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14. ABSTRACT- Gap junctions (GJ) are conglomerations of cell-cell channels that are formed by a family of 21 distinct proteins, called connexin (Cx)s. The Cxs transmembrane proteins and are designated according to molecular mass. They are assembled into GJs through many steps (Figure 1). Communication through GJs is crucial for maintaining homeostasis [1;2]. Impaired, or loss of, Cx expression has been documented in the pathogenesis of various carcinomas [1;3-5]. Moreover, many studies have shown that over-expression of Cxs in tumor cells attenuates the malignant phenotype in vivo and in vitro, reverses the changes associated with epithelial to mesenchymal transformation (EMT), and induces differentiation [3;4;6]. For example, Cx32 is expressed in the liver, lung, and exocrine glands, and knock out studies have shown that the incidence of carcinogen induced tumors in these mice is higher [7-9]. Moreover, mutations in several Cx genes have been characterized in inherited diseases associated with aberrant proliferation and differentiation [1;10]. These studies support the notion that Cxs act as tumor suppressors. Despite this the molecular mechanisms by which GJs are assembled and disassembled are poorly understood.					
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## Table of Contents

	<u>Page</u>
Introduction.....	3
Body.....	4
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusion.....	5-7
References.....	8-10
Appendices.....	

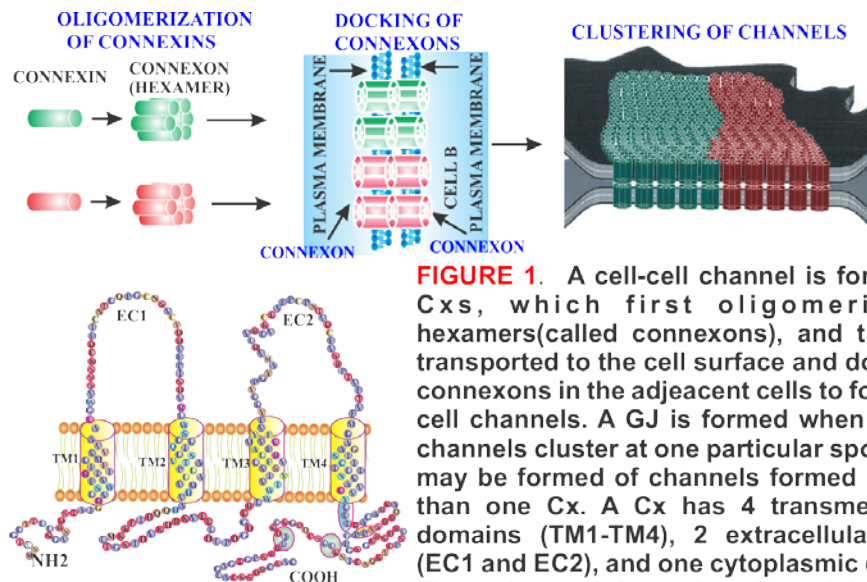
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1. Introduction:

Gap junctions (GJ) are conglomerations of cell-cell channels that are formed by a family of 21 distinct proteins, called connexin (**Cx**)s. The Cxs transmembrane proteins and are designated according to molecular mass. They are assembled into GJs through many steps (**Figure 1**). Communication through GJs is crucial for maintaining homeostasis [1;2]. Impaired, or loss of, Cx expression has been documented in the pathogenesis of various carcinomas [1;3-5]. Moreover, many studies have shown that over-expression of Cxs in tumor cells attenuates the malignant phenotype *in vivo* and *in vitro*, reverses the changes associated with epithelial to mesenchymal transformation (**EMT**), and induces differentiation [3;4;6]. For example, Cx32 is expressed in the liver, lung, and exocrine glands, and knock out studies have shown that the incidence of carcinogen induced tumors in these mice is higher [7-9]. Moreover, mutations in several Cx genes have been characterized in inherited diseases associated with aberrant proliferation and differentiation [1;10]. These studies support the notion that Cxs act as tumor suppressors. Despite this the molecular mechanisms by which GJs are assembled and disassembled are poorly understood.

