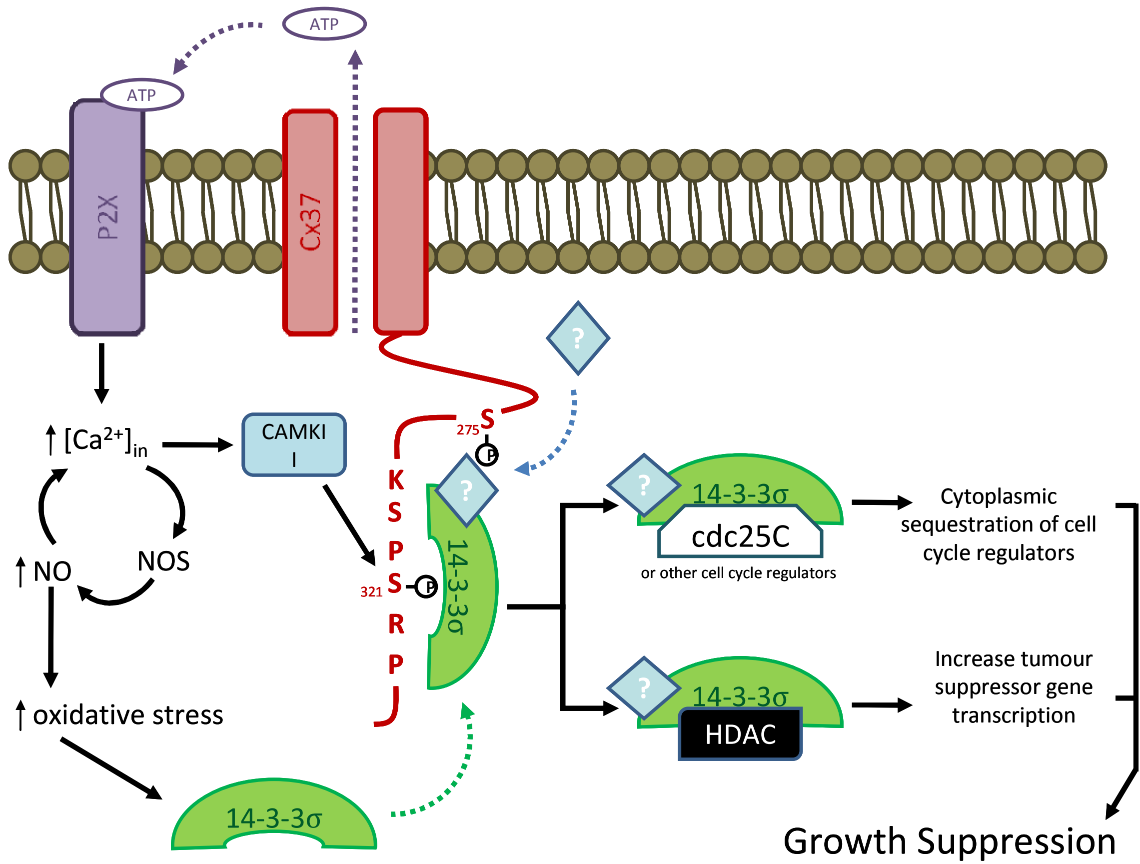


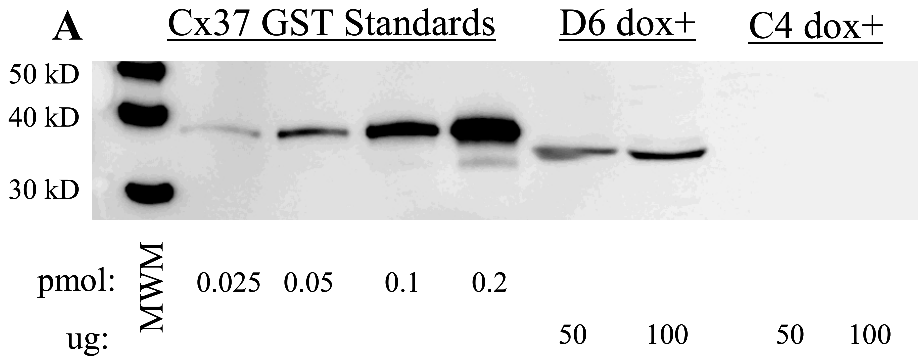
Fig. 1. Burt Lab's proposed Cx37 growth suppression mechanism. Image courtesy of Jennifer Fang, Tasha Nelson and Miranda Good.

