C-terminal lysines modulate Connexin32 turnover
and its ability to suppress growth of Neuro-2a cell cultures

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Abstract

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The extent of gap junction (GJ)-mediated coupling can be modulated through GJ channel gating. However, the amount of connexin protein available for incorporation into GJ, efficiency of channel assembly, trafficking to the cell surface, and disassembly also contribute to the regulation of cell-cell communication. In addition to their function in GJ, connexins also regulate a variety of physiological processes by forming hemichannels that are involved in paracrine signaling (Sanchez, Orellana et al. 2009) and through interactions with other proteins in the cytoplasm (Francis, Xu et al. 2011) and at the plasma membrane (Fowler, Akins et al. 2013). These other connexin functions are also likely to be influenced by the channel assembly dynamics, trafficking, and fast turnover of connexin proteins. The aim of this work was to determine if post-translational modifications, such as lysine acetylation, regulate connexin function through the fine-tuning of protein turnover or some other aspect of GJ dynamics.

We chose to focus specifically on Cx32 for several reasons. Cx32 is an important regulator of neuronal myelination and loss of Cx32 GJ function is a common cause of the demyelinating neuropathy, Charcot-Marie-Tooth disease. Most studies addressing post-translational modifications of connexins focus on Cx43, which shares little sequence homology
with Cx32 in the domains that are most-often subject to post-translational modification. We surmised that our results could be compared to what is known about Cx43 in order to determine if shared post-translational modifications regulate evolutionarily divergent connexins in similar ways.

Here we show that Cx32 is an acetylated protein and that acetylated Cx32 is found in the cytoplasm and in the plasma membrane, where it is incorporated into GJ. For many proteins, acetylation has been implicated in pathways that modulate protein turnover (Caron, Boyault et al. 2005), thus we tested whether acetylation could regulate Cx32 protein level, resulting in the modulation of Cx32 functions. Our results demonstrate that acetylation is a positive regulator of Cx32 protein level, which increases the amount of Cx32 at the cell surface. Inhibition of the cytoplasmic deacetylase, HDAC6, results in hyperacetylation and accumulation of Cx32, which is dependent upon cytoplasmic C-terminal lysines.

Mutational analysis revealed that these C-terminal lysines influence the ubiquitination and turnover rate of Cx32 protein. Comparison of the subcellular localization of WT Cx32 to that of mutants that either abolish acetylation sites while maintaining the original amino acid charge (K→R) or mimic constitutive acetylation (K→Q) suggests that acetylation does not simply alter lysine occupancy, thus preventing Cx32 ubiquitination and subsequent turnover. Instead, it seems likely that acetylation modulates protein-protein interactions that influence the amount of Cx32 in the plasma membrane and the role of Cx32 as a regulator of growth of cell cultures. K→Q Cx32 accumulates at the cell-surface more than WT Cx32, while K→R behavior resembles that of WT. Further, K→Q Cx32 suppresses the expansion of N2a cell cultures, whereas WT and K→R Cx32 do not. Interestingly, none of the mutations resulted in detectable alterations of cell-cell communication, suggesting that the suppression of cell culture growth
observed when cells expressed the K→Q mutant may be independent of cell-cell communication. These results suggest that Cx32 acetylation is a positive regulator of Cx32 mediated suppression of proliferation or enhancement of pro-apoptotic signaling and provide rationale for future studies to determine which protein-protein interactions are modulated through Cx32 acetylation.
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Chapter 1

Introduction
Initial Characterization of Gap Junctions

A fundamental property of the tissues within the body of any multicellular organism is the ability for individual cells to coordinate complex physiological processes in order to act as a single, larger unit. Cell-cell junctions are protein complexes that mediate this coordination. Definitive evidence of direct cell-cell contact was first presented in 1962 by Dewey and Barr, who used electron microscopy to investigate the structure of smooth muscle cells (Dewey and Barr 1962). They wondered how individual cells could act as though they were electrically connected and thus hypothesized that there must be a physical link between the cytoplasm of adjacent cells. Electron microscopy revealed that neighboring cells made multiple contacts through their cell membranes and that the membranes were not fused in such a way that their cytoplasm was continuous. This meant that the cells were discrete, but likely to interact with each other through the points of membrane contact, which Dewey and Barr termed the nexus. Dewey and Barr hypothesized that the nexus included structures that allow molecules to pass between the cytoplasm of neighboring cells.

Initially studies did not distinguish between the various types of junctions that appeared to form at points of contact between adjacent cells (Dewey and Barr 1964). However, detailed ultrastructural studies of epithelia revealed that several different types of contacts exist between neighboring cells. Tight junctions were identified as fusions between the membranes of adjacent cells, which form a sealed barrier between the cells and the extracellular space. Adherens junctions were identified as a zone of diffuse and homogenous material, later identified as actin intermediate filaments and their binding partners, that forms connections between the cytoskeletons of adjacent cells. Desmosomes were identified as discrete cytoplasmic plaques, converged upon by cytoplasmic fibrils (later identified as intermediate filaments) (Farquhar and...
Palade 1963) (Farquhar and Palade 1965). While these cell-cell contacts seemed likely to allow adjacent cells to coordinate certain physical processes, they did not seem to fit Dewey and Barr’s description of the nexus, in that they were unlikely to allow molecules to pass between the cytoplasm of the cells. This function seemed most likely to be mediated by structures that were located within the plasma membrane and thus could form pores between the cells.

Further characterization of cell-cell contacts at the nexus revealed that action potentials in smooth muscle could be propagated in vitro between cells that were aligned over an artificial gap that induced nexus formation (Dewey and Barr 1962; Barr, Berger et al. 1968). Small ions were also shown to travel between adjacent epithelial cells, meaning that nexus function was not limited to excitatory cells (Loewenstein and Kanno 1964). Fluorescein sodium was identified as a small molecule tracer that passed between cells, but not into the extracellular space after microinjection (Kanno and Loewenstein 1964). This meant that the nexus was not strictly involved in ion transport, but rather, it was potentially mediating the diffusional transport of a variety of small molecules between neighboring cells.

Efforts to identify the nexus continued to rely on a combination of thin sections and freeze fracture preparations as samples for structural analyses by electron microscopy. Using lanthanum as a tracer to penetrate the extracellular space, Brightman and Reese showed that a 20-30 Å gap exists between adjacent cells from a variety of tissues (Brightman and Reese 1969). Hexagonal particles were visible along these gaps as they were outlined by the lanthanum. Freeze fracture preparation of samples for electron microscopy allowed the separation of the membranes of contacting cells in order to reveal the subunits of the nexus. The nexus, which has since been referred to as the gap junction (GJ), was thus shown to be composed of hexagonal macromolecular structures that appeared to be aligned between the neighboring cells (McNutt
and Weinstein 1970). Subsequent studies defined GJ as a polygonal lattice of subunits found within the cell membrane (Figure 1.), along a 20 Å gap between adjacent cells (Goodenough and Revel 1970; Amsterdam, Josephs et al. 1976). The hexagonal particles could be arranged at varying densities and sometimes unopposed on the neighboring cell membrane. The electron density of these particles was similar to that of the cytoplasm, rather than the membrane or extracellular space, further suggesting that GJ were cytoplasmic bridges (Goodenough and Revel 1970).

The unique morphology and low solubility of gap junctions were exploited in order to isolate the constituent proteins. Membranes containing GJ were purified by density gradient centrifugation and the use of high ionic strength detergent. Then the presence of GJ was confirmed by electron microscopy and X-ray defraction (Goodenough and Stoeckenius 1972). Initially the yield of this GJ purification was too low to carry out detailed biochemical studies of the GJ proteins, but subsequent improvements in solubilization and fractionation lead to the identification of a major GJ component from rat hepatic tissue by gel electrophoresis (Goodenough 1974). This protein was named connexin, since it connects cells through nexus contacts.

**Connexins: the Building Blocks of Gap Junction Channels**

Studying GJ mediated cell-cell communication in a variety of cell types provided evidence for the existence of multiple, distinct GJ proteins. While the earliest studies of GJ mediated coupling focused on electrical coupling of excitatory cells and whole tissues, researchers eventually showed that cultured fibroblasts also produced functional GJs, a discovery that allowed GJs to be studied extensively in vitro (Gilula, Reeves et al. 1972). Using cultured
cells and in vitro GJ communication assays, GJs were shown to be permeable to small, polar molecules (Flagg-Newton, Simpson et al. 1979). Later it became clear that GJs could mediate the diffusion of specific complements of hydrophilic molecules such as nucleotides, small ions, water, and metabolites (reviewed in (Kumar and Gilula 1996)). It has been hypothesized that gene duplication and subsequent mutations gave rise to the variety of connexin genes that are found in mammalian cells(Willecke, Eiberger et al. 2002).

Once several connexin cDNAs had been cloned, it became possible to study the function of each domain within a connexin molecule. All connexin proteins are four pass transmembrane proteins (see Figure 2. for basic topology). Two highly conserved extracellular loops mediate the alignment and docking of adjacent channels through disulfide bonds(Rahman and Evans 1991; Rahman, Carlile et al. 1993). Short cytoplasmic N-termini are implicated in GJ oligomerization, gating and trafficking(Oh, Abrams et al. 2000; Purnick, Oh et al. 2000; Rouan, Lo et al. 2003; Gemel, Lin et al. 2006; Kyle, Minogue et al. 2008). The cytoplasmic loop impacts channel selectivity and gating (Wang, Li et al. 1996; Wang, Martínez et al. 2005; Xu, Kopp et al. 2012). Hydrophobic transmembrane residues form the pore itself and thus are important for determining the permeability of the GJ channel (Zhou, Pfahnl et al. 1997; Pfahnl and Dahl 1998; Skerrett, Aronowitz et al. 2002; Kronengold, Trexler et al. 2003). The cytoplasmic C-termini mediate interactions with other cytoplasmic proteins, including cytoskeletal elements, the components of other types of cell-cell junctions, and signaling proteins (Kumar and Gilula 1996; Yeager and Nicholson 1996; Giepmans and Moolenaar 1998; Giepmans, Verlaan et al. 2001; Giepmans, Verlaan et al. 2001; Shaw, Fay et al. 2007). While the amino acid sequences of N-termini, transmembrane domains, and extracellular loops of connexins are highly conserved, the
cytoplasmic loops and C-termini are more divergent. The sequences found in these divergent domains confer unique permeability and interacting factor compliments for each connexin.

Six connexin subunits oligomerize in order to form a hemichannel, which is transported to the cell surface and inserted in the plasma membrane. Each of the cells within a coupled cell pair then contributes a hemichannel and the GJ is formed when the adjacent hemichannels align to form a pore between neighboring cells (Yeager and Nicholson 1996; Yeager, Unger et al. 1998). The hemichannels are composed of either a single type or a combination of two compatible connexins. GJ may are then composed of the same hemichannel types or hemichannels with different compositions (Swenson, Jordan et al. 1989; Werner, Levine et al. 1989). Each connexin combination gives rise to a GJ with particular permeability characteristics, which can be enhanced or reduced depending on the connexin combination (Beyer, Gemel et al. 2001; Martinez, Hayrapetyan et al. 2003; Gemel, Valiunas et al. 2004; Martinez, Maripillán et al. 2011). Tissue specific GJ permeability thus depends upon connexin specific oligomerization properties and cell type specific connexin expression profiles.

Molecules pass through open GJ channels by passive diffusion unless GJ gating is induced by stimuli such as increased transjunctional voltage, intracellular acidification, or increased intracellular [Ca2+] (Dahl 1996). Residues in the N-terminal (Verselis, Ginter et al. 1994), extracellular1 (Rubin, Verselis et al. 1992), transmembrane2 (Suchyna, Xu et al. 1993) and C-terminal (Moreno, Chanson et al. 2002) domains of various connexins have been implicated in voltage gating. Connexins vary in their pH sensitivity, but gating due to cellular acidification is generally facilitated by the intracellular loop and C-terminal domains (Ek-Vitorin, Calero et al. 1996; Francis, Stergiopoulos et al. 1999; Eckert 2002). Chemical gating of GJ occurs in the presence of high concentrations of Ca2+, which results in the loss of
calmodulin binding in some cases (Dodd, Peracchia et al. 2008) (Hertzberg and Van Eldik 1987). Interestingly, electrophysiological analyses of GJ activity, correlated with high resolution image analysis of GJ size revealed that only a small fraction of total GJ at the cell surface are open at any point in time (Curti, Hoge et al. 2012). This suggests that GJ mediated cell-cell communication is regulated in large part by pathways that are independent of the aforementioned GJ gating. Also, it suggests that GJ associated connexins may be involved in physiological pathways that function independently of cell-cell communication (Figure 3.). These possibilities are discussed throughout this thesis.

**Connexin Hemichannels**

Connexins exist in several distinct cellular pools, which include GJ, hemichannels within the plasma membrane, and hemichannels within the secretory system and cytoplasm. While most work to date has focused upon the physiological consequences of GJ function, there is mounting evidence that non-junctional hemichannels are also physiologically relevant. Connexin hemichannels are permeable to a variety of molecules such as glutamate (Orellana, Froger et al. 2011), NAD+ (Okuda, Nishida et al. 2013), prostaglandin E2 (Cherian, Siller-Jackson et al. 2005), Ca2+ (De Bock, Wang et al. 2012), and ATP (Kang, Kang et al. 2008), which are involved in paracrine signaling. The passage of molecules through connexin hemichannels regulates processes such as apoptotic cell death (Vinken, Decrock et al. 2010; Kameritsch, Khandoga et al. 2013) and bone remodeling in response to mechanical stress (Jiang and Cherian 2003). It also seems likely that some studies that have identified GJ as regulators of processes such as tumorigenesis overlooked the regulatory contributions of hemichannels.
A number of recent studies have shown that connexin hemichannels are involved in the regulation of apoptosis. Vinken and colleagues observed that during apoptosis GJ-mediated coupling decreased, despite increased plasma membrane associated Cx32, suggesting a shift from GJ to hemichannel Cx32 function apoptosis (Vinken, Decrock et al. 2010). Inhibition of Cx32 hemichannels decreased cell death in response to pharmacological activators of apoptosis, providing evidence that Cx32 hemichannels are positive regulators of apoptosis. A decrease in junctional Cx43 and GJ-mediated communication has also been observed during apoptosis in lens epithelium and cultured fibroblasts (Theiss, Mazur et al. 2007). Inhibition of Cx43 hemichannels by antibody binding, mimetic peptides, or general pharmacological inhibition of GJ attenuated pro-apoptotic Ca2+ signaling in HeLa cells transfected with Cx43 cDNA, and HL-1 or freshly isolated cardiomyocytes, which both express endogenous Cx43 (Verma, Hallett et al. 2009).

Cx43 hemichannels have been implicated in bone remodeling, which results from mechanical stress and is important for healthy bone maintenance. Cx43 hemichannels are permeable to prostaglandins, which are paracrine signaling molecules that regulate bone remodeling. Osteocyte processes have been shown to act as mechanotransducers that open connexin hemichannels (Burra, Nicolella et al. 2010). Mechanical loading of osteocytes induces prostaglandin release via Cx43 hemichannels(Siller-Jackson, Burra et al. 2008). Inhibition of Cx43 channel activity results in decreased prostaglandin release when cells are subjected to shear stress. Cx43 protein level and localization also seems to be modulated by shear stress, since cell surface Cx43 level increases in response to shear stress, which results in increased prostaglandin release (Cherian, Siller-Jackson et al. 2005). The plating density of Cx43 expressing osteocytes
also modulates prostaglandin release. As cell density decreases and Cx43 is shifted from GJ to hemichannels, prostaglandin release increases.

Connexin hemichannels are also involved in tissue damage that occurs as a consequence of ischemia and reperfusion. Metabolic inhibition is used to model ischemia and reperfusion in vitro. Induction of hemichannel opening in HeLa cells expressing exogenous Cx32 occurred as a result of metabolic inhibition (Sanchez, Orellana et al. 2009). Ischemia also induced closing of GJ and opening of hemichannels in cardiac myofibroblasts in vitro (Johansen, Cruciani et al. 2011). Since cell death is associated with the flux of Ca2+ and other signaling molecules through connexin hemichannels that occurs with reperfusion, Wang et al. hypothesized that treating ischemic mice with a hemichannel specific inhibitor could protect against tissue injury during reperfusion (Wang, De Vuyst et al. 2013). GAP19 was developed and tested for Cx43 hemichannel specificity and does not inhibit GJ activity. Treatment with GAP19 inhibited the opening of hemichannels after metabolic inhibition in HeLa cells expressing exogenous Cx43, reduced cell death due in cardiomyocytes subjected to hypoxia, and also reduced infarct size in vivo in mouse hearts after ischemia and reperfusion. Taken together, these data provide evidence that metabolic regulation of connexin hemichannels is likely to be a common theme amongst members of the connexin protein family.