**Aberrant Expression of Connexins in Prostate Tumors.**

Connexin32 is expressed in the luminal epithelial cells of the human prostate whereas Cx43 is expressed in the basal cells. In earlier studies we analyzed the distribution of Cx32 and Cx43 in 23 normal prostates, 43 benign prostatic hyperplasia specimens, 60 primary and 20 metastatic prostate tumors in archival and frozen sections. We found that epithelial cells from prostate tumors showed alterations with regard to sub-cellular localization of Cx32 and Cx43 *in vivo* and *in vitro*. In invasive tumors, Cxs remained intracellular and failed to assemble into GJs whereas in well differentiated prostate tumors both Cxs formed GJs at cell-cell contact areas [11-13]



**FIGURE 1.** A cell-cell channel is formed by Cxs, which first oligomerize as hexamers (called connexons), and then are transported to the cell surface and dock with connexons in the adjacent cells to form cell-cell channels. A GJ is formed when several channels cluster at one particular spot. A GJ may be formed of channels formed of more than one Cx. A Cx has 4 transmembrane domains (TM1-TM4), 2 extracellular loops (EC1 and EC2), and one cytoplasmic domain. The amino and the carboxyl termini face the cytoplasm.

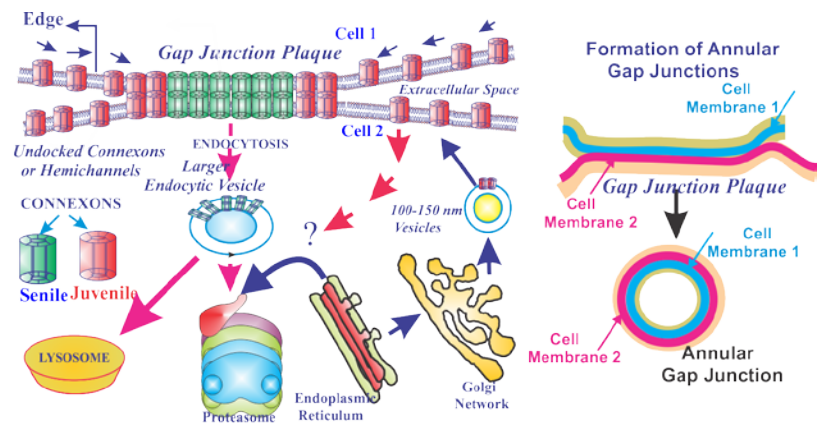
**Connexins are Prostate Tumor Suppressors.** Significantly, we showed that retrovirus-mediated expression of Cx32 and Cx43 into Cx-deficient and indolent and androgen-responsive PC cell line, LNCaP, induced the formation of GJs, restored junctional communication, inhibited growth *in vitro*, triggered differentiation, and retarded malignancy *in vivo* [13]. On the other hand, reintroduction of the same Cxs into an invasive, androgen-independent PC cell line, PC-3, resulted in Cx intracellular accumulation with no effect on growth [13]. Intracellular accumulation of Cxs was caused by defective GJ assembly and transient transfection of  $\alpha$ -catenin, a Cad associated protein deleted in PC-3 cells, induced GJ assembly [14]. Our subsequent studies showed that in androgen expressing LNCaP cells, androgens regulated the formation and degradation of GJs by controlling the expression level of Cx32 and Cx43 posttranslationally [15]. In the absence of androgens, a major fraction of Cx32 was degraded by the endoplasmic reticulum associated degradation whereas in their presence this fraction was rescued from degradation [15]. Our results also showed that degradation of Cx32 caused intracellular accumulation of tight junction associated protein, occludin, concomitant with its loss from the areas of cell-cell contact [15]. These finding identified Cxs as the downstream target of the signaling initiated by androgens.

## 2. Body

The proposed studies had two aims. In aim 1 we proposed to explore the molecular mechanisms by which formation of gap junctions retards cell growth in vivo and in vitro. The two questions addressed were: 1. Is the passage of small molecules through gap junctions required to retard tumor growth and invasion? 2. Does the formation of gap junctions retard tumor growth by inducing the assembly of other junctional and signaling complexes? In aim 2 we proposed to elucidate the molecular mechanisms by which E-cadherin and N-cadherin modulate gap junction assembly differentially. We had hypothesized that E-cadherin will facilitate gap junction assembly by preventing endocytosis of connexins whereas N-cadherin will disrupt the assembly by inducing endocytosis.

## Key Research Accomplishments

1. We have identified a key motif in connexin43 that regulates its endocytosis by clathrin-mediated pathway.
2. Endocytosis of connexin43 is regulated through phosphorylation and dephosphorylation of serine 279 and 282 via clathrin-mediated pathway.
3. We have identified two dileucine-like motifs in the cytoplasmic tail of connexin32 that regulate its endocytosis and control gap junction formation.
4. Retroviral-mediated expression of Cx32, in which the two dileucine-like motifs have been mutated, in androgen-responsive human prostate cancer cell line, LNCaP, results in the formation of large gap junctions.