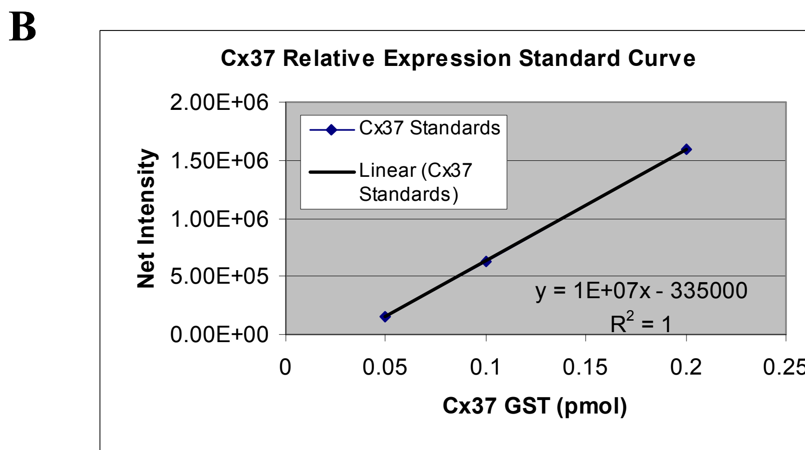
Mutant	Grows	Growth Suppressive	14-3-3σ Mechanism
Δ318		X	
Δ273	X		X
Δ273-317		X	X
Δ247	X		X
ΔS319,321A	X*		X
ΔS319,321D		X*	
S319A		X	X
S275D		X*	
S275,319D		X*	X
S275, 319A	X*		
S275,319,328D	X*		

* indicates preliminary data

Table 1. Proliferative properties of Cx37 mutants from the Burt Lab. Last column denotes which mutants' growth effects are consistent with the hypothesis that 14-3-3σ binding to Cx37 results in a growth suppressed state in Rin cells. Mutants Δ273, Δ247 and ΔS319, 321A show that removal or alteration of the putative binding site for 14-3-3σ results in growth. Thus, they fit the 14-3-3σ hypothesis which requires the presence of the putative binding site on the Cx37 C-terminus to have an effect in the underlying mechanism of Cx37-mediated growth suppression. The other mutants consistent with the hypothesis demonstrate that removal or alterations of other amino acids in the Cx37 C-terminus, apart from the putative 14-3-3σ binding site, continue to allow for Cx-37 mediated growth suppression in Rin cells. The remaining table entries show that there is variation present which suggest the role of other key amino acids (S275 is postulated to be necessary) and the environment surrounding the binding site.