**Connexin Interacting Partners**

Connexins are also involved in protein-protein interactions that have physiological consequences that are independent of GJ or hemichannel functions. For example, Cx43 regulates cell motility in a manner that seems to be independent of cell-cell communication and hemichannel function (Francis, Xu et al. 2011). Ablation of the Cx43 gene results in neonatal
lethal heart defects that have been linked to a cell motility defect in neural crest cells (Xu, Francis et al. 2006). This cell motility defect can be reversed by expression of exogenous Cx43. Expression of a Cx43 mutant that forms non-functional GJ also rescues the motility defect, while expression of Cx43 lacking a microtubule binding motif does not (Francis, Xu et al. 2011).

Cx43 also interacts with other junctional proteins, enhancing the formation of cell-cell junctions in a way that may be independent of GJ or hemichannel function. For example, Cx43 interacts with tight junction proteins ZO-1 and Occludin-1 and may be required for tight junction maintenance of the blood-brain barrier (Nagasawa, Chiba et al. 2006). These interactions occur through a PDZ motif found in the C-terminus of Cx43, a region of the protein that is not involved in GJ function (Giepmans, Verlaan et al. 2001). Interaction of Cx43 with β-catenin is associated with increased adherens junction localization of β-catenin and decreased transcription factor activity (Ai, Fischer et al. 2000). N-cadherin, another adherens junction protein, has been isolated as part of a multi-protein complex with Cx43 (Wei, Francis et al. 2005). Depletion of N-cadherin protein in cultured fibroblasts prevented Cx43 GJ formation, and depletion of Cx43 resulted in reduced N-cadherin associated with adherens junctions. The interaction of a desmosomal protein, Plakophilin-2, with Cx43 acts as a positive regulator of cell adhesion in an in vitro model of the blood-testis barrier (Li, Mruk et al. 2009). Taken together, these studies suggest that GJ and other cell-cell junctions interact physically in order to promote cell-cell adhesion as a whole.

Less is known about the interacting partners of Cx32. Cx32 lacks the PDZ motif that is found in Cx43 and other proteins that bind to scaffold proteins such as ZO-1. However, Cx32 co-immunoprecipitates with complexes containing tight junction proteins including Zo-1, occludin, and claudin-1 (Kojima, Kokai et al. 2001). These proteins also colocalize with Cx32 in freeze-
fracture replicas of cultured rat hepatocytes. Expression of exogenous Cx32 in a hepatocyte cell line that lacked endogenous connexins induced tight junction formation, suggesting that the interactions between Cx32 and tight junction proteins may function in the formation or stabilization of tight junctions (Kojima, Sawada et al. 1999). Cx32 also interacts with the membrane associated scaffold protein, Dlgh-1 (Discs large homology-1), which can act as a tumor suppressor (Duffy, Iacobas et al. 2007). Dlgh-1 plasma membrane localization and protein level were enhanced by expression of exogenous Cx32 in a connexin deficient cell line and decreased in hepatocytes derived from Cx32 null mice compared to those from Cx32 expressing mice.

A recent proteomic study led to the isolation of liver Cx32 associated with endoplasmic reticulum resident, cytoplasmic, plasma membrane associated, and mitochondrial proteins (Fowler, Akins et al. 2013). Cx26 was associated with Cx32 at the plasma membrane and loss of Cx32 resulted in reduced Cx26 protein, suggesting that Cx32 stabilizes Cx26 protein. Among the isolated interacting proteins, sideroflexin-1, is a mitochondrial protein that was isolated with both mitochondrial and plasma membrane associated Cx32. Based upon the comparison of sideroflexin-1 localization in Cx32 null and Cx32 WT hepatocytes, the authors suggested that Cx32 interacts with mitochondrial sideroflexin-1 at the plasma membrane, resulting tethering of mitochondria to the plasma membrane. This is a novel interaction that could explain the detection of mitochondrial Cx43 localization as well (Boengler, Dodoni et al. 2005; Trudeau, Muto et al. 2012). The interactions between connexins and mitochondrial proteins could provide a link between connexins and cellular metabolism that is independent of connexin channel function and instead dependent upon the subcellular localization and cytoplasmic amino acid sequences of the connexins.
Cx32 as a Regulator of Nervous System and Liver Homeostasis

Cx32 is an important regulator of neuronal and liver homeostasis. The physiological role of Cx32 expression has been studied in the most detail in hepatocytes and glia where it is most abundant. However, Cx32 is also expressed in the lungs, kidney, prostate, stomach, and small intestine (Paul 1986; Mehta, Perez-Stable et al. 1999; King and Lampe 2004). Cx32 mediated cell-cell communication plays a role in sympathetic nerve function in the liver (Nelles, Butzler et al. 1996), as well as peripheral nerve function(Scherer, Xu et al. 1998). Mutations that reduce or eliminate Cx32 function result in peripheral neuropathy(Abrams, Freidin et al. 2001). In the liver, Cx32 has been implicated as a regulation of processes such as tumorigenesis (Temme, Buchmann et al. 1997) and sensitivity to chemical toxicity (Patel, Milwid et al. 2012).

Chromosomal mapping of mutations associated with the hereditary demyelinating neuropathy, Charcot-Marie-Tooth disease(CMT), identified Cx32 as a regulator of peripheral nerve physiology(Bergoffen, Scherer et al. 1993). CMT is a common neurodegenerative disease with symptoms that include muscle wasting and sensory loss in the distal extremities. Mutations in a large and varied group of genes have been identified in CMT patients. Cx32 mutations cause a dominant X-linked form of CMT and represents roughly 10% of total CMT patients(Kleopa, Abrams et al. 2012). CMT associated Cx32 mutations are usually point mutations that effect hemichannel formation and trafficking the cell surface, Cx32 stability, or GJ gating (Ressot, Gomes et al. 1998).

Analysis of Cx32 expression in oligodendrocytes and Schwann cells revealed that the timing and localization of Cx32 expression are regulated in parallel with other myelin-related proteins(Scherer, Deschenes et al. 1995). Both oligodendrocytes and Schwann cells are glial
cells, which function in the maintenance of neuronal myelinated sheaths. Oligodendrocytes maintain neurons in the Central Nervous System, while Schwann cells function in the Peripheral Nervous System. The existence of functional GJ within the myelin sheath that is formed by Schwann cells has been demonstrated ex vivo (Balice-Gordon, Bone et al. 1998). Further, Cx32 GJ have been detected between myelin layers, where they may provide short-distance radial pathways for the transfer of metabolites and water through the myelin sheath (Meier, Dermietzel et al. 2004). These observations suggest that Schwann cell GJs may be involved in maintaining the myelin sheath, independent of coupling with other cells. Consistent with this possibility, Cx32 null mice develop normally but suffer from demyelination of peripheral nerves over the course of their lives (Scherer, Xu et al. 1998). Based upon the peripheral nerve specific phenotype observed in Cx32 null mice, it seemed likely that loss of Cx32 function in Schwann cells is the underlying cause of CMTX-1. This hypothesis was supported by the observation that expression of a Cx32 transgene driven by a Schwann cell specific promoter was sufficient to prevent peripheral nerve demyelination in Cx32 null mice (Scherer, Xu et al. 2005).

Cx32 does not play an essential role during liver development, as evidenced by normal liver development in Cx32 null mice (Evert, Ott et al. 2002). While the livers of Cx32 null mice are structurally normal, further investigation revealed that sympathetic nerve stimulation of bile secretion is impaired in Cx32 null mice (Temme, Stumpel et al. 2001). Glucose mobilization by the liver is significantly lower in Cx32 null mice than WT, also due to a defect in sympathetic nerve stimulation of the liver (Nelles, Butzler et al. 1996). Further, Cx32 function has been implicated in hepatic stress hormone response, which depends upon coordination of Ca2+ signaling (Correa, Guerra et al. 2004). Ca2+ signaling is not inhibited in Cx32 null cells, but there is a marked delay in the propagation of Ca2+ release signals in the absence of Cx32. Loss
of Cx32 expression is also associated with an increased incidence of chemical and radiation induced tumorigenesis in the liver (Tsuda, Asamoto et al. 1995).

GJ have been implicated in pro-inflammatory pathways such as the DNA-triggered innate immune response (Patel, King et al. 2009). The DNA-triggered innate immune response refers to the sensing of exogenous DNA and subsequent activation of immune cells, which secrete cytokines and antiviral molecules. The inflammatory response is propagated across a tissue via GJ communication and thus, inhibition of GJs reduces inflammation but could also enhance infection. While this study did not specify which GJ were involved in the propagation of inflammatory signals, other work has implicated Cx32 as a negative regulator of vascular inflammation through its regulation of cytokine expression (Okamoto, Akiyama et al. 2011). Still other work has implicated Cx32 in the propagation of tissue damage that occurs with tissue damage in the liver. Inhibition of Cx32 with a novel specific inhibitor has been shown to reduce liver damage that results from drug toxicity (Patel, Milwid et al. 2012). Transgenic mice expressing a dominant negative Cx32 mutant are also resistant to chemically induced liver damage (Asamoto, Hokaiwado et al. 2004).

Taken together, these studies show that Cx32 is implicated in a variety of physiological processes which are cell type and context dependent. Additionally, Cx32 GJ are likely to regulate tissue homeostasis through pathways that are independent of cell-cell communication in some cases.

**Cx32 as a Regulator of Cellular Proliferation**

Several different experimental approaches have generated data that suggest a role for Cx32 in tumor suppression. Analyses of human and murine tumor sample support the claim that
Cx32 is a tumor suppressor protein. Reduced Cx32 expression has been detected in both murine and human gastric carcinomas, where Cx32 expression level relates inversely to the severity of the tumor (Jee, Nam et al. 2011). Cx32 expression was significantly reduced after carcinogen exposure in mouse models of liver tumorigenesis (Fitzgerald, Mesnil et al. 1989). A study following the course of chemically induced tumor progression in mouse livers revealed that Cx32 protein level is inversely related to tumor cell proliferation rate (Tsuda, Asamoto et al. 1995). Cx32 expression is also correlated with human lung cancer prognosis; higher Cx32 expression levels correspond to better prognosis (Zhang, Li et al. 2009).

Loss of GJ by cancer cells was first observed in hepatocytes over 40 years ago (Loewenstein and Kanno 1967). Genetic ablation of Cx32 increased spontaneous, chemical, and radiation induced tumorigenesis in the liver when compared to Cx32 expressing tissue (Temme, Buchmann et al. 1997). Further investigation of radiation induced tumorigenesis revealed that the overall tumor load was increased in Cx32 null mice, compared to wild-type, and that liver, lung, intestinal, lymph, and adrenal tumors were most prevalent (King and Lampe 2004). Thyroid tissue in the Cx32 null mouse also exhibited increased cellular proliferation under growth promoting conditions, which could be inhibited by restoration of Cx32 expression (Prost, Bernier-Valentin et al. 2008). Irradiation resulted in increased activation of the oncogenic MAP kinase pathway in Cx32 null mice, compared to the WT (King and Lampe 2004). When Cx32 null mice were crossed with mice lacking the p27 tumor suppressor gene, the resulting mouse (p27 and Cx32 null) was even more susceptible to radiation induced tumor development than the Cx32 null mouse (King, Gurley et al. 2005). This result further implicated the MAP kinase pathway, which p27 is a part of, as a target of Cx32 mediated tumor suppression.
Connexin deficient cell lines have also been used to study the growth suppressive function of Cx32. For example; normal prostate cells and well differentiated prostate tumor cells have higher Cx32 expression levels than poorly differentiated prostate tumor cells, suggesting that loss of Cx32 expression may be important for prostate tumor progression (Mehta, Perez-Stable et al. 1999). Forced expression of Cx32 in the poorly differentiated prostate tumor cells slowed growth. Similarly, another study established the existence of an inverse relationship between Cx32 protein level and proliferation in a rat hepatoma cell line (Edwards, Jondhale et al. 2008). Other tumor-derived cell lines from tissues such as liver and kidney have also been reported to express lower levels of Cx32 than their non-tumorigenic counterparts (Ma, Ma et al. 2002; Fujimoto, Sato et al. 2005).

The tumor suppressive role of Cx32 is likely context-dependent since there are several documented cases where Cx32 expression fails to reduce tumor development or even seems to enhance cellular proliferation. For example, expression of Cx32 in the GJ-deficient HeLa cell line is not sufficient to suppress tumorigenic growth (Omori and Yamasaki 1999). In another study, expression of a dominant negative Cx32 mutant in liver cells reduced GJ mediated cell-cell communication, but did not increase the incidence of spontaneous tumor formation. In fact, in this context loss of Cx32 function enhanced tissue regeneration after hepatic injury, suggesting that Cx32 can function in both positive and negative regulation of proliferation (Omori, Zaidan Dagli et al. 2001). In a mouse model of hepatic tumor development, animals were treated with phenobarbital to promote tumor formation and Cx32 null mice showed reduced tumor formation compared to WT (Moennikes, Buchmann et al. 2000; Schwarz, Wanke et al. 2003). This result suggests that Cx32 is involved in phenobarbital induced tumorigenesis.
Spurious expression of Cx32 was detected in some human granulosa cell tumors (Lucke, Siebert et al. 2010). These tumors are derived from ovarian tissue and usually express Cx43, which is lost or reduced in the tumors, and not Cx32. Perhaps the aberrant expression of Cx32 does not permit cell-cell communication and instead alters cytoplasmic signaling or the Cx32 containing GJ allow cell-cell communication that promotes proliferation. Another recent report suggests that a shift from plasma membrane to cytoplasmic localization of Cx32 may promote proliferation in adenocarcinoma cells (Kanczuga-Koda, Koda et al. 2010). A shift from junctional to cytoplasmic Cx32 localization has also been detected in mouse gastric metaplasia (Jee, Nam et al. 2011) and hepatic cancer stem cells (Kawasaki, Omori et al. 2011). Experiments that tested the role of Cx32 mediated cell-cell coupling in Schwann cell growth factor response showed that Cx32 is involved in a proliferative response that is independent of its role in GJ coupling (Freidin, Asche et al. 2009), suggesting that Schwann cell proliferation may also be enhanced by a cytoplasmic Cx32 pool.

Taken together, these studies suggest that Cx32 is involved in several distinct pathways that regulate cellular proliferation. Loss of Cx32 expression in tissues that usually express a high level of GJ associated Cx32 is associated with increased proliferation. This suggests that Cx32 mediated cell-cell communication may be a negative regulator of proliferation. Aberrant expression of Cx32 in a tissue where it is not the primary connexin may enhance proliferation. This could involve altered regulation of GJ coupling and/or cytoplasmic signaling through Cx32. Finally, the cytoplasmic pool of Cx32 may be a positive regulator of proliferation under normal physiological conditions and during tumorigenesis.
**Post-translational modifications of Cx32**

Phosphorylation of Cx32 was first detected based on the observation that treating cells with membrane permeant cAMP derivatives increased cell-cell communication between hepatocytes in vitro. This finding prompted the incubation of hepatocytes with 32-P, which allowed the detection of phosphorylated Cx32 (Saez, Spray et al. 1986). Following the detection of Cx32 phosphorylation, several candidate kinases were tested for their ability to increase Cx32 phosphorylation. cAMP-dependent kinase, protein kinase C, and Ca2+/calmodulin-dependent kinase II phosphorylated Cx32, but not Cx26 in vitro (Saez, Nairn et al. 1990). Peptide mapping of the phosphorylation sites indicated that phosphorylation occurred primarily on serine residues and that they are located in the C-terminus of Cx32. Protein kinase C mediated phosphorylation of Cx32 was shown to prevent proteolysis in vitro, but the physiological relevance remains unclear (Elvira, Diez et al. 1993). Tyrosine phosphorylation of Cx32, mediated by epidermal growth factor receptor tyrosine kinase, has also been detected in vitro (Diez, Elvira et al. 1998).

Increased Cx32 serine phosphorylation was detected after treatment with nafenopin, which is a hepatocarcinogen (Elcock, Deag et al. 2000). This increase in phosphorylation was associated with decreased Cx32 GJ function, while Cx32 protein level and localization were unaltered, suggesting that the phosphorylation was causing GJ closure. Treatment with a serine kinase inhibitor, but not a tyrosine kinase inhibitor, prevented the decrease in cell-cell coupling that was caused by nafenopin treatment. These findings are similar to what was shown for cAMP activated Cx32 phosphorylation. Other studies have linked Cx32 MAP kinase activity to Cx32 protein level and GJ localization, but it is unclear if Cx32 is regulated directly by its own phosphorylation or the phosphorylation of an interacting protein(Kojima, Yamamoto et al. 2004). Overall, the literature addressing the physiological consequences of Cx32 phosphorylation is
lacking very many data that show direct interactions between Cx32 and kinases in vivo. However, it is clear that phosphorylation influences Cx32 GJ function.