**Figure 2.** Assembly and Disassembly of GJs. Cxs are short lived proteins with a half life of 2-5 h. Connexons (see Figure 1) traffic to the plasma membrane (PM) in 100-150 nm particles, diffuse laterally and dock with their counterpart connexons in the PM of apposed cells. Juvenile connexons (red) are recruited to the periphery of the GJ plaque while senile connexons (green) are pinched off from the middle as double membrane vesicles, also called annular GJs, into either one or the other cell. Alternatively, an entire GJ plaque is also endocytosed in its entirety into one or the other cell (left).

## Reportable Outcomes

### Previous Report:

1. We generated mutants of E-cadherin and N-cadherin in which critical amino acid, tryptophan (W), in the fifth extracellular domain of both cadherins was mutated to alanine (A). These mutants were tagged with green (EGFP) as well as with red (mCherry) fluorescent proteins.
2. Expression of mutant E-cadherin and N-cadherin induced a weak cell-cell adhesion when expressed in cadherin-null cells compared to wild-type cadherins.
3. We demonstrated that gap junctions were endocytosed by clathrin-dependent and –independent endocytosis.
4. Upon internalization, gap junctions were degraded by autophagy.
5. Endocytosis of Cxs appeared to be one of the key determinants in regulating the formation of gap junctions.
6. Retroviral expression of N-cadherin in E-cadherin-expressing androgen-responsive human prostate cancer cell line, LNCaP, induced a scattered phenotype.

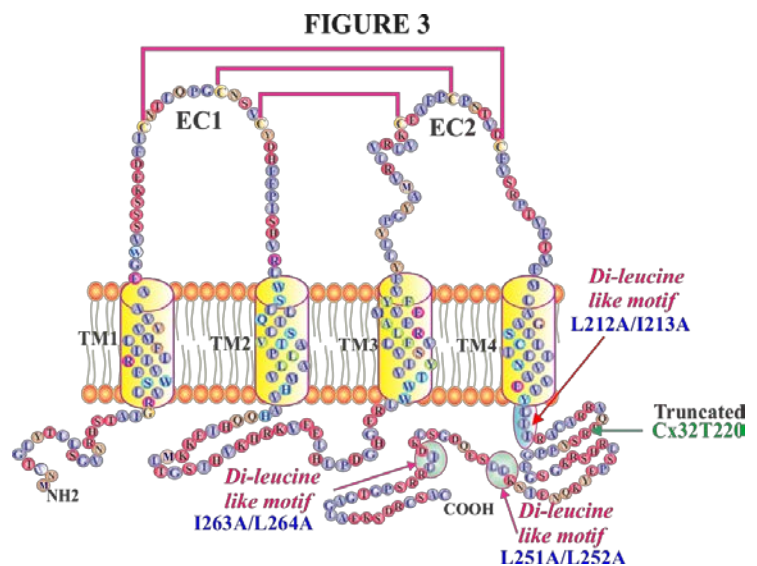
## Current Report:

It is as yet unknown how a bi-cellular structure, such as a GJ or a GJ plaque, is endocytosed [1;10;16;17]. Connexins are short-lived proteins and both the assembly of Cxs into GJs and their disassembly are multi-step processes, which are poorly understood (Figures 1 & 2). A GJ can be endocytosed into one or the other cell, either in its entirety — also called annular GJ — by autophagy [18], or as a fragment pinched off from the center of the plaque as a double membrane vesicle, and degraded in the lysosome [19-21]. Alternatively, undocked connexons may be endocytosed by clathrin mediated endocytosis (**CME**) or non-clathrin mediated endocytosis (**NCME**) (**Figure 2**).

## Construction of Wild-Type Cx32 and Its Mutants:

To address the role of cytoplasmic tail of Cx32, we generated a Cx32 mutant, Cx32T220, from which the entire cytoplasmic tail, comprising of the last 63 amino acids, had been deleted (**Figure 3**, green arrow marks the point of truncation). We also found that the cytoplasmic tail of Cx32 harbored three dileucine-like motifs that resemble the consensus motif **[DE]XXXL[LI]** (hydrophobic amino acid residues are shown in bold red whereas acidic residues are shown in bold black). The dileucine-like motifs have been shown to regulate the internalization of many trans-membrane proteins from the cell surface by the clathrin-mediated pathway [22]. These dileucine-like motifs are shown in **Figure 3** (the three motifs are indicated by the red arrows).