Relative Expression of Cx37



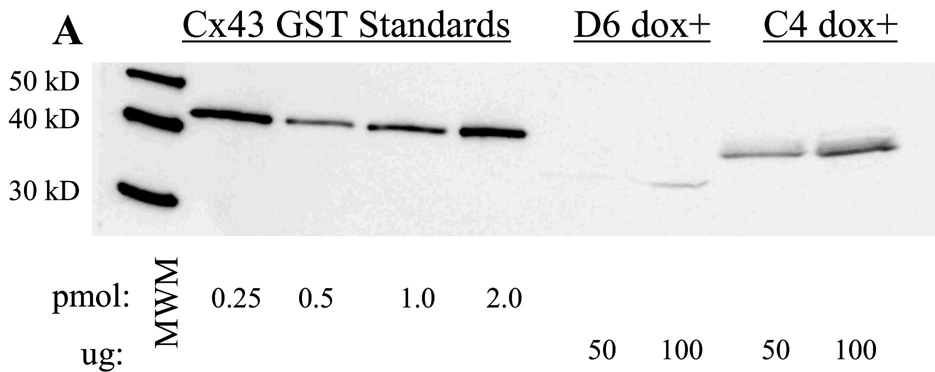


C

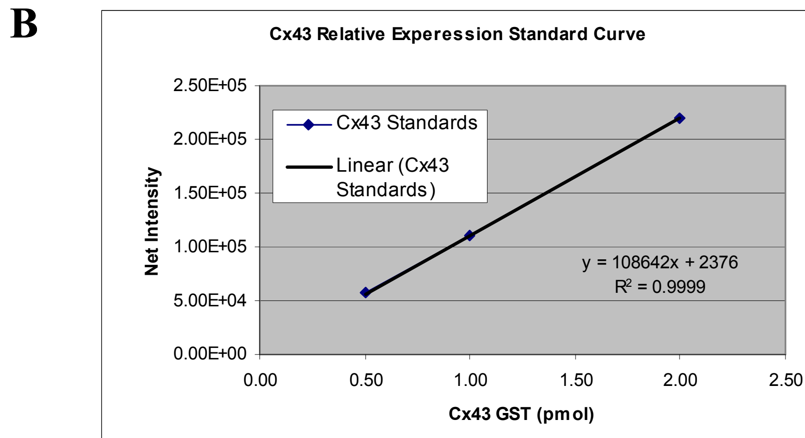
Sample	Calculated pmol of Cx37	Cx37 pmol/ug
i43/i37 D6 50ug	4.95E-02	1.0E-03
i43/i37 D6 100ug	5.45E-02	5.5E-04
43tet/37 C4 50ug	3.30E-02	6.6E-04
43tet/37 C4 100ug	3.35E-02	3.4E-04

Fig. 2. Western blot shows relative expression of Cx37 protein compared to Cx37-GST standards in clones i43/i37 D6 and 43tet/37 C4 following induction with doxycycline for 24h. The D6 clone expresses significantly more Cx37 expression than the C4 clone (A). pmol indicates the picomoles of Cx37-GST protein loaded in each lane; µg indicates the amount of total protein for the indicated cell line loaded in each lane. (B) Densitometry was performed on the Cx37-GST bands and used to create a standard curve of Cx37 band intensity vs. pmol of Cx37-GST. A linear regression trendline was fit to the data and the resulting equation was used to calculate the expression of Cx37 in the clonal cell lines. (C) The 43tet/37 C4 clone has very low expression of Cx37, which is virtually undetectable on a Western blot. The i43/i37 D6 clone has high expression of Cx37 (1.0×10^{-03} pmol/µg) upon induction (dox+). The iRin37 cell line expresses Cx37 in a functional range of 8.21×10^{-03} to 4.23×10^{-04} pmol/µg.

Relative Expression of Cx43



B



C

Sample	Calculated pmol of Cx43	Cx43 pmol/ug
i43/i37 D6 50ug	-9.00E-03	-1.80E-04
i43/i37 D6 100ug	8.00E-02	8.00E-04
43tet/37 C4 50ug	4.80E-01	9.60E-03
43tet/37 C4 100ug	9.00E-01	9.00E-03

Fig. 3. Western blot shows relative expression of Cx43 protein compared to Cx43 GST standards in clones i43/i37 D6 and 43tet/37 C4 upon induction (dox+). The C4 clone expresses significantly more Cx43 than the D6 clone, the opposite of what was seen for Cx37 in these same cell lines (A). Using the net intensity of the GST-Cx43 standards, a standard curve was created and fit by linear regression (B). The resulting equation was used to calculate the relative expression of Cx43 in the clonal cell lines. The 43tet/37 C4 clone has very high expression of Cx43 (in the range of 9.00×10^{-03} pmol/µg to 9.60×10^{-03} pmol/µg) while the i43/i37 D6 clone has low expression of Cx43 (maximum shown at 8.00×10^{-04} pmol/µg) upon induction (dox+) (C).