Ubiquitination, which entails the covalent attachment of one or more molecules of the 76-amino acid ubiquitin protein, has been implicated in the regulation of Cx32 stability. Roughly 50% of newly synthesized wild type Cx43 and Cx32 are degraded prior to trafficking to the plasma membrane under steady state conditions (Kelly, VanSlyke et al. 2007). Kelly et al. showed that these connexin molecules are degraded through ER-associated degradation (ERAD), which is mediated by the 26-S proteasome, after translocation of target proteins from the ER. Thus, treatment with proteasome inhibitors, increases GJ-mediated cell-cell communication by stabilizing a large amount of connexin protein early the transport pathway. Interestingly, mild cytosolic stress, such as brief hyperthermia or exposure to mild oxidative denaturants, results in increased connexin stability and cell-cell communication (VanSlyke and Musil 2002). This is specific to cytosolic stress, as treatment with inducers of ER stress, such as DTT, does not stabilize the nascent connexin molecules. The authors of this study hypothesized that the connexins are stabilized under mild cytosolic stress conditions because proteasomes are preferentially targeting misfolded cytosolic proteins, and thus a larger portion of newly synthesized connexins escape degradation and are ultimately incorporated into GJs.

Currently, it is unknown how cell surface Cx32 is internalized and degraded. However, many recent studies have addressed the internalization of cell surface Cx43 and its regulation by Ubiquitination. Nedd4, an E3 ubiquitin ligase, was initially shown to associate with Cx43 and act as a negative regulator of GJ associated Cx43 level (Leykauf, Salek et al. 2006). Subsequent experiments have established Nedd4 as acting directly on Cx43 in order to regulate its internalization via mono-ubiquitination (Girao, Catarino et al. 2009). Nedd4 mediated
ubiquitination of Cx43 results in its association with the ubiquitin binding protein, EPS15, which ultimately leads to Cx43 internalization (Girao, Catarino et al. 2009). A recent study has suggested that EPS15/Nedd4 mediated Cx43 degradation occurs by macroautophagy (Bejarano, Girao et al. 2012). Live cell imaging and ultrastructural data suggest that connexins from the cell surface are frequently degraded by macroautophagy (Lichtenstein, Minogue et al. 2011; Fong, Kells et al. 2012), while other studies have implicated the endocytic pathway (Naus, Hearn et al. 1993; Leithe, Kjenseth et al. 2009; Catarino, Ramalho et al. 2011). Given that not all of the cell-surface localized connexin is incorporated into GJ plaques, perhaps GJs and connexin hemi-channels are internalized and degraded through separate pathways. This is a particularly intriguing possibility since GJs are internalized as large, double membrane vesicles by one member of the cell-pair (Amsterdam, Josephs et al. 1976; Jordan, Chodock et al. 2001; Piehl, Lehmann et al. 2007), whereas a hemichannel would be enclosed in a single membrane.

SUMOylation entails the covalent attachment of ubiquitin-like proteins to a substrate, which often regulates substrate degradation. Two SUMOylation sites have been identified in Cx43 (Kjenseth, Fykerud et al. 2012). They are located at K144 and K237, which are both cytoplasmic, but membrane proximal residues. Mutations at these residues decreased CX43 protein level overall and especially at cell-cell junctions, suggesting that SUMOylation is a negative regulator of Cx43 turn-over. Cx43 K144, which is located in the cytoplasmic loop, shares a high level of sequence homology with Cx32 K124. The authors predicted that K124 of Cx32 is also a SUMOylation site and that this post-translational modification is another common post-translational modification of connexin protein family overall.

Prenylation, which is the addition of prenyl lipid groups, is another reported post-translational modification of Cx32 (Huang, Sirkowski et al. 2005). This lipidation occurs at
consensus sites and can modulate membrane protein stability and/or function through the modulation of protein-protein and protein-membrane interactions. Cx32 has two consensus prenylation sites, C280 and S281. WT Cx32 prenylation was demonstrated in cultured cells by incorporation of 3H- mevalonolactone, whereas Cx32 C280G and S281x mutants were not labeled with the 3H-mavalonolactone. Microscopic investigation revealed that the prenylation deficient Cx32 mutants did not appear to have trafficking or stability defects. Further, both of the mutants seemed to rescue the neuronal defects associated found in Cx32 null mice, suggesting that prenylation is not necessary for Cx32 function in myelinating Schwann cells.

While Cx32 was the first GJ protein identified, nearly 40 years ago, we know relatively little about the post-translational modifications that modulate its physiological activity. The studies referenced here suggest that there is much more to be learned about post-translational modifications of Cx32. These studies also indicate that most post-translational modifications of connexins occur on cytoplasmic residues in the most variable regions of connexin proteins, and thus it is unlikely that we can simply assume that what we know about Cx43 post-translational modifications can be assumed true for other connexins.

**Lysine acetylation is a common post-translational modification with diverse consequences**

The experiments described in this thesis were designed around the central hypothesis that lysine acetylation of Cx32 contributes to the fine-tuning of Cx32 function. Lysine acetylation is a reversible, covalent, modification that occurs post-translationally on epsilon-amino groups. Lysine acetylation is distinct from N-terminal acetylation that occurs co-translationally on alpha-amino groups. Note that this thesis deals exclusively with lysine acetylation when referring to ‘acetylation’.
While acetylation was initially assumed to occur primarily within the nucleus, it is becoming increasingly obvious that lysine acetylation is a common regulator of proteins in all cellular compartments. Recent proteomic screens have identified acetylated lysine residues in thousands of proteins that are involved in diverse physiological processes (Kim, Sprung et al. 2006; Shepard, Tuma et al. 2010; Lundby, Lage et al. 2012; Pehar, Lehnus et al. 2012). Further, one of these studies provides evidence for the existence of tissue specific consensus acetylation sites (Lundby, Lage et al. 2012). This result suggests that tissue specific networks of enzymes and interacting factors would be involved in selecting proteins for acetylation, thus it follows that acetylation could act as a tissue specific regulator of the biochemical properties of a potentially acetylated protein.

Acetylation is regulated directly by acetyltransferases that covalently attach the acetyl group from acetyl-Co-A to the target substrate and deacetylases (referred to as histone deacetylases or HDACs regardless of substrate specificity) which reverse the modification. We are just beginning to amass information about the specificities of acetyltransferases that modify proteins other than histones. Acetyltransferases such as PCAF (Blanco-Garcia, Asensio-Juan et al. 2009), CBP (Kwok, Liu et al. 2006), and Tip60 (Hass and Yankner 2005) shuttle between the nucleus and cytoplasm and have substrates in both compartments. Endoplasmic reticulum resident acetyltransferases such as ATase1 and ATase2 have also been identified (Ko and Puglielli 2009). Mitochondria also contain an abundance of acetylated proteins (Kim, Sprung et al. 2006) and thus the discovery of mitochondrial acetyltransferases is likely forthcoming. Deacetylases fall into two major catagories; NAD+ dependent Sirtuins and Zn2+ dependent HDACs. Sirtuins often deacetylate proteins that are involved in metabolic regulation and have been detected in the nucleus, cytoplasm and recently in the mitochondria (Schlicker, Gertz et al. 2006).
The HDAC family includes four classes that are defined by their homology to the yeast deacetylases RPD3 (class I, IV) and HDA1 (class IIa, IIb, IV). While the HDACs were initially assumed to be nuclear proteins, it is becoming increasingly clear that most of them shuttle between the nucleus and cytoplasm.

Acetylation alters diverse biochemical properties of proteins through a variety of mechanisms (Figure 4.). Enzymatic activity (Lundby, Lage et al. 2012), subcellular localization (Gay, Calvo et al. 2003), and protein-protein interactions (Levy, Wei et al. 2004) can all be regulated by acetylation. Here we focus on the role of acetylation as a regulator of protein turnover. Protein turnover can be regulated by acetylation through competition with ubiquitination for lysine occupancy (Gronroos, Hellman et al. 2002; Ito, Kawaguchi et al. 2002), alteration of the higher order structure of protein-protein interaction sites (Choudhary, Kumar et al. 2009), or through interaction with acetyltransferases or deacetylases that have intrinsic ubiquitin-binding or –ligase activities (Hook, Orian et al. 2002; Linares, Kiernan et al. 2007).

Reduced acetylation has been detected in cancer cells, where it can impact tumorigenesis through diverse mechanisms. For example, mutations and deletions of the p300 acetyltransferase gene occur frequently in B-cell Non-Hodgkins Lymphoma and result in decreased acetylation and stability of the tumor suppressor protein, p53 (Pasqualucci, Dominguez-Sola et al. 2011). Another recent report identified an acetylation site that inhibits the activity of lactate dehydrogenase A, a protein that is often overexpressed in tumor cells, contributing to an increase in glucose metabolism that enables increased proliferation (Zhao, Zou et al. 2013). This acetylation which regulates cellular metabolism and proliferation was notably decreased in pancreatic tumor cells, suggesting that reduced acetyltransferase or increased deacetylase activity was contributing to the tumorigenicity of the cells. Overexpression of the microtubule
deacetylase, HDAC6, results in decreased microtubule acetylation and increased cell motility in vitro (Hubbert, Guardiola et al. 2002). Interestingly, decreased microtubule acetylation has been detected in prostate cancer cells compared to normal prostate cells, implicating MT acetylation in tumor cell biology as well (Soucek, Kamaid et al. 2006). In contrast to these examples of decreased acetylation, some recent studies suggest that global hyperacetylation is associated with pathological states such as cardiomyopathy (Colussi, Rosati et al. 2011; Vadvalkar, Baily et al. 2013). As we learn more about the regulators of non-histone protein acetylation, it seems likely that both positive and negative regulators of acetylation will continue to be tested as targets for novel drug development to combat a variety of diseases.

**Objectives of this study**

While many membrane proteins have long half-lives of 24 hours or more (Chu and Doyle 1985), connexins have reported half-lives for 30 minutes to 5.0 hours in tissue and cultured cells (Fallon and Goodenough 1981; Zhang and Nicholson 1989; Laird 1991; Beardslee, Laing et al. 1998). This discrepancy between the half-lives of other gated channel proteins and connexins is surprising and suggests that GJ mediated communication level is not strictly dependent upon the regulation of channel gating. Alterations in connexin gene transcription or protein stability could result in significant changes in GJ activity by altering the amount of connexin protein that is available for incorporation into GJ plaques. Alterations in trafficking or interactions with interacting proteins could also modulate GJ function and/or non-junctional connexin functions. Post-translational modifications are often involved in the fine tuning of protein function through the regulation of protein stability and protein-protein interactions, and are thus likely to be important regulators of connexins within GJ and in other physiological contexts.
Here we report that Cx32 is an acetylated protein and show the results of experiments that were conducted in order to determine the physiological significance of Cx32 acetylation. Specifically, we asked whether acetylation could modulate the turn-over, subcellular localization, and/or GJ function of Cx32. Pharmacological inhibition of deacetylase activity was utilized as a tool to enrich for acetylated Cx32 and also implicated HDAC6 as a regulator of Cx32 turn-over, through modulation of Cx32 acetylation. Our results suggest that acetylation is a positive regulator of Cx32 protein stability. Analysis of Cx32 mutants that mimicked constitutive acetylation suggested that reducing Cx32 turn-over through acetylation increases the amount of Cx32 at the cell surface. Increasing cell surface Cx32 levels did not result in a detectable increase in GJ-mediated intercellular communication, but was accompanied by reduced cellular proliferation in our model system. These results provide the framework for future investigation of protein-protein interactions that are modulated by Cx32 acetylation and physiological events that may influence Cx32 acetylation level.
Chapter 1- Figures
Figure 1. Electron microscopy revealed the ultrastructure of gap junctions. Goodenough and Revel (1970) identified GJ as hexagonal particles (right image) that align at varying densities along a 20 Å gap between adjacent cells (left image).
Figure 2. Schematic of connexin protein topology. (A.) All connexin proteins contain four transmembrane domains (labeled TM 1-4), two extra cellular loops, and three cytoplasmic domains that include the N- and C-termini. The positions of Cx32 cytoplasmic lysines, which may be subject to acetylation, are indicated. (B.) Pairs of hexameric connexin channels align at cell-cell borders to form pores between neighboring cells (depicted in purple).
Figure 3. **Connexins mediate direct cell-cell communication and influence a variety of physiological processes.** Members of the connexin protein family have been implicated in diverse physiological processes such as those depicted above. Connexin dependent pathways are regulated either through GJ mediated intercellular communication, the passage of molecules through unpaired hemichannels, or through connexin interactions with intracellular proteins that are independent of channel function altogether.
Figure 4. Protein turnover is regulated by acetylation through competition with ubiquitination for lysine occupancy (top), alterations of interaction sites for binding partners that regulate turnover (middle) or through interaction with acetyltransferases or deacetylases that have intrinsic ubiquitin-binding or –ligase activities (bottom).
Chapter 2

Connexin 32 acetylation is regulated by HDAC6
Introduction

Gap junctions are dynamic structures (Lauf, Giepmans et al. 2002; Flores, Nannapaneni et al. 2012; Wayakanon, Bhattacharjee et al. 2012), composed of short-lived (Fallon and Goodenough 1981; Yancey, Nicholson et al. 1981; Laird 1991) connexin proteins. The level of GJ-mediated communication between cells is dependent upon the amount of connexin protein available for incorporation into hemichannels, as well on efficient assembly and trafficking of the hemichannels. Post-translational modifications alter the biochemical properties of many proteins and thus fine-tune their involvement in physiological processes. GJ-mediated cell-cell communication is an example of a process that is modulated by post-translational modifications. For example, connexin phosphorylation has been implicated in the regulation of GJ gating (Mitra, Xu et al. 2012), assembly (Musil, Cunningham et al. 1990; Musil and Goodenough 1991; Lampe 1994), and disassembly (Lau, Kanemitsu et al. 1992; Leithe and Rivedal 2004). This work highlights the role of another post-translational modification, lysine acetylation, as a potential regulator of connexin protein stability and GJ assembly.

Reversible, covalent, post-translational modifications serve as regulators of protein structure and function. Among these modifications, ubiquitination, neddylation, sumoylation, methylation, and acetylation all occur on lysine residues, which have accessible epsilon-amino groups (Yang and Seto 2008). The consequences of lysine modifications vary according to the amino acid sequence surrounding the modification site and the charge and size of the modifying moiety.

Ubiquitination entails the enzymatic attachment of a single or polymeric chain of the 76-amino acid peptide, ubiquitin, to the substrate protein. This modification promotes protein
degradation (reviewed in (Kornitzer and Ciechanover 2000)), regulates endocytosis and protein sorting (reviewed in (Haglund and Dikic 2012)), and modulates protein-protein interactions (reviewed in (Zhang 2003)). Neddylation and sumoylation refer to the addition of small, ubiquitin-like proteins to substrates, which also modulate protein-protein interactions by altering secondary and tertiary protein structure (reviewed in (Rabut and Peter 2008), (Geiss-Friedlander and Melchior 2007)). Methylation and acetylation involve the addition of small functional groups to lysine, again resulting in altered interactions with other proteins that are mediated by changes in confirmation and charge of the modified protein (reviewed in (Yang and Seto 2008; Zhang, Wen et al. 2012)). These altered protein-protein interactions regulate protein stability (Ianari, Gallo et al. 2004; Geng, Liu et al. 2012), enzymatic activity (Vethantham, Rao et al. 2008; Lirussi, Antoniali et al. 2012), localization (Ishfaq, Maeta et al. 2012; Kjenseth, Fykerud et al. 2012), complex formation (Chan, Krstic-Demonacos et al. 2001; Kovacs, Murphy et al. 2005; Lee, Lee et al. 2008), and other features of protein function.

Many post-translational modifications of lysine residues were initially identified on histones (e.g.: (Gershey, Vidali et al. 1968; Goldknopf, Taylor et al. 1975; Goldknopf and Busch 1977)). Histone modifications influence transcriptional regulation by altering the electrostatic interactions between histones and DNA, in turn altering transcription factor access to the chromatin (reviewed in (Li, Nagaraja et al. 1993)). It eventually became evident that many transcription factors undergo post-translational modification of lysines as well, with acetylation and ubiquitination being the most common (Kuras, Rouillon et al. 2002; Gay, Calvo et al. 2003; Ianari, Gallo et al. 2004; Akiyama, Kamitani et al. 2005).

Ubiquitination has emerged as an important regulator of degradation of cytoplasmic proteins (Jennissen and Laub 1988; Laszlo, Doherty et al. 1990; Watson, Laszlo et al. 1991).
Acetylation, however, was initially assumed to be rare among proteins residing outside of the nucleus. Alpha-tubulin was the first cytoplasmic acetylated protein found to be acetylated, in 1985 (L'Hernault and Rosenbaum 1985), followed by the occasional identification of acetylation sites on widely studied proteins such as p53 (Li, Luo et al. 2002). It was not until proteomic technologies improved in the past decade that hundreds of other cytoplasmic and mitochondrial acetylated proteins were identified (Kim, Sprung et al. 2006; Choudhary, Kumar et al. 2009). Acetylation of cytoplasmic proteins has been implicated in the regulation of a variety of processes such as microtubule-mediated regulation of cell motility (Tran, Marmo et al. 2007) and chaperone-mediated protein folding (Scroggins, Robzyk et al. 2007).