**Figure 3. A schematic diagram of Cx32 and the location of the truncation and the 3 dileucine-like motifs.** The site of truncation mutant and 3 dileucine-like motifs are indicated by the arrows. TM1-TM4 = Four transmembrane domains. EC1, EC2 = Extracellular domain. L= leucine, I=Isoleucine and A=alanine. In L212A/I213A the leucine at position 212 and isoleucine at position 213 were mutated to alanine. Similar strategy was used to generate mutants L251A/I252A and L263A/I264A



To explore the role of these motifs in the trafficking, assembly and endocytosis of Cx32, we also generated the following Cx32 mutants in which these motifs were mutated. These mutants were L212A/I213A, L251A/I252A and L263A/I264A in which leucine and isoleucine at the indicated amino acid residues were mutated to alanine using site-directed mutagenesis (**Figure 3**).

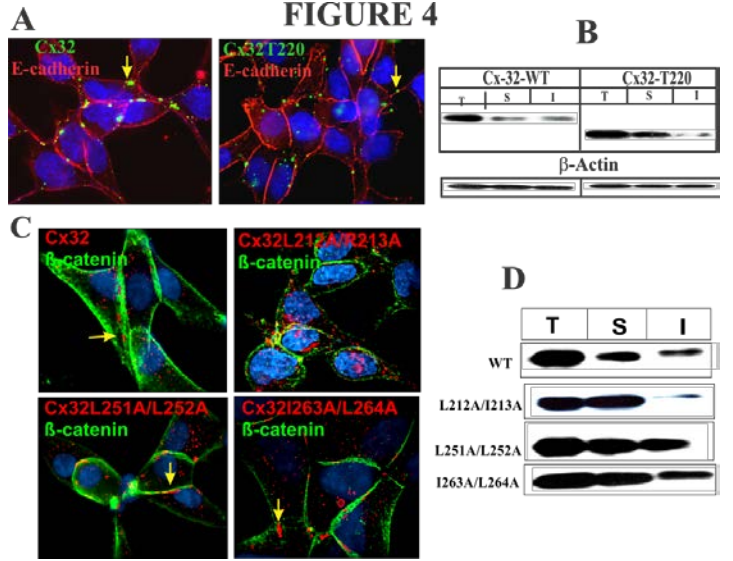
## Expression of Cx32 and Its Mutants and Gap Junction Assembly

Human LNCaP cells neither express Cx32 nor form functional GJs [23]. We introduced WT-Cx32 and various mutants into early passage LNCaP cells using recombinant retroviruses as described in our earlier published studies [24;25]. Western blot analysis of infected cells showed that they expressed wild-type Cx32 as well as the engineered mutants abundantly (**Figure 4, B**, left lanes). To examine if mutants were assembled into GJs, we immunostained infected cells with the antibody against the cytoplasmic loop of Cx32. Our results showed the following: 1. Compared to wild-type Cx32, mutant Cx32T220 —from which the entire cytoplasmic tail had been deleted — formed smaller GJs (**Figure 4 A**, Cx32 in green, arrows). 2. Mutants L251A/I252A and L263A/I264A — in which the dileucine-like motifs involved in the clathrin-mediated endocytosis have been mutated — formed larger GJs when compared to those formed by wild-type Cx32 (**Figure 4 C**, Cx32 in red,



arrows). 3. Intriguingly, mutant L212A/I213A failed to assemble into GJs and remained scattered as discrete vesicular puncta throughout the cytoplasm (**Figure 4 C**, Cx32 in red).