Co-Immunoprecipitation

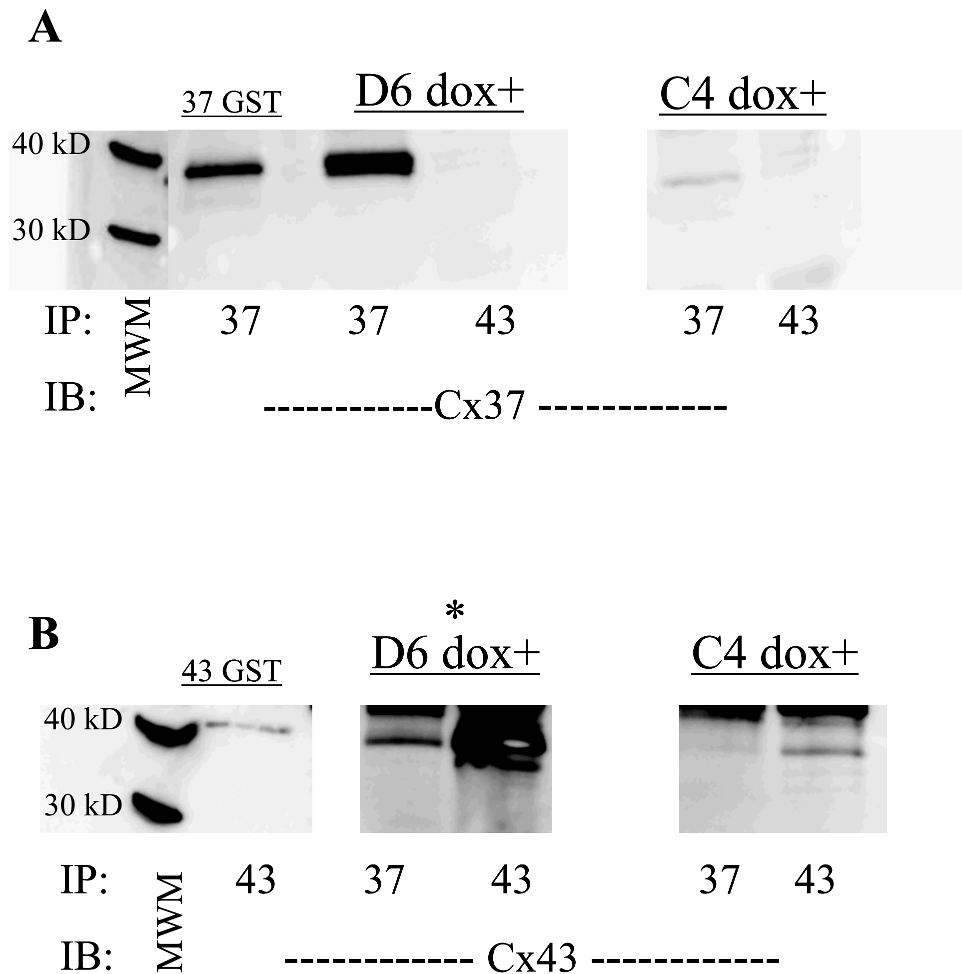


Fig. 4. Protein was isolated from the D6 and C4 clones after induction (dox+) using a non-denaturing lysis buffer. The lysate was then immunoprecipitated (IP) with Cx37 or Cx43 antibody. The samples were then immunoblotted (IB) with either Cx37 (A) or Cx43 (B) antibodies. The Cx37 IB shows expression of Cx37 in the 37IP lanes but Cx43 is not detected. The Cx43 IB shows Co-IP in the D6 clone with bands present in both 37 and 43 IP. The C4 clone is positive for Cx43 and Co-IP should not be ruled out (B).