Acetylation regulates protein turnover through several distinct mechanisms. In many cases there is competition between acetylation and ubiquitination of the same lysine, such that acetylation prevents ubiquitin-mediated proteasomal degradation of the protein (Gronroos, Hellman et al. 2002; Ito, Kawaguchi et al. 2002; Jin, Jeon et al. 2004; van Loosdregt, Vercoulen et al. 2010). Acetylation can also act as a signal that modulates protein-protein interactions in ways that either decrease (Jacob, Lund et al. 2001; Goel and Janknecht 2003; Suryaraja, Anitha et al. 2013) or increase (Jiang, Wang et al. 2011) recruitment of degradation machinery. Here we suggest that K231 and K260 of Cx32 are potential acetylation sites and play a role in regulating Cx32 turn-over. Furthermore, we implicate HDAC6 as a regulator of Cx32 acetylation and stability, but show that it does not alter Cx43 acetylation. Taken together, these results suggest that acetylation is likely to be a post-translational modification that is common to many connexin proteins, but that the regulation of connexin acetylation varies amongst members of the connexin protein family.
Results

**Cx43 localization, but not acetylation is altered by loss of HDAC6 activity.**

Our laboratory has used HDAC6 +/+ and -/- mouse embryonic fibroblasts (MEFs) in experiments investigating the role of microtubule acetylation in cell motility and vesicle transport((Tran, Marmo et al. 2007) and Salam et al, in prep). Meanwhile HDAC6 has been implicated as a major regulator of the acetylation of cytoplasmic proteins, with an expanding list of substrates (Hubbert, Guardiola et al. 2002; Kovacs, Murphy et al. 2005; Zhang, Yuan et al. 2007). As we worked to develop antibodies against pan-acetyl lysine, we became interested in detecting and studying the acetylation of cytoplasmic proteins that are often overlooked in large scale proteomic screens due to poor solubility under common lysis conditions. Connexins are one such group of proteins, as they require an ionic detergent such as SDS in order to become solubilized.

Since the HDAC6 +/+ and -/- MEFs express a readily detectable level of Connexin43 (Cx43), we began by assessing the acetylation of Cx43 in these cells. Cell extracts prepared in the presence of phosphatase inhibitors revealed that both phosphorylated (Figure 1A. upper band) and non-phosphorylated (Figure 1A., lower band) Cx43 are acetylated under steady state conditions in HDAC6 +/+ MEFs. Phosphorylation decreases the electrophoretic mobility of Cx43 and is detected on GJ associated Cx43, whereas the unphosphorylated Cx43 band is derived from the newly synthesized protein(Musil, Cunningham et al. 1990). This suggests that acetylated Cx43 exists in the cytoplasm as well as GJ.

Since we detected acetylated Cx43 in the cytoplasmic pool, we hypothesized that the cytoplasmic deacetylase, HDAC6, could regulate Cx43 acetylation. Acetylation can modulate
protein localization (Gay, Calvo et al. 2003; Ishfaq, Maeta et al. 2012), so we hypothesized that the localization of Cx43 in HDAC6 -/- or HDAC6 inhibited WT MEFs would differ from control WT MEFs. Immunostaining revealed that the localization of Cx43 is dramatically altered when HDAC6 activity is lost either through HDAC6 deletion (Figure 1B. HDAC6 -/-) or pharmacological inhibition (Figure 1B., HDAC6 +/-, 18h TubA). Loss of HDAC6 activity results in increased cytoplasmic and especially perinuclear localization of Cx43. Based upon these results, we hypothesized that Cx43 acetylation was regulated by HDAC6 activity and the loss of HDAC6 activity would increase Cx43 acetylation. However, probing Western blots of Cx43 IPs with antibodies against pan-acetyl lysine revealed that Cx43 was not hyperacetylated when HDAC6 activity was lost (Figure 1C.), suggesting that the altered Cx43 localization that we observed was not the result of increased Cx43 acetylation.

Cx32 is an acetylated protein

While we were investigating Cx43 acetylation, another group simultaneously published a fairly extensive study of Cx43 acetylation (Colussi, Rosati et al. 2011). At that time, we shifted our focus to the acetylation of Cx32. The Cx43 acetylation sites proposed by Colussi et al. are not conserved in Cx32. Comparison of the regulation of the two divergent connexins by the same post-translational modification seemed likely to provide valuable information about connexin acetylation as a general regulatory mechanism.

We are the first to report that Cx32 is acetylated (Figure 2A.). We detected both acetylation and ubiquitination of total Cx32 immunoprecipitated from N2a cells transfected with Cx32 plasmids. These post-translational modifications of Cx32 exist at readily detectable levels in cells that have not been treated with drugs or otherwise perturbed. A pilot experiment carried
out by Ranju Kumari and Elliot Hertzberg revealed that broad inhibition of Class I and II HDACs with TrichostatinA (TSA) resulted in the accumulation of Cx32 protein (Figure 2B.). TSA treatment also resulted in increased acetylation of Cx32 (Chapter 3. Figure 1B.) and thus we hypothesized that Cx32 acetylation could influence Cx32 stability. Moving forward, we began to use HDAC inhibition as a tool to study the physiological role of Cx32 acetylation.

Acetylation can regulate protein folding (Costantini, Ko et al. 2007) and subcellular localization (Gay, Calvo et al. 2003; Ishfaq, Maeta et al. 2012) and in doing so, is often restricted to a particular pool of a protein of interest. Information about the localization of an acetylated protein could reveal details about the particular role of its acetylation. We carried out cell surface biotinylation experiments in order to determine if acetylated Cx32 was clearly enriched or excluded from the cell surface.

Cell surface biotinylation, fractionation based upon solubility in 1% Triton-X (to separate Triton-X insoluble GJ from other Cx32 pools), followed by immunoprecipitation of Cx32 from plasma membrane (biotinylated) and cytoplasmic (non-biotinylated) fractions was carried out in order to determine which subcellular pools contained acetylated Cx32. Probing each fraction with antibodies against pan-acetyl-lysine revealed that acetylated Cx32 was present in Triton-X soluble and insoluble fractions of both cytoplasmic and plasma membrane associated pools (Figure 3A.). Quantification of normalized acetylated Cx32: total Cx32 ratios from three independent experiments indicated that acetylated Cx32 was not enriched in any particular fraction, but instead was found throughout the cellular Cx32 pool (Figure 3B.). It is worth noting that most of the Triton-X insoluble Cx32 that we detected by cell-surface biotinylation is unlikely to have been GJ associated because extracellular residues would not have been easily accessible to the biotinylation reagent when incorporated in GJ. Instead, the biotinylated, cell-
surface localized, acetylated Cx32 was likely in hemichannels that may have eventually become incorporated into GJ plaques. Our data should be reassessed alongside studies that determine the fate of Triton-X insoluble hemichannels, in order to determine if they are likely to become incorporated into GJ.

Treatment with Brefeldin-A (BFA) results in the accumulation of secretory proteins in the endoplasmic reticulum. These include ER-resident proteins and nascent proteins that were in the ER and Golgi at the time of treatment (Sciaky, Presley et al. 1997). We treated Cx32 expressing cells with BFA for 12 hours in order to determine if Cx32 acetylation was dependent upon exit from the secretory system, meaning that it was likely to occur at the cell surface. We found that Cx32 remained acetylated after BFA treatment (Figure 3C.), suggesting that acetylation may occur prior to exit from the secretory system. This is in agreement with the results shown in Figure 3A. and 3B., which indicate that acetylated Cx32 is not restricted to the plasma membrane.

**HDAC6 activity regulates Cx32 protein level and post-translational modifications**

Cx32 protein accumulated in the perinuclear region when cells were treated with TSA overnight (Figure 4A.). HDAC6 inhibition by overnight TubastatinA treatment (+TubA) results in a similar Cx32 distribution (Figure 4B.). The subcellular distribution of Cx32 in both cases is similar to that of Cx43 when HDAC6 activity is lost (compare Figure 4. to Figure 1B.). Inhibition of HDAC6 activity via TubastatinA treatment resulted in decreased ubiquitination of Cx32 (Figure 5A.). This was consistent with the accumulation of Cx32 protein that accompanied TSA treatment, since HDAC6 is among the large cohort of deacetylases inhibited by TSA (Figure 2B.) and TubastatinA treatment (Chapter 3. Figure 1C.).
HDAC6 has been co-immunoprecipitated with substrates such as HSP90 (Kovacs, Murphy et al. 2005), cortactin (Zhang, Yuan et al. 2007), and tubulin (Zhang, Li et al. 2003), thus we hypothesized that Cx32 interacts with HDAC6. We probed Cx32 immunoprecipitates from lysates that lacked ionic detergent for interactions between Cx32 and HDAC6 (Figure 5B.). HDAC6 and Cx32 were expressed in N2a cells from exogenous cDNAs for these experiments. We found that HDAC6 and Cx32 co-immunoprecipitate and that their interaction is significantly reduced in the presence of the TubastatinA (Figure 5C.), which has been reported to inhibit the C-terminal catalytic site of HDAC6 (Haggarty, Koeller et al. 2003). This TubastatinA induced reduction of Cx32-HDAC6 interaction suggests that the interaction occurs through the C-terminal catalytic site of HDAC6 and is dependent upon Zn2+ binding to HDAC6 (TubastatinA chelates the Zn2+). A similar loss of interaction due to TSA exposure, has been reported for the association of HDAC6 and HSP90 (Kovacs, Murphy et al. 2005).

Depletion of HDAC6 through expression of HDAC6 shRNA also resulted in an accumulation of Cx32 protein (Figure 6A.). HDAC6 shRNA expression causes a minor accumulation of acetylated Cx32 (Figure 6B.), as compared to Pharmacological inhibition of HDAC6, which increases Cx32 acetylation level appreciably (Chapter 3. Figure 1E.). However, depletion of HDAC6 through shRNA expression (Figure 6C.) resulted in a clear reduction in Cx32 ubiquitination. It is possible that the pharmacological inhibition caused nearly complete inhibition of HDAC6, whereas shRNA expression did not reduce HDAC6 expression enough to generate a detectable increase in acetylation, given the constraints of available pan-acetyl-lysine antibodies. Another possibility is that other HDACs compensate for HDAC6 after prolonged reductions in expression (shRNA expression was for 48 hours, compared to 12 hour
pharmacological inhibition). This could also be happening in the case of Cx43 and the HDAC6 -/- MEFs.

**C-terminal lysines influence Cx32 turnover**

Cx32 has 3 cytoplasmic domains; the N-terminus, cytoplasmic loop, and C-terminus. There are 9 lysines located in these domains and several of them were mutated to arginine in order to abolish potential cytoplasmic acetylation sites, in order to test the hypothesis that Cx32 accumulation that accompanied TSA treatment was due to altered Cx32 acetylation. K→R mutations at K231 and K260 prevented the accumulation of Cx32 in response to TSA treatment (Figure 7A.). We carried out several experiments to determine if K231 and K260 are major acetylation sites in Cx32 and to begin to determine the role of Cx32 acetylation.

Probing Western blots of Cx32 immunoprecipitates with pan-acetyl lysine antibodies revealed that Cx32 K231+260R(2R) was still acetylated (Figure 7B.). Since acetylation and ubiquitination often occur on the same lysines, we also probed for ubiquitination and found that Cx32 2R was ubiquitinated to roughly WT levels (Figure 7C.). However, the accumulation of ubiquitinated Cx32 in response to proteasome inhibition through MG132 treatment was reduced in 2R compared to WT (Figure 7C., +MG132). Based upon this result and the earlier observation that loss of HDAC activity resulted in Cx32 accumulation, we hypothesized that K231 and K260 were involved in modulating Cx32 turn-over.

We carried out 35-S pulse-chase experiments in order to compare the turn-over of Cx32WT and 2R (Figure 8A.). We found that there was significantly more Cx32 2R remaining after 1.5 hours chase, as compared to WT, confirming that K231 and/or K260 influence Cx32 turn-over (Figure 8B.). This increase in Cx32 stability was not accompanied by altered
subcellular distribution of Cx32 (Figure 8C., top panels and 8D.). Furthermore, Cx32 2R resembled WT after overnight HDAC6 inhibition (Figure 8C., bottom panels). Taken together, these results implicated K231 and K260 in the regulation of Cx32 stability, but failed to clarify the role of Cx32 acetylation.
Chapter 2- Figures
Figure 1.

A.

B.

C.
Figure 1. HDAC6 regulates Cx43 localization independent of acetylation.

(A.) Cx43 acetylation was detected by immunoprecipitating endogenous Cx43 from HDAC6 +/- MEFs in the presence of phosphatase inhibitors. Western blots were probed with antibodies against pan-acetyl lysine (AcK) and Cx43.

(B.) Cx43 was detected by immunofluorescence in HDAC6 +/- MEFs, HDAC6 +/- MEFs, and HDAC6 +/- MEFs with HDAC6 inhibited for 18 hours by treatment with 20 µM TubastatinA. (C.) Cx43 acetylation in each of these cases was assessed by immunoprecipitating Cx43 and then probing Western blots of the immunoprecipitates with antibodies against Cx43 and pan-acetyl lysine (AcK).
Figure 2.

A.

WB: Myc

AcK Cx32 Ub

30kDa

Input IP Myc-tag

B.

CX32: WT

TSA: - +
Figure 2. Cx32 accumulates in response to HDAC inhibition. (A.) Cx32 post-translational modifications were detected by probing Western blots of anti-Myc-tag immunoprecipitates of Cx32-Myc/His from N2a cells transfected with pcDNA3.1-Cx32-Myc/His with antibodies against pan-acetyl lysine (AcK) and pan-ubiquitin (Ub), as well as Cx32 and Myc-tag as loading controls. (B.) N2a cells expressing WT Cx32 and several K→R mutants of cytoplasmic residues were treated for 18 hours with DMSO (-) or 1μM TSA (+) and Cx32 protein was detected by Western blotting. This experiment was conducted by Ranju Kumari and Elliot Hertzberg.
Figure 3.

A.

TX Sol:  S  S  I  I  
Fract:  C  PM  C  PM  

IP Cx32 WB AcK
IP Cx32 WBCx32

B.

Normalized AcCx32 (A.U.)

C.

C  +BFA
IP Cx32, WB Cx32
IP Cx32, WB AcK
Figure 3. Acetylated Cx32 is found in the cytoplasm and at the cell surface. (A.) N2a cells expressing pIRESegfp-Cx32 were subjected to cell-surface biotinylation. Biotinylated cell extracts were prepared in a buffer containing 1% Triton X-100 (S) and Triton insoluble (I) proteins were sedimented and solublized in a buffer containing SDS. Strepavidin based isolation of biotinylated proteins was used to separate cytosolic (C) and plasma membrane (PM) proteins, followed by immunoprecipitation of Cx32 in order to detect acetylated and total Cx32 by Western blotting. (B.) Average intensities of AcCx32, normalized over total Cx32, are plotted for 3 independent experiments +/- S.E.M. Student’s T-test was used for statistical analysis, which revealed no significant differences between the amounts of AcCx32 found in the different fractions. (C.) N2a cells expressing Cx32-Myc/His were treated for 12 hours with 5 µg/ml Brefeldin-A (BFA) or DMSO vehicle (C). Cx32 immunoprecipitates were probed for Cx32 and pan-acetyl lysine (AcK) content.
Figure 4.

A. Control +TSA (16h)

B. Control +TubA (16h)
Figure 4. Cx32 accumulation after TSA and TubastatinA treatment are similar. N2a cells were transfected for 56 hours with pcDNA3.1Myc/His-Cx32, then treated overnight with a broad class I and II HDAC inhibitor, TSA (A. +TSA), or the HDAC6 specific inhibitor, TubastatinA (B. TubA), or DMSO (A, B Control). Cx32 immunostaining was imaged by confocal microscopy at 63x magnification and 6x 0.29 μm slice Z-projections are shown here. Scale bars = 25 μm.
Figure 5.

A.

TubA: - +

[Image: IP Cx32, WB Poly-Ub]

[Image: IP Cx32, WB Cx32]

B.

- + TubA

[Image: HDAC6 in]

[Image: Tubulin in]

[Image: IP:Cx32, WB HDAC6]

[Image: IP:Cx32, WB: Cx32]

C.

[Graph: Co-IP HDAC6:Cx32 (AU)]

C  T *
Figure 5. HDAC6i increases Cx32 acetylation and reduces polyubiquitination. (A.) Polyubiquitination of Cx32 after HDAC6i was assessed by transfecting N2a cells with Cx32-Myc/His and then treating for 6 hours with 20 µM TubastatinA (+ TubA) or DMSO(-TubA), then Cx32 was immunoprecipitated and Western blots of immunoprecipitates were probed with antibodies against Cx32 or poly-ubiquitin. Note that the amount of Cx32 in the +TubA sample is clearly increased while poly-ubiquitin is slightly decreased as compared to DMSO treated, meaning that overall poly-ubiquitination was greatly decreased by HDAC6 inhibition. (B.) Cx32 association with HDAC6 was also assessed by co-immunoprecipitation in the presence of DMSO (-TubA) or 20 µM TubastatinA (+TubA). N2a cells expressing Cx32-Myc/His and HDAC6-FLAG were treated overnight with DMSO or TubastatinA 48 hours post-transfection for these experiments. Western blots were probed with antibodies against Myc-tag (Cx32), FLAG-tag (HDAC6) and beta-tubulin. (C.) Average ratio of HDAC6:Cx32 in IP is plotted +/- S.E.M. and Student’s T-test indicated that there is a significant difference between the amount of HDAC6 in +TubA and –TubA Co-Ips (*) p<0.05.
Figure 6.