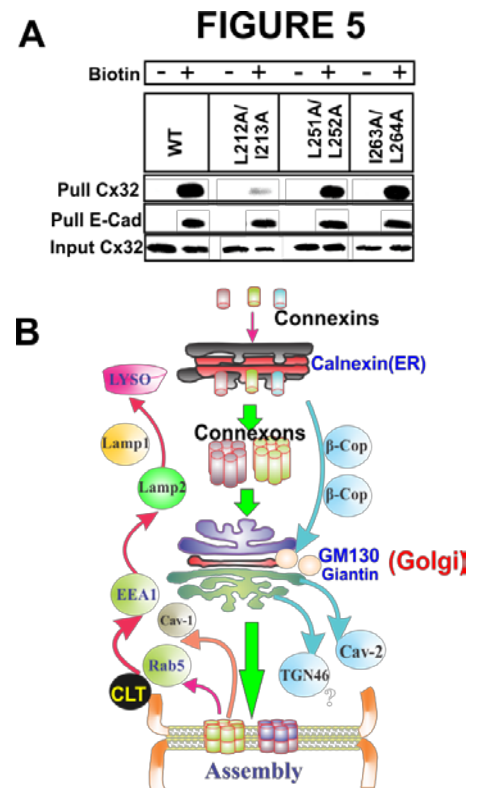
**Figure 4. Assembly of wild-type and mutant Cx32 in LNCaP cells.** **A.** Cx32 (green) and E-cadherin (red) in LNCaP cells expressing WT (left panel) and truncated Cx32T220 (right panel). Note smaller size of GJs (indicated by the arrows) of Cx32T220. **C.** Immunostaining of WT-Cx32 and the mutants (red) and  $\beta$ -catenin (green) in LNCaP cells. Note that mutant L212A/I213A fails to form GJs whereas mutants L251A/L252A and L263A/I264A form larger GJs. **B, D.** Detergent-solubility of WT-Cx32, Cx32T220, Cx32L212A/I213A, Cx32L251A/L252A, and L263A/I264A in LNCaP cells. Note that compared to WT-Cx32, mutants Cx32T220 and Cx32L212A/I213A are more soluble in TX100. T=total cell lysate, S=TX100-soluble fraction and I=TX100-insoluble fraction. The nuclei are shown blue.



Quantitative analysis, using the state of the art imaging software (Volocity), showed that the average size of GJs formed by the mutant Cx32T220 was 2-3 fold smaller ( $n = 70$ ) whereas the average size of the GJs formed by the mutants L251A/L252A and L263A/L264A was 2-3 fold larger (data not shown).

To substantiate the immunocytochemical data, we determined the assembly of Cx32 into GJs biochemically by Western blot analysis of total and TX100-soluble and TX100-insoluble fractions. This assay is based on the principle that Cxs, which are not assembled into GJs are not solubilized in TX100 and vice versa [26].

**Figure 5. Cx32 mutants traffic normally to cell surface in LNCaP cells.** **A.** Cells were biotinylated as described [27]. Biotinylated proteins from total cell lysates were pulled down by Streptavidin and analyzed by immunoblotting. The blots were probed for Cx32 and E-cadherin (E-cad). For input, 10  $\mu$ g of total protein was used. Note that compared to WT-Cx32, mutant Cx32L212A/I213A is biotinylated poorly whereas all other mutants are biotinylated robustly. **B.** A flow chart of the endocytic and secretory pathways and the markers used to identify the endocytic and secretory compartments.