Proliferation Curves

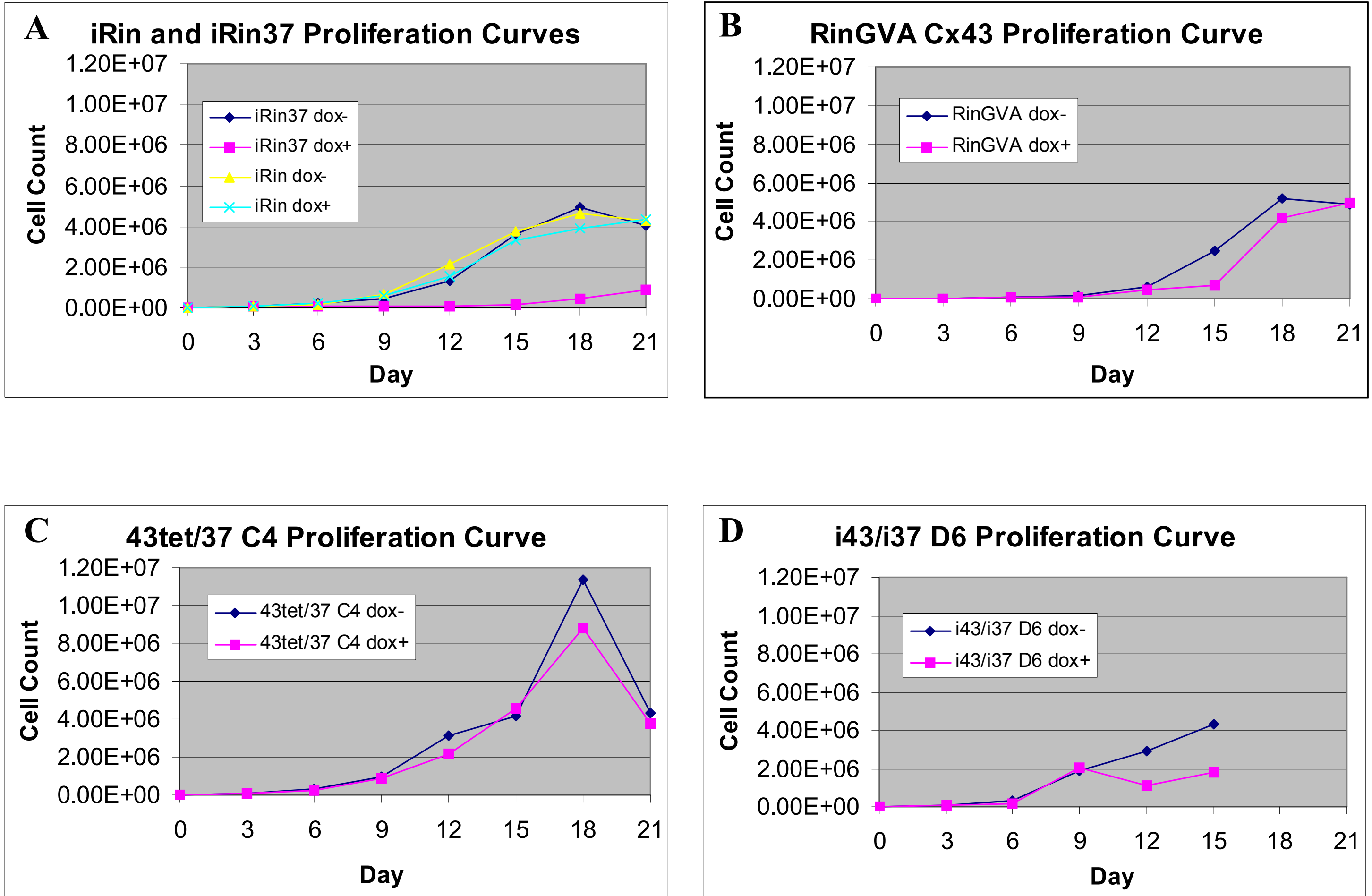


Fig. 5. Proliferation curves of the C4 and D6 clones were run for 21 and 15-days respectively and are compared to the Burt Lab data on iRin37, iRin and GVA cell lines. (A) Upon induction (dox+), the iRin37 cells' growth is suppressed compared to the control iRin cells and iRin37 dox-. (B) RinGVA cells express Cx43. There does not appear to be a significant difference between dox+ induction and dox- in these cells suggesting expression of Cx43 does not have a growth suppressive effect on Rin cells. (C) The C4 clone, which has high expression of Cx43 and low expression of Cx37, appears to grow comparably in the presence vs. absence of dox, suggesting that Cx37 is not exerting a potent growth suppressive effect in these cells. This clone appears to have a much higher cell count compared to the iRin and iRin 37 controls, which may be due to constitutive Cx43 expression. (D) The D6 clone has high expression of Cx37 and low expression of Cx43. It appears to grow more similarly to the iRins and iRin37s. At day 12 there is a decrease in the cell count for the dox+ cells compared to the dox- cells. Growth in the dox+ cells does reach a higher cell count than in the iRin37 dox+ cells however, this may reflect an attenuation of Cx37's growth suppressive effect by Cx43 expression.