A.

Scr HDAC6 shRNA

![Image showing Western Blot for Cx32, Tubulin, and AcTubulin]

B.

Scr HDAC6 shRNA

![Image showing IP Cx32 and WB AcK, IP Cx32 and WB Cx32]

C.

Scr HDAC6 shRNA

![Image showing IP Cx32 and WB Pan-Ub, IP Cx32 and WB Cx32]
Figure 6. HDAC6 KD results in Cx32 protein accumulation and reduced ubiquitination. (A.) N2a cells were co-transfected with plasmids encoding Cx32-Myc/His and shRNAs against HDAC6 or a random sequence (Scr). Cx32-Myc/His was isolated by Co2+ affinity binding 48 hours post-transfection and post-translational modifications were assessed by Western blotting. Crude extracts were blotted and probed for Cx32, tubulin, and acetylated tubulin. Hyperacetylation of tubulin was used to confirm that HDAC6 level had been reduced by shRNA expression because the steady state level of HDAC6 expression in N2a cells is difficult to detect. Cx32 acetylation was detected by probing the blot of affinity isolated Cx32 with antibodies against pan-acetyl lysine (AcK, B.) and ubiquitination was detected by probing similar blots with antibodies against ubiquitinated lysine (Pan-Ub, C.).
### A.

<table>
<thead>
<tr>
<th>CX32:</th>
<th>WT</th>
<th>K104R</th>
<th>K121R</th>
<th>K124R</th>
<th>K231R</th>
<th>K244R</th>
<th>K260R</th>
<th>K276R</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA:</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

### B.

- Cx32: WT 2R
- IP Cx32
- WB AcK
- IP Cx32
- WB Cx32

### C.

<table>
<thead>
<tr>
<th>Cx32:</th>
<th>WT</th>
<th>2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG132:</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- IP Cx32
- WB Pan-Ub
- IP Cx32
- WB Cx32
Figure 7. Post-translational modifications of Cx32 cytoplasmic lysines are regulated by TSA treatment. (A.) N2a cells expressing WT Cx32 and several K→R mutants of cytoplasmic residues were treated for 18 hours with DMSO (-) or 1µM TSA (+) and Cx32 protein was detected by Western blotting. Red boxes denote K→R mutants that did not accumulate with TSA treatment. This experiment was conducted by Ranju Kumari and Elliot Hertzberg. (B.) Acetylation and (C.) ubiquitination of WT Cx32 and K231+260R (2R) Cx32 were detected by probing Western blots of Cx32 immunoprecipitates with antibodies against pan-acetyl lysine (AcK) (B.) and pan-ubiquitin (C.) respectively. N2a cells were transfected with Cx32-Myc/His WT or 2R for 48 hours in both experiments. Cells were treated with DMSO (- MG132) or 10 µM MG132 (+MG132, proteasome inhibitor) for 6 hours prior to lysis when probing ubiquitination.
Figure 8.

A.

\[
\begin{array}{c|c|c|c|c}
\text{T(h):} & 0 & 0.5 & 1.0 & 1.5 \\
\hline
\text{WT} & \text{35-S Cx32} & & & \\
& \text{Cx32-Myc} & & & \\
\hline
\text{2R} & \text{35-S Cx32} & & & \\
& \text{Cx32-Myc} & & & \\
\end{array}
\]

B.

\[
\begin{array}{c|c|c}
& \text{WT} & 2R \\
\hline
\text{35-S Cx32 Remaining t=1.5h} & \text{%} & \\
\hline
& 30 & 70 \\
\end{array}
\]

*
Figure 8. Continued

C.

WT  2R

Normalized Cx32 at cell-cell border (AU)

D.
Figure 8. K231 and K260 modulate Cx32 turn-over, but not localization.

(A.) Turn-over of Cx32 WT and K231 +260R (2R) was assessed by 35-S pulse-chase assay (as described in methods). Immunoprecipitated 35-S Cx32 was detected by Western blotting and autoradiography. The Western blot was exposed directly to a storage phosphor screen overnight and then phosphorimaged (representative phosphorimage shown). Then the blot was probed with an antibody against Myc-tag and the 35-S signal for each sample was normalized over Cx32-Myc load. (B.) Mean normalized 35-S remaining after 1.5 hour chase are plotted +/- S.E.M. (n=3) and Student’s t-test was used for statistical analysis. (*) p<0.05 as compared to WT. (C.) N2a cells expressing WT or 2R Cx32-Myc/His were grown on glass coverslips for 48 hours and then treated overnight with 20 µM TubastatinA (+TubA) or DMSO (-TubA) and fixed. Cx32-Myc/His was detected by immunostaining of Myc-tag, followed by confocal microscopy at 63x magnification. Images are Z-projections of 6 slices that were most in focus at points of cell-cell contact. (D.) Mean Cx32 staining intensity at cell-cell junctions normalized against cytoplasmic Cx32 staining intensity +/- S.E.M. are plotted for control (-TubA) images (n=20 cell pairs).
Discussion

Here we have shown that Cx32 is an acetylated protein and that Cx32 acetylation is regulated by HDAC6. Acetylated Cx32 is found in the cytoplasm and at the cell surface, suggesting that acetylation is a common post-translational modification of Cx32. Further, both ubiquitination and acetylation can be detected in the same Cx32 immunoprecipitate, without the use of pharmacological inhibitors to prevent the reversal of either modification. Pharmacological or genetic manipulation of HDAC6 activity resulted in Cx32 protein accumulation and decreased ubiquitination, suggesting that acetylation may be a regulator of Cx32 stability. Interestingly, pharmacological or genetic inhibition of HDAC6 activity did not alter Cx43 acetylation, suggesting that the acetylation of Cx32 and Cx43 are regulated through different pathways.

Despite the fact that Cx43 acetylation did not change due to altered HDAC6 activity, we observed a reduction in Cx43 at points of cell-cell contact in HDAC6 -/- MEFs, as well as in HDAC6 +/- MEFs treated with TubastatinA. A study by Shaw et al. demonstrated that microtubule dependent delivery of Cx43 to cell-cell junctions is important for proper Cx43 GJ formation(Shaw, Fay et al. 2007). Recent work in our lab has shown that microtubule based intracellular transport becomes less efficient when HDAC6 activity is lost (Salam et al., in prep), so perhaps the altered localization of Cx43 that we observed was due to a transport defect that is independent of Cx43 acetylation.

We used HDAC inhibitors as a tool to promote the accumulation of acetylated Cx32 in order to study the consequences of Cx32 acetylation. We found that overnight HDAC inhibition caused Cx32 protein to accumulate in the perinuclear region of N2a cells. The accumulation of
Cx32 protein occurred when HDAC6 was specifically inhibited with TubastatinA or as a result of broad class I and II HDAC inhibition with TSA. Both inhibitors likely altered many aspects of overall cellular physiology since they alter the acetylation of numerous substrates. Later experiments were carried out with shorter periods of HDAC inhibition, which stabilized Cx32 protein but did not result in global protein trafficking defects (see Chapter 3. of this thesis).

Since we observed an accumulation of Cx32 protein after TSA treatment, we hypothesized that acetylation is a regulator of Cx32 stability. Screening a number of cytoplasmic K→R mutants for the ability to prevent Cx32 protein accumulation in the presence of TSA identified K231 and K260 as putative acetylation sites. The Cx32 K231+K260→R (2R) mutant turned over more slowly than WT and showed reduced ubiquitination, providing support for the hypothesis that acetylation modulates Cx32 stability. However, we found that 2R Cx32 acetylation was not appreciably reduced when compared to WT. 2R Cx32 also still seemed to accumulate after HDAC6 inhibition. Furthermore, we had not screened all 9 of the cytoplasmic lysines found in Cx32 and the screen had been carried out with TSA instead TubastatinA. We concluded that K231 and K260 do influence Cx32 stability and are likely acetylation sites, but there are acetylation sites elsewhere in Cx32 as well.
Materials and Methods

Cell Lines

WT and HDAC6 null mouse embryonic fibroblasts were generously provided by Dr. Tso-Pang Yao from Duke University. Experiments with transfected cells were carried out in Neuro-2a (N2a) cells (ATCC #CCL-131), which lack endogenous connexins. N2a cells are derived from a mouse neuroblastoma and can be cultured under conditions that either promote or prevent differentiation into a neuronal cell type. All cells were maintained in DMEM supplemented with 10% FBS at 37°C and 5% CO2, in a humidified environment.

Plasmids

Rat Cx32 cDNA in pIRES2eGFP was obtained from Elliot Hertzberg. Rat Cx32 cDNA was cloned into BamHI/ApaI sites of pcDNA3.1/Myc-His A. K→R and K→Q mutations were made using Genetaylor site directed mutagenesis kit (Invitrogen). HDAC6-FLAG plasmid was generously provided by Dr. Tso-Pang Yao from Duke University.

Western blotting

2% SDS and 100mM DTT were added to samples for Western blotting and samples were not heated above 25°C. Proteins were separated on 10% polyacrylamide denaturing gels and transferred to nitrocellulose membranes. Blocking and antibody dilution was carried out in PBS containing 2% BSA and 0.05% Tween.

Western blots were probed with mouse anti-Cx32 clone 7c6.6 (Elliot Hertzberg) 1:1000, rabbit anti-Cx32 L1 (Elliot Hertzberg) 1:1000, rabbit anti-Myc tag (AbCam) 1:2000, rabbit anti-acetylated proteins (Cell Signaling Technologies) 1:1000, mouse anti-ubiquitinated proteins
clone P4G7 (Covance) 1:200, anti-FLAG M2 antibody (Invitrogen) 1:1000, rabbit anti-FLAG (AbCam) 1:1000, and mouse anti-beta-tubulin clone 3F3-G2 at 1:2000.

Anti-Cx32 L1 antibody was used at 1:100 for immunoprecipitation and rabbit anti-Myc-tag (Abcam) was used at 1:200.

Western blots were probed with goat anti-rabbit IRdye800 and goat anti-mouse IRdye700 secondary antibodies (LiCor) and digital images were obtained with the Odyssey imaging system (LiCor).

**Drugs and other chemicals**

TubastatinA was obtained from Chemie Tek (catalog # CT-TUBA) and dissolved in DMSO prior to further dilution in medium. All chemicals were obtained from Sigma Aldrich, unless otherwise indicated.

**Transfections**

An 80-90% confluent 10cm plate of N2a cells was transfected with 12 µg DNA, 24 µl PLUS reagent and 36µl Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. Antibiotic free medium was used for transfections and all subsequent culture steps. Transfection mix was removed from cells 4-6 hours post-transfection and cells were subcultured or plated on coverslips for microscopy.

**Immunofluorescence**

Cells were grown on acid washed glass coverslips for 48 hour prior to 15 minute, room temperature fixation with Histochoice (Sigma Aldrich). Coverslips were washed with PBS prior to fixation and all subsequent washes were with PBS supplemented with 0.05% Tween. Blocking
and antibody dilutions were carried out in PBS supplemented with 0.05% Tween and 2% BSA. Cx32 was detected with rabbit anti-Myc-tag or anti-Cx32 LI diluted 1:500 for 1 hour at room temperature. Alexa-568 goat-anti-mouse secondary antibody (Invitrogen) was used at 1:1000 in blocking buffer for 15 minutes at room temperature. The coverslips were mounted on glass slides in Fluoromount (Invitrogen) with 1µg/ml DAPI.

**Imaging**

A Zeiss LSM 700 confocal microscope system was used to obtain Z-stacks at 63x magnification. All images were obtained with a 1 airy unit pinhole. Laser power, filter settings, beam splitter, and scan mode were the same for all images. Brightness and contrast were adjusted using ImageJ software, all images were adjusted equally. Cx32 at cell-cell contacts was quantified in a single Z-slice where the points of cell-cell contact for a particular cell pair were most in focus, as determined by alignment of Cx32 staining and edges of phase image. Fluorescence intensity along the contacting edges was quantified by linescan analysis in ImageJ, using a 10 pixel, segmented line.

**Cell lysate preparation for IP and Co2+ purification**

Cells were scraped into PBS and collected by centrifugation at 9000 rpm in an Eppendorf microcentrifuge. PBS was aspirated and cells were lysed in 470 µl lysis buffer (PBS supplemented with 1% Triton-X 100 and protease inhibitors). Then 30 µl 20% SDS was added and lysate was mixed well and diluted with 1ml of lysis buffer and passed through a 26.5 gauge needle. Debris were removed by centrifugation at 45 krpm at 4 C/15 minutes. 100 µl of 50% slurry of Hispur Co2+ coated agarose beads (Pierce) were used to isolate His tagged Cx32, which was eluted with lysis buffer containing 300mM imidazole.
**35-S Met/Cys Pulse-Chase Assay**

Cells were transfected 48 hours prior to experiments. Each experimental sample consisted of confluent 10cm plate of transfected cells. Cells were rinsed with 3x 4ml PBS and then starved in DMEM lacking serum, methionine, and cysteine for 1h. The 35-S Met/Cys pulse was carried out for 20 minutes. Radioactive pulse medium contained 50 mCi/ml of 35-S Met/Cys (Expre35-S35-S labeling mix, Perkin Elmer) in serum and Met/Cys free DMEM and 4ml/10cm plate was used for each sample. After radioactive pulse, each plate was washed with 3x 4ml PBS and complete DMEM with an additional 20mM Met/Cys and 10% FBS was added for indicated chase times.

Cell lysates were prepared as described above and Cx32-Myc/His was isolated with Co2+-agarose resin. Co2+ purified Cx32 was analyzed by Western blotting. 35-S labeled Cx32 was detected by exposing the blots overnight to a storage phosphor and then imaging with a Storm Phosphorimager (GE Life Sciences). Then the blots were probed with antibodies against the Myc tag as a loading control. 35-S Cx32 and Cx32-Myc/His bands were quantified using image-J and normalized 35-S Cx32 values were obtained for each sample by dividing 35-S Cx32 band intensity by Cx32-Myc/His band intensity.

**Cell surface biotinylation**

Biotinylation experiments were carried out 48 hours post-transfection. Confluent 60mm plates of transfected cells were used for each experimental sample. Cell surface proteins were labeled with 2x 2.5 ml of 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS supplemented with Ca2+ and Mg2+ for 30 minutes, gently shaking in an ice bath. After biotinylation cells were rinsed 4x in PBS supplemented with 100mM Glycine and lysed. Biotinylated proteins were
isolated by binding to Neutravidin-agarose beads (Thermo Scientific) and then eluted with SDS-PAGE sample buffer and subjected to Western blotting. Biotinylated Cx32 was detected by probing biotinylated proteins with an antibody against Myc-tag.
Chapter 3

C-terminal lysines modulate Cx32 turnover and ability to suppress growth of cell cultures
**Introduction**

Gap junctions (GJ) have been implicated in diverse physiological processes such as embryonic patterning (Warner, Guthrie et al. 1984; Ewart, Cohen et al. 1997; Dobrowolski, Hertig et al. 2009; Hatler, Essner et al. 2009; Rhee, Zhao et al. 2009), synaptic transmission (Barr, Berger et al. 1968; Brightman and Reese 1969; Alvarez-Maubecin, Garcia-Hernandez et al. 2000; Houades, Koulakoff et al. 2008), tumor suppression (Mehta, Perez-Stable et al. 1999; Edwards, Jondhale et al. 2008), and cardiac conduction (Smith, Green et al. 1991; Mureli, Gans et al. 2013), all of which are dependent upon appropriate intercellular communication. Despite—or perhaps because of— the broad physiological significance of GJ-mediated intercellular communication, many questions remain about how it is regulated.

GJ-mediated intercellular communication refers to the passage of small molecules between the cytoplasm of adjacent cells, allowing cells to coordinate a variety of physiological processes such as cell division, migration, and differentiation (reviewed in (Dbouk, Mroue et al. 2009)). GJ are permeable to a variety of molecules less than 1 kDa in size including metabolites, second messengers, and ions. Connexins are the protein subunits that GJ are composed of. There are 21 different connexins expressed in mammals and the connexin composition of a particular GJ determines its permeability (Willecke, Eibberger et al. 2002; Söhl and Willecke 2004). There is a high level of sequence homology amongst the different connexin gene products, with the exception of the cytoplasmic loop and C-terminal domains. The cytoplasmic loops of connexins are involved in channel gating and are important determinants of GJ permeability (Wang, Li et al. 1996). Connexin C-termini vary substantially in length and in their capacity to mediate interactions with the cytoskeleton (Giepmans, Verlaan et al. 2001; Chakraborty, Mitra et al. 2010; Saidi Brikci-Nigassa, Clement et al. 2012), other junctional complexes (Wei, Francis et al. 2005),
and other cytoplasmic proteins (reviewed in (Dbouk, Mroue et al. 2009)). The C-terminal sequences of connexins have also been implicated in voltage (Moreno, Chanson et al. 2002), pH (Ek-Vitorin, Calero et al. 1996; Francis, Stergiopoulos et al. 1999; Eckert 2002), and chemical (Yeager and Nicholson 1996) gating of GJ channels. While C-terminal truncation of connexins does not alter single channel conductance, the truncated connexin may accumulate in the cytoplasm indirectly reducing overall GJ-mediated cell-cell communication (Barrio, Castro et al. 1999; Castro, Gomez-Hernandez et al. 1999; Maass, Shibayama et al. 2007; Johnson, Reynhout et al. 2012).