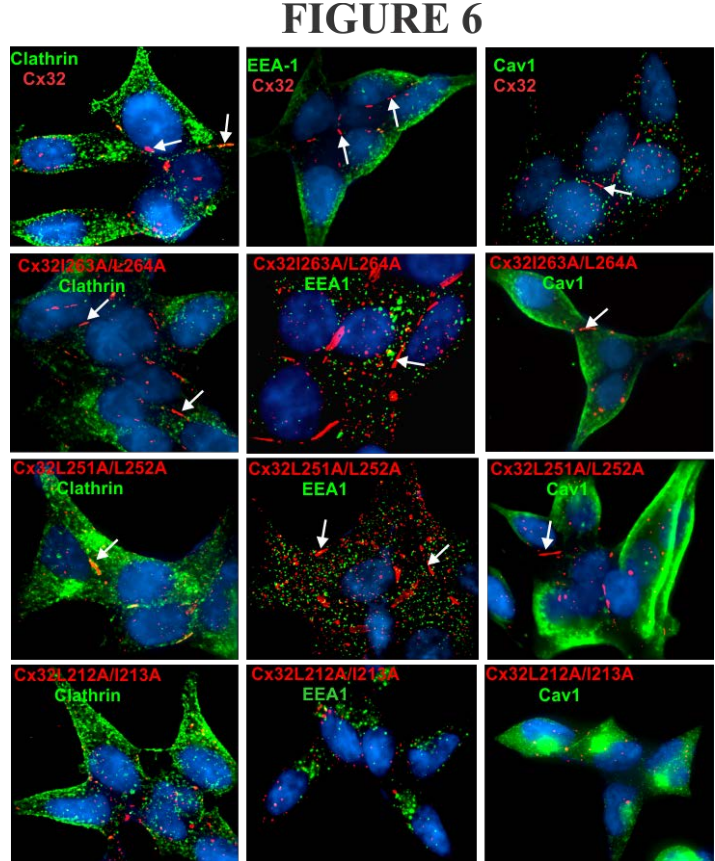


Biochemical assay showed that a significant fraction of WT-Cx32 and mutants L251A/L252A and I263A/L264A remained detergent-insoluble whereas mutant Cx32T220 and L212A/I213A were not robustly resistant to TX100 extraction, substantiating the immunocytochemical data (**Figure 4, C, D**, see also **Figure legend**). Taken together, the data shown in **Figure 4** suggest the following: 1. The cytoplasmic tail of Cx32 determines the size of the GJs and hence its robust assembly. 2. The two dileucine like motifs, L251A/L252A and I263A/L264A, increase GJ size, possibly by preventing its endocytosis by the clathrin-mediated pathway. 3. The third dileucine-like motif, L212A/I213A, likely controls the trafficking of Cx32 to the cell surface.

## Trafficking of Wild-type Cx32 and Its Mutants

To examine whether WT-Cx32 and its various mutants trafficked normally to the cell surface, we used cell-surface biotinylation as well as markers for the secretory and the endocytic compartments to assess their subcellular fate (**Figure 5**). Using biotinylation of E-cadherin (E-cad), a cell-surface protein, as a positive control, we found that WT-Cx32 and mutants L251A/L252A and I263A/L264A were biotinylated significantly whereas mutant L212A/I213A could not be significantly biotinylated (**Figure 5**). These data suggested that despite abundant expression mutant L212A/I213A either trafficked poorly to the cell surface and/or was targeted to other subcellular compartments

**Figure 6. Both WT-Cx32 and Cx32 mutants fail to colocalize with the endocytic markers.** LNCaP cells expressing WT-Cx32 and the indicated mutants were immunostained for Cx32 (red), clathrin, EEA1 and caveolin-1 (Cav-1, green). Some GJs are marked by the white arrows. Note that neither WT-Cx32 nor mutants Cx32L212A/I213A, Cx32L251A/L252A, and L263A/L264A colocalize with the endocytic markers shown in **Figure 5 B**.



Intriguingly, we also found that both WT-Cx32 and mutants L251A/L252A and L263A/L264A failed to co-localize with clathrin [28], with an early endocytic marker EEA1 [29], and with caveolin 1 (Cav 1) [30], which are the makers for the endocytic pathways (**Figure 6**). Also, no discernible co-localization was observed with GM130, a cis-Golgi-resident protein [31], Giantin, a Golgi-associated structural protein [32] and Caveolin 2 (Cav-2) [30], which are the makers for the secretory compartments (data not shown; but see **Figure 5 B** for markers). In contrast, significant colocalization was observed with the lysosomal marker, Lamp2 [33;34] (not shown). Taken together, these data suggest that although the cytoplasmic tail of Cx32 harbors endocytic motifs that could potentially mediate its internalization by the clathrin-mediated pathway, the endocytic itinerary of both wild-type Cx32 and its various mutants seemed to be nonconventional compared to other transmembrane proteins at least in the cell line used in the present study [22]. Also, these experiments identified a new dileucine-like motif in Cx32 that likely controls the trafficking to the cell surface and governs its assembly into GJs.

## Subcellular Fate of Mutant Cx32 L212A/I213A

We explored further the fate of mutant L212A/I213A in LNCaP cells. We asked the question: What is the secretory and endocytic itinerary of this mutant? We wished to investigate whether or not it traffics to the cell surface via endoplasmic reticulum and Golgi and Trans-Golgi network. Hence, we immunostained LNCaP cells expressing mutant L212A/I213A with calnexin, an ER-resident protein, with GM130, a cis-Golgi-resident protein [31], Giantin, a Golgi-associated structural protein [32], with TGN46, a protein associated with the TGN, a late secretory station [35], with Caveolin 2 (Cav-2) [30], and  $\beta$ -COP [36], which are the makers for the secretory compartments [36]. We also immunostained these cells with Lamp2, which is a marker for the late endosome, to test if it is directly sorted to the lysosomes from ER/Golgi as has been observed for some other secretory proteins [36] (see **Figure 5 B** for markers). As is evident from the data shown in **Figure 6**, this mutant did not co-localize with any of the markers used to trace its secretory itinerary.



## Parmender P. Mehta, Ph.D.

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**Parmender P. Mehta, Ph.D.**

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**Parmender P. Mehta, Ph.D.**

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