14-3-3σ Expression, Co-Immunoprecipitation and GST Pull-Down

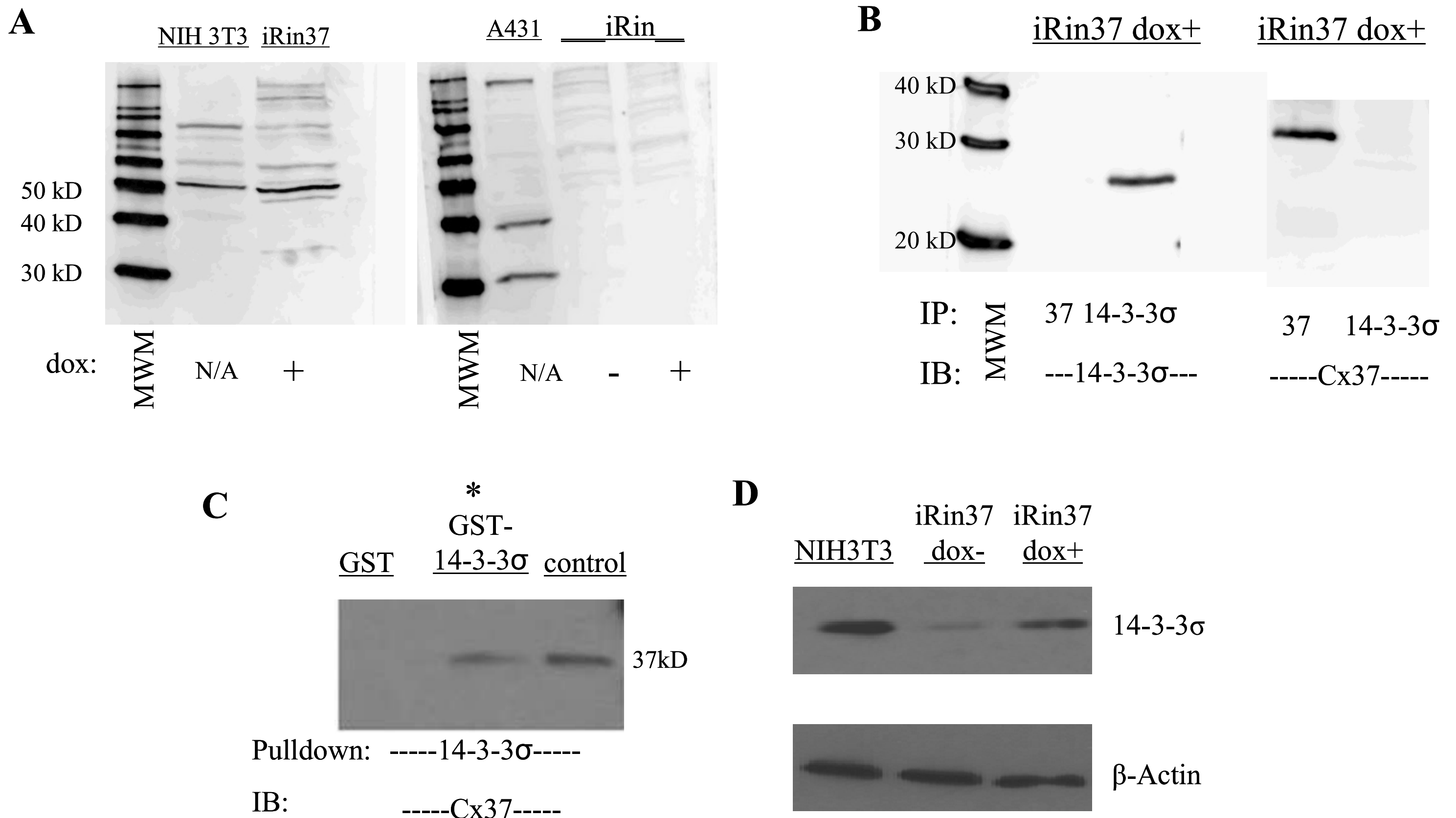


Fig. 6. 14-3-3σ is a scaffolding protein that is thought to play a role in the growth suppressive effects of Cx37. According to the manufacturer of anti-14-3-3σ, Santa Cruz Biotechnology, 14-3-3σ should run at 30kD on a Western Blot. Their control, the A431 lysate, does run at 30kD. The NIH 3T3 lysate should act as a control also but there is no band at 30kD; however, there is multiple banding present. The iRin37 cells show a similar multiple banding pattern to the NIH 3T3s. There is not presence of 14-3-3σ in the iRins with or without dox induction (A). Immunoprecipitation (IP) of iRin37 dox+ cells with anti-Cx37 and anti-14-3-3σ and subsequent immunoblotting (IB) with anti-14-3-3σ reveals expression of 14-3-3σ in iRin37s. We did not show co-immunoprecipitation of the proteins (B). Collaboration with the lab of J. Martinez (University of Arizona Cancer Center) provided a more sensitive assay for binding of Cx37 to 14-3-3σ. The GST pull-down assay shows that Cx37 can bind to 14-3-3σ (C). In another collaborative effort with the J.Martinez lab, they were able to show that with dox induction of Cx37 expression, there is increased expression of 14-3-3σ. The β-actin bands show that unequal loading of total protein cannot explain the observed increase in 14-3-3σ following dox induction because similar amounts of protein were loaded into each lane (D).