Each GJ is composed of two hexameric connexin hemichannels. The connexin hemichannels align within the extracellular space of adjacent cells in order to form a pore between the cells (Kumar and Gilula 1996). The aligned hemichannels are located in accretions that are referred to as GJ plaques. GJ plaques are dynamic structures that are constantly being repopulated at their outer margins, while older hemichannels are removed from the center (Lauf, Giepmans et al. 2002; Thomas, Jordan et al. 2005; Flores, Nannapaneni et al. 2012). The balance between hemichannel delivery to and removal from the plasma membrane thus regulates the amount of GJ-mediated cell-cell communication and other connexin dependent physiological processes occurring within the cell.

In this study, we focus on the role of C-terminal residues as modulators of Cx32 protein level, which as a consequence regulates the amount of Cx32 at the plasma membrane. Most of what is known about post-translational modifications of connexins and interactions between connexins and cytoplasmic proteins has been deduced by studying Cx43. We chose to focus on Cx32 because the variability of C-terminal sequences of connexins raises questions about whether or not Cx43 regulation through C-terminal residues and post-translational modifications
is conserved among the connexin family as a whole. Further, Cx32 plays an important role in neuronal myelination (Scherer, Xu et al. 1998) and can also regulate proliferation (King and Lampe 2004; Prost, Bernier-Valentin et al. 2008), so understanding pathways that regulate Cx32 GJ formation is inherently valuable. Cx32 is the most abundantly expressed connexin in Schwann cells and is mutated in the X-linked demyelinating neuropathy, Charcot Marie Tooth syndrome (CMTX) (Bergoffen, Scherer et al. 1993). CMTX associated mutations are found throughout the Cx32 gene and result in diverse alterations of Cx32 protein, which ultimately cause defects in Cx32 trafficking and GJ formation, or GJ gating (Ressot, Gomes et al. 1998; Abrams, Freidin et al. 2001; Wang, Chang et al. 2004).

The quantity of connexin protein available for incorporation into hemichannels and eventually into GJ plaques could be regulated through a variety of mechanisms. We report here that Cx32 is acetylated and since acetylation is often a regulator of protein stability (reviewed in Caron, Boyault et al. 2005), we hypothesized that Cx32 acetylation might regulate Cx32 turnover. Connexins have short half-lives compared to other membrane proteins (Yancey, Nicholson et al. 1981; Chu and Doyle 1985; Beardslee, Laing et al. 1998) and recent studies have revealed that GJ plaques are highly dynamic (Flores, Nannapaneni et al. 2012; Johnson, Reynhout et al. 2012). Further, GJ channels have a low open probability overall, with a small fraction actively involved in GJ-mediated cell-cell communication at any instant (Bukauskas, Bukauskiene et al. 2001; Curti, Hoge et al. 2012). This suggests that the replenishment of hemichannels to GJ plaques must be tightly regulated in order to maintain the proper level of coupling between cells. Post-translational modifications such as acetylation may regulate the amount of connexin at the cell surface, indirectly regulating GJ-mediated intercellular communication as well. Here we identified the C-terminal lysines of Cx32 as likely acetylation
sites and showed that altering their charge stabilizes Cx32 protein, increases the amount of Cx32 localized in the plasma membrane, and results in reduced growth of cell cultures. Additionally, we show that C-terminal lysines are dispensable for Cx32 channel function and suggest that increasing plasma membrane associated Cx32 regulates proliferation or apoptosis through a mechanism that may be independent of GJ mediated communication.

Results

**HDAC6 inhibition causes Cx32 protein accumulation**

Post-translational acetylation regulates the turnover of many proteins (Li, Luo et al. 2002; Ianari, Gallo et al. 2004; Costantini, Ko et al. 2007; Geng, Liu et al. 2012), thus deacetylase inhibitors are often employed to promote the accumulation of acetylated proteins. Cx32 protein accumulated when cells were treated with a broad spectrum deacetylase inhibitor, TrichostatinA (TSA) (Figure1A.) This accumulation of Cx32 protein was accompanied by an increase in Cx32 acetylation (Figure 1B.), implying that acetylation could be a regulator of Cx32 turnover.

HDAC6 is inhibited by TSA and has several known cytoplasmic substrates (Hubbert, Guardiola et al. 2002; Kovacs, Murphy et al. 2005; Zhang, Yuan et al. 2007). Unlike other deacetylases inhibited by TrichostatinA, HDAC6 is actively retained in the cytoplasm (Verdel, Curtet et al. 2000; Bertos, Gilquin et al. 2004) and HDAC6 inhibition does not alter histone acetylation or other modes of transcriptional regulation (Haggarty, Koeller et al. 2003). We hypothesized that HDAC6 inhibition would result in the accumulation of acetylated Cx32 protein, recapitulating the results of TSA treatment, independent of any transcriptional perturbations that may have occurred as a result of broad HDAC inhibition.
Cx32 protein accumulated in cells that were treated with the HDAC6 inhibitor, TubastatinA (Figure 1C.). This could have been due to increased Cx32 expression or decreased Cx32 turnover. We carried out 35-S pulse-chase experiments to compare the turnover of Cx32 in cells that had been treated with DMSO (control) or TubastatinA (TubA). 35-S labeled Cx32 was detected by autoradiography and total Cx32 in each sample was determined by Western blotting (Figure 1D.). Since the amount of Cx32 increased in the presence of TubastatinA, we normalized the 35-S band intensity over total Western blot Cx32 band intensity and then reported the percentage of normalized 35-S Cx32 remaining at t=1.5 hours. There was nearly twice as much Cx32 protein remaining after a 1.5 hour chase when cells were treated with TubastatinA (Figure 1E.). This result supports the hypothesis that Cx32 accumulation in response to HDAC6 inhibition was due to stabilization of Cx32 protein and not an increase in Cx32 expression. Cx32 immunoprecipitates probed with pan-acetyl-lysine (AcK) antibodies revealed that acetylation increased as Cx32 protein accumulated due to HDAC6 inhibition (Figure 1F.). Again, the total amount of Cx32 in the HDAC6 inhibited sample increased, but normalized acetylated Cx32 band intensity over total Cx32 band intensity ranged from a 2-9 fold increase over the control in 3 independent experiments.

After we had shown that total Cx32 turnover is reduced by HDAC6 inhibition, we wondered if we were detecting the stabilization of primarily cell-surface localized Cx32 or if HDAC6 was modulating the turnover of Cx32 in multiple subcellular compartments. If Cx32 acetylation reduced the internalization of the cell-surface Cx32 pool, then it would subsequently reduce lysosomal degradation of Cx32 protein. It is also possible that nascent Cx32, which would be cytoplasmic compartments, could be stabilized by acetylation through the inhibition of its proteasomal degradation. Cell-surface biotinylation experiments were carried out in order to
determine if acetylated Cx32 was accumulating preferentially in the cytoplasm or at the cell surface. This was assessed by comparing the internalization of cell surface Cx32 in cells that had either been treated with TubastatinA or DMSO.

Cell surface proteins were labeled with biotin after 3 hours of pretreatment with TubastatinA or DMSO and then either lysed or incubated in medium that again contained either TubastatinA or DMSO for an additional 1.5 hours. Biotinylated and total Cx32 were measured by Western blotting and ratios of biotinylated: total Cx32 were determined (Figure 2A.). TubastatinA treatment reduced the fraction of biotinylated: total Cx32 in the cell at the initial time point of the experiment (Figure 2B., top graph), but we also observed a larger fraction of biotinylated Cx32 remaining after the 1.5 hour chase in the presence of TubastatinA (Figure 2B., bottom graph). Taken together, these results suggest that Cx32 accumulated in the cytoplasm and at the cell surface after HDAC6 inhibition. Immunofluorescent staining and confocal imaging confirmed these results. Cx32 staining in both cytoplasmic and cell surface pools was visibly increased after 6 hours of TubastatinA treatment (Figure 2C.).

C-terminal lysines are implicated in HDAC6 inhibitor response

We hypothesized that acetylation sites in cytoplasmic domains of Cx32 are the most likely to be involved in modulating its turnover, since protein turnover depends upon interactions with cytoplasmic proteins. Connexins have three cytoplasmic domains; the N-terminus, cytoplasmic loop, and C-terminus. Cx32 has 9 lysines located in these domains, thus we made single point mutants of each cytoplasmic lysine and tested them for resistance to the HDAC inhibitor induced accumulation that we observed with WT Cx32. Lysine to arginine mutations (K→R) result in a non-acetylatable residue, while conserving the positive charge of lysine.
TubastatinA treatment resulted in the equivalent accumulation of WT and cytoplasmic loop K→R mutant Cx32 proteins(Figure 3A.). However, C-terminal K→R mutations reduced the accumulation of Cx32 protein in response to HDAC6 inhibition, as compared to WT Cx32 (Figure 3A.,B.). C-terminal lysine to glutamine mutations (K→Q), which act as an acetylation mimetic, also abrogated the HDAC6 inhibitor response (Figure 3B.).

Single point mutations of Cx32 C-terminal K→R did not result in a detectable reduction in Cx32 acetylation (data not shown), so we made constructs with all 5 C-terminal lysines mutated to arginine (5R) and glutamine (5Q) and probed immunoprecipitates for acetylation. We found that 5R Cx32 was still acetylated, but that this acetylation did not increase in response to TubastatinA treatment (Figure 3C.). This suggests that the observed increase in Cx32 acetylation and accumulation of Cx32 protein in response to HDAC6 inhibition was dependent upon C-terminal lysines. The acetylation level of 5Q Cx32 was visibly lower than WT, but increased in response to HDAC6 inhibition (Figure 3C.). This suggests that a subset of the C-terminal lysines could be acetylation sites, but that there may be other secondary acetylation sites that are detected after HDAC6 inhibition under these experimental conditions.

Ubiquitination of 5R Cx32 was reduced when compared to WT (Figure 3D.); thus we hypothesized that its turnover would also be reduced compared to WT. 35-S pulse-chase experiments indicated that Cx32 5R turns over more slowly than WT (Figure 4.). Replacement of C-terminal lysines with glutamine (5Q), in order to mimic constitutive acetylation, slowed down Cx32 turnover to an even greater extent than K→R mutation (Figure 4.). Unlike Cx32 5R, Cx32 5Q acetylation was visibly decreased when compared to WT (Figure 3C.) and its ubiquitination was decreased when compared to both WT and 5R Cx32 (Figure 3D.).
together, results recapitulated what we found for WT Cx32 when HDAC6 was inhibited and thus provide further support for the hypothesis that acetylation modulates Cx32 protein level.

**C-terminal lysines regulate Cx32 level at the cell surface**

Cell surface biotinylation experiments indicated that C-terminal lysines are dispensable for the delivery of Cx32 to the cell surface (Figure 5A.). Confocal imaging of immunostained N2a cells expressing WT, 5R, and 5Q Cx32 corroborated these results (Figure 5B. –TubA and Figure S1.). Quantification of fluorescence intensity at points of cell-cell contact suggested that similar amounts of 5R and WT Cx32 are incorporated into GJ (Figure 5B. –TubA and Figure S1.). However, the fluorescence intensity of 5Q Cx32 was increased at points of cell-cell contacts, compared to WT and 5R (Figure 5B. –TubA and Figure S1.). Neither 5R nor 5Q Cx32 protein accumulated at the cell surface in response to 6 hours of TubastatinA treatment, whereas WT did (Figure 5B, +TubA and Figure S1.). This confirmed our earlier Western blot results that implicated the C-terminal lysines in the HDAC6 inhibitor induced accumulation of Cx32 protein. Note that WT Cx32 with HDAC6 inhibition, and the acetylation mimetic mutant, 5Q Cx32, both increase the level of Cx32 at the cell surface compared to the WT Cx32 control. This provides further evidence that the 5Q mutant phenocopies Cx32 acetylation.

**C-terminal lysines are dispensable for GJ function**

These experiments were carried out by Dr. Charles Abrams at SUNY Downstate. Dual whole cell recordings of trans-junctional conductance indicated that 5R and 5Q Cx32 formed functional GJ (Figure 6A.). Both of the Cx32 mutants generated trans-junctional voltages that were comparable to WT Cx32, indicating that cells expressing all three constructs were well coupled. These experiments were conducted with transiently transfected cells that expressed high
levels of Cx32 and it is unclear if it would be possible to detect an increase in cell-cell coupling above the already high WT level in these experiments. A three-fold increase in the amount of DNA used in transfections did not cause a significant increase in cell-cell coupling for any of the constructs, suggesting that the expression of Cx32 in all cases was too high to detect increases in coupling (Figure 6B.).

The voltage gating profile of Cx32 5R resembled WT Cx32, indicating that the mutations did not cause any major disruptions in Cx32 protein structure (Figure 6C.). We are in the process of completing the voltage gating experiments for comparison of 5Q and WT Cx32, but we anticipate that they will also be similar due to the fact that we have already shown that 5Q Cx32 forms functional GJ.

Acetylation is a modulator of Cx32 mediated suppression of cell culture expansion

Cx32 has been shown to act as a tumor suppressor in a variety of cell types, particularly when they are poorly differentiated (Mehta, Perez-Stable et al. 1999; Omori, Zaidan Dagli et al. 2001; Edwards, Jondhale et al. 2008; Prost, Bernier-Valentin et al. 2008; Jee, Nam et al. 2011). Tumor suppression by Cx32 expression could be the result of increased apoptotic cell death or decreased cell division. We hypothesized that increasing the cell surface pool of Cx32 would slow growth either by increasing cell-cell coupling, increasing hemichannel mediated paracrine signaling, or through the modulation of interactions between Cx32 and cytoplasmic proteins.

Equal numbers of N2a cells were transfected with pIRES2egfp, pIRES2egfp-WT, 5R, or 5Q Cx32 and then were counted 72 hours post-transfection (Figure 6A.). We found that after 72 hours there were over 50% less 5Q Cx32 expressing cells compared to either pIRES2egfp,
pIRES2egfp-WT Cx32, or 5R Cx32 (Figure 6B.). This could be the result of reduced cell division or increased cell death. Since we know that both mutants form functional GJ with WT gating properties (Figure 5.), it seems unlikely that this growth suppression is due to toxicity associated with overexpression of Cx32. Therefore, we conclude that 5Q Cx32 suppresses the growth of N2a cell cultures either through negative regulation of cell cycle progression or positive regulation of apoptosis.
Chapter 3 - Figures
Figure 1.

A.

<table>
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<tr>
<td>40kDa</td>
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</table>

IP Cx32, WB AcK

B.

TSA: - +

IP Cx32, WB AcK

C.

TubA: - +

Tubulin

Cx32
Figure 1. Continued

D.

\[
\begin{array}{c|c|c|c|c}
\text{t(h):} & 0 & 0.75 & 1.5 & 3.0 \\
30 \text{ kDa} & - & - & - & - \\
35-S & - & - & - & - \\
30 \text{ kDa} & + & + & + & + \\
\end{array}
\]

E.

![Bar graph showing % 35-S Cx32 at 1.5h with TubA](image)

F.

\[
\begin{array}{c|c|c}
\text{TubA:} & - & + \\
30 \text{ kDa} & \text{IP:Cx32} & \text{WB:Cx32-Myc} \\
30 \text{ kDa} & \text{IP:Cx32} & \text{WB:AcK} \\
\end{array}
\]
Figure 1. HDAC6 inhibition reduces turn-over and increases acetylation of Cx32. (A.) HeLa cells expressing pIRESeGfpCx32 were treated overnight with the indicated concentrations of TSA and then lysed and subjected to Western blotting. Blots were probed with antibodies against Cx32 and beta actin as a loading control. (B.) Acetylation of Cx32 in N2a cells expressing pIRESeGfp-Cx32 after overnight treatment with 1 uM TSA was assessed by probing Cx32 immunoprecipitates with antibodies against pan-acetyl lysine (AcK). (C.) N2a cells were transfected with pcDNA3.1Myc/His-Cx32 for 48 hours and then cells were treated for 6 hours with the HDAC6 inhibitor, TubastatinA (20 µM) (C.+TubA) or DMSO (C.-TubA). Cx32 and beta tubulin were detected by Western blotting. (D.) The turnover of Cx32 with (D.+TubA) and without (D.-TubA) HDAC6 inhibition was assessed by 35-S pulse-chase. For pulse chase experiments, Cx32-Myc/His transfectants were pretreated with drug during 1 hour amino acid starve and throughout the experiment. Cells were pulsed with 35-S Met/Cys for 30 minutes and then either lysed or chased with complete medium with extra Met/Cys. Cx32-Myc/His was recovered by Co2+ affinity purification, followed by Western blotting. Western blots were exposed directly to a storage phosphor, which was subsequently phosphorimaged. (E.) 35-S intensity was normalized over total Cx32-Myc load and percent of 35-S Cx32 remaining after 1.5 hours was quantified from 4 independent experiments and means +/- S.E.M. are plotted here. Student’s T-test was used for statistical analysis. (*) p<0.05 as compared to DMSO control. (F.) Cx32 acetylation after overnight HDAC6 inhibition (F.+TubA) or DMSO (F.-TubA) treatment was assessed by immunoprecipitating Cx32-Myc/His and probing blots of immunoprecipitates with antibodies against pan-acetyl-lysine (AcK) or Cx32.
Figure 2.

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30 kDa

Total Cx32-Myc

Bio Cx32-Myc

B.
Figure 2. Continued

C.  

\(-\text{TubA}\)  \(+\text{TubA}\)
Figure 2. HDAC6 inhibition results in the accumulation of both cytoplasmic and cell surface Cx32. (A.) N2a cells were transfected for 48 hours with pcDNA3.1Myc/His-Cx32 and then used in cell-surface protein biotinylation experiments. Cells were pretreated for 3 hours with either TubastatinA (A.+TubA) or DMSO (A.-TubA) and cell surface proteins were labeled with biotin for 30 minutes. After biotinylation cells were either lysed or chased for 1.5 hours with complete medium. Biotinylated proteins were isolated by binding to streptavidin and subjected to Western blotting. Biotinylated Cx32 was detected by probing biotinylated proteins with an antibody against Myc-tag. Total Cx32 was also blotted and probed with the Myc-tag antibody. (B.) Biotinylated Cx32 as a fraction of total Cx32 for each sample at t=0 is plotted here (B. left graph), as well as fraction of biotinylated Cx32 remaining after 1.5 hour chase (B. right graph), both +/- S.E.M. Student’s T-test was used to determine statistical significance of differences between DMSO and TubastatinA treatments, (*)p<0.05. (C.) Confocal microscopy was used to observe the cellular localization of Cx32 after a short (6 hour) TubastatinA treatment. Cx32 antibody staining was carried out in N2a cells 48 hours post-transfection with pIRES2egfp- WTCx32. Confocal imaging was used to obtain Z-stacks of 0.29µm slices (63X). Images shown are maximum intensity projections of 5 slices that were most in focus at points of cell-cell contact. Scale bars = 25µm and arrows indicate points of cell-cell contact. Cells were incubated with either DMSO (C.-TubA) or 20µM TubastatinA (C.+TubA) for 6 hours prior to fixation.
Figure 3.

A.

Cx32: WT  K103R  K104R  K121R  K124R

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Cx32-Myc

Tubulin

B.

Cx32: WT  5Q  5R

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Cx32-Myc

Tubulin
Figure 3. Continued

C.

<table>
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<tr>
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30 kDa
IP Cx32, WB AcK

30 kDa
IP Cx32, WB Cx32

D.

<table>
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<tr>
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<th>WT</th>
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230 kDa
IP Cx32-Myc/6xHis
WB Pan-Ub

60kDa

30 kDa
IP Cx32-Myc/6xHis
WB Myc

E.

![Graph showing Cx32 Ub/Cx32 total (% Of WT)]
Figure 3. C-terminal lysines are implicated in HDAC6i response. (A.) Single K→R mutations were made at each cytoplasmic lysine in pcDNA3.1Myc/His-Cx32. N2a cells were transfected with plasmids encoding WTCx32 or one of the 9 cytoplasmic K→R mutants for 48 hours, followed by overnight treatment with 20 uM TubastatinA (A. +TubA) or DMSO (A. -TubA). Cells were then lysed in buffer containing 2% SDS and subjected to Western blotting. Blots were probed with antibodies against the Myc-tag to detect Cx32 or beta-tubulin as a loading control. (B.) Multi-site mutants were constructed such that all 5 cytoplasmic, C-terminal lysines were mutated either to arginine (5R) or glutamine (5Q). N2a cells expressing WT, 5R, or 5Q Cx32 were treated with 20uM TubastatinA for 6 hours, 48 hours post-transfection. Cx32 and beta-tubulin were again detected by Western blotting. (C.) Acetylation of WT, 5R, and 5Q Cx32 was assessed by immunoprecipitating Cx32 and probing the immunoprecipitates with antibodies against Cx32 or pan-acetyl-lysine (AcK). One of 3 representative experiments is shown. (D.) Ubiquitination of WT, 5R, and 5Q Cx32 was assessed by probing Western blots of Co2+ affinity purified Cx32 Myc/His with antibodies against pan-ubiquitin and Myc-tag. (E.) Ub-Cx32 immunoreactivity was normalized over Cx32-Myc and means of fraction WT Cx32-Ub are plotted +/- S.E.M. for independent experiments (n=3).
Figure 4.

A.

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</table>

30 kDa ---- 35-S Cx32
25kDa ----
30 kDa ----

WB Cx32-Myc

B.

% 35-S Cx32 remaining at 1.5h

- WT
- 5R
- 5Q

*
Figure 4. C-terminal lysines influence Cx32 turn-over. (A.) N2a cells were transfected for 48 hours with pcDNA3.1Myc/His- WTCx32, 5RCx32, or 5QCx32 and then pulsed with 35-S Met/Cys for 5 minutes followed by lysis or 1.5 hour chase in complete medium. Cx32-Myc/His was isolated by affinity purification with Co2+ agarose and detected by Western blotting and autoradiography. The Western blot was exposed directly to a storage phosphor screen overnight and then phosphorimaged (representative phosphorimage shown). Then the blot was probed with an antibody against Myc-tag and the 35-S signal for each sample was normalized over Cx32-Myc load. (B.) Mean normalized 35-S remaining after 1.5 hour chase are plotted +/- S.E.M. (n=3) and Student’s t-test was used for statistical analysis. (*) p<0.05 as compared to WT. Note that the apparent half-life of Cx32 in these experiments is lower than reported by other groups and our experiment in Figure 1. because the 35-S labeling was carried out for a shorter time (10 minutes versus 20-30 minutes) and thus the detection limit was reached sooner.
Figure 5.

A. 

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<th>5Q</th>
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B. 

<table>
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<tr>
<td>+TubA</td>
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C. 

avg grey value at cell-cell contact (au)
Figure 5. C-terminal lysines influence Cx32 localization and HDACi response. (A.) Cell surface biotinylation was performed in order to confirm the presence of WT, 5R, and 5Q Cx32 at the cell surface. N2a cells were biotinylated 48 hours after transfection with pcDNA3.1Myc/His-WT, 5R, or 5Q Cx32. Biotinylated proteins were isolated by binding to streptavidin and then detected by Western blotting. Biotinylated and total Cx32 were detected by probing Western blots with an antibody against the Myc-tag. (B. and C.) The amount of Cx32 at points of cell-cell contact was measured by quantifying the fluorescence intensity of Cx32 antibody staining of N2a cells 48 hours after transfection with pIRES2egfp- WTCx32, 5RCx32, or 5QCx32. (B.) Confocal imaging was used to obtain Z-stacks of 0.29µm slices (63X). Images shown are maximum intensity projections of 6 slices that were most in focus at points of cell-cell contact. Cells were incubated with either DMSO (-TubA) or 20µM TubastatinA (+TubA) for 6 hours prior to fixation. Scale bars are 25µm and arrows indicate points of cell-cell contact. (C.) Anti-Cx32 fluorescence intensity at points of cell-cell contact was measured as described in methods. Average fluorescence intensities at cell-cell contacts for each set of images is plotted here +/- S.E.M. (n=15-23 cell pairs for each group. Student’s T-test was used for statistical analysis of means as compared to WT and * p<0.05
Experiment and analysis conducted by C. Abrams.
Figure 6. C-terminal lysine mutants form functional GJ. (A.) To evaluate the channel forming ability of the 5R and 5Q mutants, we measured electrical coupling between cell pairs with dual whole-cell recordings. Mean junctional conductance (Gj) for empty pIRESegfp (I), WT, 5R, and 5Q Cx32 are plotted +/- S.E.M. (B.) Cells in experiments plotted here were transfected with 3-fold more DNA than in A. (C.). Representative Average normalized Gj-Vj relations for 5R and WT Cx32 channels are shown in. Both cells of a pair were voltage clamped at the same voltage, then cell 1 (was stepped in 20 mV increments from Vj= –100 to Vj =+100 mV, and junctional currents (Ij) were recorded from cell 2. The average normalized instantaneous (open triangles) and steady-state (filled squares) junctional conductance (Gj) at each Vj were calculated from current traces and fit as described in the Methods. Solid lines represent fits to Cx325R; broken lines represent fits to data for Cx32WT homotypic and Cx32WT/Cx30WT cell-cell channels. Representative Average normalized Gj-Vj relations for 5R Cx32 channels. Both cells of a pair were voltage clamped at the same voltage, then cell 1 (was stepped in 20 mV increments from Vj= –100 to Vj =+100 mV, and junctional currents (Ij) were recorded from cell 2. The average normalized instantaneous (open triangles) and steady-state (filled squares) junctional conductance (Gj) at each Vj were calculated from current traces and fit as described in the Methods. Solid lines represent fits to Cx325R; broken lines represent fits to data for Cx32WT homotypic and Cx32WT/Cx30WT cell-cell channels.
Figure 7.

A. egfp at 72h
- pIRES2egfp
- pIRES2egfp-WT Cx32
- pIRES2egfp-5R Cx32
- pIRES2egfp-5Q Cx32

B. Bar graph showing nuclei per image:
- pIRES2egfp-WT Cx32
- pIRES2egfp-5R Cx32
- pIRES2egfp-5Q Cx32

* indicates significant difference.
Figure 7. Cx32 5Q suppresses growth of N2a cells. (A.) Equal numbers of N2a cells were transfected with pIRES2egfp, pIRES2egfp-WT Cx32, 5RCx32, or 5QCx32 and plated on glass coverslips after 6 hours. Cells were allowed to grow for 72 hours, then were fixed and mounted for imaging. (B.) Cells were imaged at 40x and nuclei were counted from 36 independent fields per transfection (n=3). Mean cell counts for 3 independent transfections were plotted with error bars +/- S.E.M. Student’s T-test was used to compare cell counts at 72 hours post-transfection. (*)p<0.05 as compared to 5Q Cx32.
Figure S1.
Figure S1. Continued
Figure S1. Continued
**Figure S1. C-terminal lysines influence Cx32 localization and HDACi response.** The amount of Cx32 at points of cell-cell contact was measured by quantifying the fluorescence intensity of Cx32 antibody staining of N2a cells 48 hours after transfection with pIRES2egfp- WTCx32, 5RCx32, or 5QCx32. Confocal imaging was used to obtain Z-stacks of 0.29um slices (63X). Cells were incubated with either DMSO (-**TubA**) or 20µM TubastatinA (+**TubA**) for 6 hours prior to fixation. Scale bars are 10µm and arrows indicate points of cell-cell contact where Cx32 staining is in focus within a single Z-slice. Images shown here are representative of those quantified in Figure 5C.
Discussion

The data presented here address the role of C-terminal lysines and their post-translational modifications as regulators of Cx32 function. Our results demonstrate that C-terminal lysines modulate the turnover of Cx32 protein. Further, our data suggest that at least a subset of these lysines is subject to acetylation. Decreasing Cx32 turnover through K→Q mutations or treatment with a deacetylase inhibitor resulted in an increase in Cx32 protein at the cell surface. Since 5Q Cx32 forms functional GJ channels, we propose a model where acetylation results in increased Cx32 channel activity, either as hemichannels or GJ, due to the stabilization of Cx32 protein. Also, 5Q Cx32 may participate in increased or new protein-protein interactions with plasma membrane or cytoplasmic proteins as a result of its increased plasma membrane localization. These protein-protein interactions and/or increased GJ or hemichannel function may contribute to the suppression of growth of cell cultures that express 5Q Cx32 by inhibiting proliferation or promoting cell death. Thus our data suggest that acetylation may act as a regulator of a variety of Cx32 dependent processes that are fine-tuned through alterations in Cx32 protein level.

We utilized deacetylase inhibitors to promote the accumulation of acetylated Cx32 and determined that HDAC6 in particular is a regulator of Cx32 acetylation. While we did not show direct interaction through in vitro deacetylation assay, we did show that even brief HDAC6 inhibition was sufficient to accumulate Cx32 protein throughout the cell and that this accumulation was accompanied by increased Cx32 acetylation. These results recapitulated our initial results with the broad type I and II deacetylase inhibitor, TSA. It is worth noting that HDAC3 and 4 have been implicated as regulators of Cx43 acetylation, which are frequently localized at the plasma membrane (Colussi, Rosati et al. 2011). We have not ruled out that
possibility that they also deacetylate Cx32. Future experiments should address whether other class I and II deacetylases also alter Cx32 acetylation in a way that impacts its stability.

Our analysis of cytoplasmic lysine mutants of Cx32 revealed that C-terminal lysines are involved in the Cx32 accumulation which results from HDAC6 inhibition. To our knowledge, other studies have not shown a role for these residues in the regulation of Cx32 turnover or GJ plaque dynamics. Other studies have shown that while Cx32 with a truncation of its C-terminal domain retains the capacity to form functional GJ, the resultant level of cell-cell coupling is inversely proportional to the number of amino acids that were truncated (Barrio, Castro et al. 1999; Castro, Gomez-Hernandez et al. 1999). Furthermore, much of the truncated Cx32 accumulates in the cytoplasm, implicating the C-terminus in the regulation of GJ assembly and/or disassembly (Ahmad, Martin et al. 2001). Our results provide further support for a model of GJ assembly that is regulated by the C-termini of connexins. Specifically, our data suggest that post-translational modifications of C-terminal lysines are implicated in the modulation of the amount of Cx32 that exists at the cell surface. This cell surface pool of Cx32 may be incorporated into GJ or may exist as hemichannels, either of which could regulate downstream physiological processes.

Interestingly, both of our 5 lysine mutants remained detectably acetylated. 5R Cx32 acetylation was roughly equivalent to WT, but did not increase in response to HDAC6 inhibition. This suggests that the 5 C-terminal lysines are indeed acetylated in response to HDAC6 inhibition, but that there are other acetylated lysines in Cx32. Other physiologically relevant Cx32 acetylation may increase when the C-terminal lysines are no longer available because the protein may be recognized as unacetylated, resulting in acetylase recruitment. Another possibility is that other lysines are spuriously acetylated when normal sites are unavailable, but
the protein is not detectably acetylated. 5Q Cx32 acetylation is visibly decreased as compared to 5R and WT, suggesting that the mutations are actually eliminating acetylation sites. The acetylation level of 5Q Cx32 increases after HDAC6 inhibition, but the protein does not accumulate, again suggesting enhancement of spurious acetylation in a region of the protein that is not involved in regulating Cx32 stability.

This work also provides evidence that acetylation alters Cx32 protein level through protein-protein interactions that are dependent upon the lysines that we mutated and not simply through elimination of ubiquitination sites. We come to this conclusion because 5Q Cx32 is increased at the cell surface compared to WT and 5R Cx32. Also, 5Q Cx32 suppresses the growth of N2a cell cultures, whereas 5R Cx32 does not. Both mutants are more stable than WT and show reduced ubiquitination, but these changes are not sufficient to alter the physiological consequences of Cx32 expression. The alterations in Cx32 stability that we observed may be due to altered protein-protein interactions that are modulated by the loss of positively charged residues, which normally occurs as a result of acetylation and is mimicked by K\(\rightarrow\)Q mutation.

Connexins are paradigmatic of a large cohort of proteins that are regulated by interactions with binding partners, which cause a particular sequence to change from an intrinsically disordered state into a functional secondary structure (Receiveur-Brechot, Bourhis et al. 2006; Stauch, Kieken et al. 2012). For example, calmodulin (CaM) is thought to stabilize the otherwise disordered structure of the Cx32 C-terminus into an alpha-helix when bound (Stauch, Kieken et al. 2012). Chemical gating of Cx32 GJ occurs in the presence of high concentrations of Ca\(^{2+}\), which results in the loss of CaM binding and the return to a disordered C-terminal state (Dodd, Peracchia et al. 2008) (Hertzberg and Van Eldik 1987). Inhibition of CaM binding or truncation of CaM binding sites resulted in cytoplasmic accumulation of Cx32, further
highlighting the regulatory role of interactions between Cx32 C-terminus and binding partners (Ahmad, Martin et al. 2001). Based on this example, we hypothesize that either the positive charge of the lysine or the presence or absence of an acetyl group may modulate interactions between the C-terminus of Cx32 and its binding partners. This could result in alterations of Cx32 structure which in turn modulate Cx32 stability and dynamics at the cell surface. Future studies comparing the binding partners of WT and 5Q Cx32 will be necessary in order to test this possibility.

While we are just beginning to understand the role of acetylation as a direct regulator of connexins, some earlier studies addressed the overall impact of deacetylase inhibitors on Cx32 and Cx43 GJ formation. TSA treatment enhanced GJ-mediated cell-cell communication and the amount of Cx32 in GJ in primary cultures of hepatocytes (Vinken, Henkens et al. 2006). These changes occurred independent of increased Cx32 transcription. Interestingly, Cx43 protein level increased after TSA treatment as well, but was localized to the nucleus with very little GJ formation. These results were recapitulated when primary cultures of mitogen stimulated hepatocytes were grown in the presence of 4-Me₂N-BAVAH, a structural analog of TSA with increased metabolic stability (Vinken, Henkens et al. 2007; Henkens, Vinken et al. 2008). Culture in the presence of Me₂N-BAVAH promoted hepatocyte differentiation, increased Cx32 GJ formation and decreased Cx43 protein level, while having no effect on Cx32 or Cx43 transcription. These studies did not address whether connexin acetylation was altered by deacetylase inhibition, but they provided evidence that deacetylase inhibition could influence GJ in varied ways, depending on their constituent connexins.

A recent report suggested that Cx43 acetylation acts as a negative regulator of GJ formation (Colussi, Rosati et al. 2011). HDAC inhibition was associated with increased
acetylation and decreased Cx43 at cell-cell junctions. K→Q mutations of putative acetylation sites also decreased the amount of Cx43 in GJs. It is important to note that the Cx43 acetylation sites identified in this study (K9, K234, and K264) do not share sequence homology with Cx32. Alignment of Cx32 and Cx43 C-terminal amino acid sequences using protein-protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that there is only one C-terminal lysine in a well conserved portion of the two proteins. It is K250 (NKLLSEQD) in Cx32 and K303 (NKQASEQN). Our work suggests that K250 of Cx32 could be an acetylation site. The possible acetylation of Cx43 K303 and its role as a regulator of Cx43 stability were not addressed in the initial characterization of Cx43 acetylation. More work is needed in order to determine if there are residues in Cx43, such as K303, which may be involved in regulating GJ-mediated cell-cell coupling in a manner that is homologous to C-terminal lysines in Cx32, or if the consequences of acetylation vary amongst members of the connexin protein family.
Materials and Methods

Cell Lines

Experiments were carried out in Neuro-2a (N2a) cells (ATCC #CCL-131), which lack endogenous connexins. N2a cells are derived from a mouse neuroblastoma and can be cultured under conditions that either promote or prevent differentiation into a neuronal cell type. Cells were maintained in DMEM supplemented with 10% FBS at 37°C and 5% CO2, in a humidified environment. These culture conditions prevent differentiation. N2a cells are particularly well suited for studying GJs because they amenable to electrophysiology and biochemical studies owing to their high transfection efficiency and small size.

Plasmids

Rat Cx32 cDNA in pIRES2eGFP was obtained from Elliot Hertzberg. Rat Cx32 cDNA was cloned into BamHI/ApaI sites of pcDNA3.1/Myc-His A. K→R and K→Q mutations were made using Genetator site directed mutagenesis kit (Invitrogen).

Western blotting

2% SDS and 100mM DTT were added to samples for Western blotting and samples were not heated above 25°C. Proteins were separated on 10% polyacrylamide denaturing gels and transferred to nitrocellulose membranes. Blocking and antibody dilution was carried out in PBS containing 2% BSA and 0.05% Tween.

Western blots were probed with mouse anti-Cx32 clone 7c6.6 (Elliot Hertzberg) 1:1000, rabbit anti-Myc tag (AbCam) 1:2000, rabbit anti-acetylated proteins (Cell Signaling Technologies)

Anti-Cx32 clone 7c6.6 (Elliot Hertzberg) was used for immunoprecipitation at 1:100.

Western blots were probed with goat anti-rabbit IRdye800 and goat anti-mouse IRdye700 secondary antibodies (LiCor) and digital images were obtained with the Odyssey imaging system (LiCor).

**Drugs and other chemicals**

TubastatinA was obtained from Chemie Tek (catalog # CT-TUBA) and dissolved in DMSO prior to further dilution in medium. All chemicals were obtained from Sigma Aldrich, unless otherwise indicated.

**Transfections**

An 80-90% confluent 10cm plate of N2a cells was transfected with 12 µg DNA, 24 µl PLUS reagent and 36µl Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. Antibiotic free medium was used for transfections and all subsequent culture steps. Transfection mix was removed from cells 4-6 hours post-transfection and cells were subcultured or plated on coverslips for microscopy.

**Immunofluorescence**

Cells were grown on acid washed glass coverslips for 48 hour prior to 15 minute, room temperature fixation with Histochoice (Sigma Aldrich). Coverslips were washed with PBS prior to fixation and all subsequent washes were with PBS supplemented with 0.05% Tween. Blocking and antibody dilutions were carried out in PBS supplemented with 0.05% Tween and 2% BSA.
Cx32 was detected with mouse anti-Cx32 clone 7C6.6 (developed by Elliot Hertzberg) diluted 1:1000 for 1 hour at room temperature. Alexa-568 goat-anti-mouse secondary antibody (Invitrogen) was used at 1:1000 in blocking buffer for 15 minutes at room temperature. The coverslips were mounted on glass slides in Fluoromount (Invitrogen) with 1µg/ml DAPI.

**Imaging**

A Zeiss LSM 700 confocal microscope system was used to obtain Z-stacks at 63x magnification. All images were obtained with a 1 airy unit pinhole. Laser power, filter settings, beam splitter, and scan mode were the same for all images. Brightness and contrast were adjusted using ImageJ software, all images were adjusted equally. Cx32 at cell-cell contacts was quantified in a single Z-slice where the points of cell-cell contact for a particular cell pair were most in focus, as determined by alignment of Cx32 staining and edges of phase image. Fluorescence intensity along the contacting edges was quantified by linescan analysis in ImageJ, using a 10 pixel, segmented line.

Images were acquired at 40x magnification for cell counting experiments. These images consisted of 4x3 image tile scans in a single Z-slice for cell counting experiments. Equal numbers of N2a cells were transfected with pIRES2egfp, pIRES2egfp-WT, 5R, or 5Q Cx32 and then replated on glass coverslips and allowed to grow for 72 hours. After 72 hours the cells were fixed and nuclei were stained with DAPI. Cells were imaged in 27 independent fields per coverslip, from 3 independent transfections and nuclei were counted manually using ImageJ software.
**Cell lysate preparation for IP and Co2+ purification**

Cells were scraped into PBS and collected by centrifugation at 9000 rpm in an Eppendorf microcentrifuge. PBS was aspirated and cells were lysed in 470 µl lysis buffer (PBS supplemented with 1% Triton-X 100 and protease inhibitors). Then 30 µl 20% SDS was added and lysate was mixed well and diluted with 1ml of lysis buffer and passed through a 26.5 gauge needle. Debris were removed by centrifugation at 45 krpm at 4 C/15 minutes. 100 µl of 50% slurry of Hispur Co2+ coated agarose beads (Pierce) were used to isolate His tagged Cx32, which was eluted with lysis buffer containing 300mM imidazole.

**35-S Met/Cys Pulse-Chase Assay**

Cells were transfected 48 hours prior to experiments. Each experimental sample consisted of confluent 10cm plate of transfected cells. Cells were rinsed with 3x 4ml PBS and then starved in DMEM lacking serum, methionine, and cysteine for 1h. The 35-S Met/Cys pulse was carried out for 20 minutes (+/-TubA experiments) or 5 minutes (WT versus 5R/5Q). Radioactive pulse medium contained 50 mCi/ml of 35-S Met/Cys (Expre35-S35-S labeling mix, Perkin Elmer) in serum and Met/Cys free DMEM and 4ml/10cm plate was used for each sample. After radioactive pulse, each plate was washed with 3x 4ml PBS and complete DMEM with an additional 20mM Met/Cys and 10% FBS was added for indicated chase times.

Cell lysates were prepared as described above and Cx32-Myc/His was isolated with Co2+-agarose resin. Co2+ purified Cx32 was analyzed by Western blotting. 35-S labeled Cx32 was detected by exposing the blots overnight to a storage phosphor and then imaging with a Storm Phosphorimager (GE Life Sciences). Then the blots were probed with antibodies against the Myc
tag as a loading control. 35-S Cx32 and Cx32-Myc/His bands were quantified using image-J and normalized 35-S Cx32 values were obtained for each sample by dividing 35-S Cx32 band intensity by Cx32-Myc/His band intensity.

**Cell surface biotinylation**

Biotinylation experiments were carried out 48 hours post-transfection. Confluent 60mm plates of transfected cells were used for each experimental sample. Cell surface proteins were labeled with 2x 2.5 ml of 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS supplemented with Ca2+ and Mg2+ for 30 minutes, gently shaking in an ice bath. After biotinylation cells were rinsed 4x in PBS supplemented with 100mM Glycine and either lysed or chased for 1.5 hours with complete medium. Biotinylated proteins were isolated by binding to Neutravidin-agarose beads (Thermo Scientific) and then eluted with SDS-PAGE sample buffer and subjected to Western blotting. Biotinylated Cx32 was detected by probing biotinylated proteins with an antibody against Myc-tag.

**Electrophysiology**

Conductance measurements were made by pulsing from $V_j=0$ to $\pm 40$ mV. Cytoplasmic bridges were excluded by demonstrating the sensitivity of $G_j$ to application of bath solution containing octanol. Values for different pairing configurations were compared using the Kruskal-Wallis test with Dunn’s multiple comparison test. For determination of steady-state junctional conductance-junctional voltage ($G_j-V_j$) relations, cells were held between each pulse at $V_j=0$ for 22.5 seconds, and cell 1 was pulsed in 20 mV increments from –100 to +100 mV for 12.5 seconds. Any resulting change in current in cell 2, the apposed cell, is attributable to junctional current. Currents are displayed with positive up for both cells; thus a positive $V_j$ step in cell 1 causes a
downward deflection in cell 2. For WT and mutants with measurable conductance at $V_j=0$ mV, voltage pulses were preceded by a short (~200 mS) pulse to -20 mV for normalization. Data were collected as described. Steady-state ($t=\infty$) conductances were determined by fitting each current trace to a sum of exponentials. Dividing the current at $t=\infty$ by the applied voltage gives the steady-state junctional conductance. For determination of Boltzmann parameters, steady-state plots were fit to the product of two Boltzmann distributions of the form:

$$G_{ss}(V) = \frac{(G_{min} + (G_{max} - G_{min})/(1+e^{A(V-V_0)})) \times (G_{min} + (G_{max} - G_{min})/(1+e^{A(V-V_0)}))}{(1+e^{A(V-V_0)})}$$

where $G_{ss}$ is the steady-state junctional conductance normalized to $V_j=0$, $G_{max}$ is the maximal normalized conductance, and $G_{min}$ is the normalized residual conductance, which in macroscopic recordings is approached as the absolute value of $V_j$ is increased, $V_0$ is the voltage at which the conductance is 1/2 of the difference between $G_{min}$ and $G_{max}$ and roughly corresponds to the voltage at which a single connexin hemichannel has an open probability of 50%, and $A$ is a parameter which reflects the slope of the $G_j-V_j$ plot and is a measure of voltage sensitivity. $A=nq/kT$ where $n$ is the effective gating charge and $q$, $k$ and $T$ have their usual meanings. The (+) and (-) designations indicate that the parameters are fits to the positive or negative limbs of the $G_j-V_j$ relation. The model makes two assumptions that may not always be met for connexin gating: 1. It assumes a two state gating process where the energy difference between the states is proportional to the applied voltage, and 2: The steady state $G_j-V_j$ arises from the gating of two independent apposed hemichannels. However, the parameters generated provide a useful basis for comparison among channels produced by various pairing
configurations of mutant and WT connexins. Homotypic pairs of Cx32WT-IRES-EGFP served as a positive control and pairs of empty vector expressing cells served as negative controls for homotypically paired mutants. For heterotypic cell pair controls, an additional “heterotypic” pairing of Cx32WT-IRES-EGFP with Cx32WT-IRES-DSRed expressing cells was performed. Pipette solution: 145 mM CsCl, 5 mM EGTA, 1.4 mM CaCl2, 5.0 mM HEPES pH 7.2; Bath solution: 150 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM Dextrose, 2 mM Pyruvate, 5 mM HEPES; pH 7.4
Chapter 4

Conclusions and Future Experiments
Summary of Conclusions

Based upon the data presented in this thesis we conclude the following;

- **Cx32 undergoes lysine acetylation.**
- **Acetylated Cx32 is found in both cytoplasmic and cell-surface associated Cx32 pools.**
- **Cx32 acetylation is regulated by HDAC6 and thus HDAC6 inhibition results in:**
  - Increased Cx32 acetylation
  - Decreased Cx32 ubiquitination
  - Reduced Cx32 turnover
  - Increased Cx32 at the cell surface
- **C-terminal lysines modulate Cx32 turnover and are likely acetylation sites.**
  - K→R mutations of C-terminal lysines result in:
    - Decreased Cx32 ubiquitination
    - Reduced Cx32 turnover
    - Cell surface Cx32 protein level resembling WT
    - Cell-cell coupling comparable to WT
    - Growth of mutant expressing culture is comparable to WT
  - K→Q mutations of C-terminal lysines result in:
    - Decreased Cx32 ubiquitination
    - Reduced Cx32 turnover
    - Increased cell surface Cx32 protein compared to WT
    - Cell-cell coupling comparable to WT
    - Growth of mutant expressing culture is reduced compared to WT
- **Based upon comparison of C-terminal K→R and K→Q mutants to WT Cx32, we conclude that acetylation enhances Cx32 protein stability through the modulation of protein-protein interactions rather than simple competition for lysine occupancy. K→R and K→Q mutations have different consequences; expression of 5Q Cx32 results in increased plasma membrane localized Cx32 and reduces the expansion of the transfected culture, compared to 5R or WT Cx32 expressing cultures.**
Here we investigated the role of acetylation as a regulator of Cx32 mediated cell-cell communication. Post-translational modifications such as phosphorylation (Laird 2005), ubiquitination (Laing and Beyer 1995), acetylation (Colussi, Rosati et al. 2011), and sumoylation (Kjenseth, Fykerud et al. 2012) have been identified as regulators of various aspects of Cx43 mediated cell-cell communication. Little is known about post-translational modifications of other connexins, such as Cx32. To our knowledge, we are the first to report that Cx32 undergoes lysine acetylation.

Analysis of putative acetylation sites indicated that C-terminal lysines are involved in the modulation of Cx32 protein level and are likely acetylation sites. Mutations that mimic constitutive acetylation of C-terminal lysines (5Q) reduced Cx32 turnover and increased the amount of Cx32 found at the cell surface. The GJ formed by 5Q Cx32 were functional and the extent of coupling between cells expressing 5Q Cx32 was the same as WT. Mutations that removed putative acetylation sites, but did not mimic acetylation (5R), also reduced Cx32 turnover. The amount of 5R Cx32 at the cell surface was similar to WT, as were GJ gating and the level of coupling between 5R expressing cells, suggesting that acetylation is not required for normal GJ function. Interestingly, expression of 5Q Cx32, but not 5R or WT Cx32, resulted in reduced expansion of cell cultures compared to cells expressing the empty vector. This result suggests that acetylated Cx32 plays a role in the regulation of cell division or cell death.

We have shown that HDAC6 is a regulator of Cx32 acetylation, which influences Cx32 protein stability. When we found that the effect of HDAC6 inhibition on Cx32 was similar to broad HDAC inhibition, we focused our efforts on understanding the HDAC6 mediated...
regulation of Cx32 because HDAC6 is most abundant cytoplasmic HDAC and we had evidence that Cx32 acetylation occurs on cytoplasmic residues (Bertos, Gilquin et al. 2004). Our cell-surface biotinylation experiments indicated that acetylated Cx32 is present in the cytoplasm and at the cell surface and that HDAC6 inhibition causes Cx32 protein to accumulate in both pools. This was result was corroborated by confocal imaging of WT Cx32 in HDAC6 inhibitor treated cells, where the intensity of Cx32 staining increased in the cytoplasm and at the cell surface, compared to vehicle treated WT Cx32. The stabilization of Cx32 that occurred as a result of HDAC6 inhibition and increased Cx32 acetylation was recapitulated by K→Q mutation of likely acetylation sites, suggesting that the observed Cx32 stabilization was a direct result of the alteration of biochemical properties of the Cx32 C-terminus.

The consequences of Cx32 acetylation differ from those of Cx43 acetylation

Acetylation was recently reported to influence the amount of Cx43 in GJs (Colussi, Rosati et al. 2011). Global acetylation was assessed in a mouse model for Duchenne’s cardiac myopathy (Mdx), where many proteins including Cx43, were found to be hyperacetylated. The hyperacetylated Cx43 in Mdx cardiomyocytes displayed a reduced junctional localization compared to WT tissue. Further evidence that acetylation regulates junctional Cx43 levels was ascertained by manipulating Cx43 acetylation in cultured fibroblasts. Broad spectrum and Class IIa specific HDAC inhibitor treatment increased Cx43 acetylation and reduced GJ associated Cx43 in the fibroblasts. This result was recapitulated by mutating putative acetylation sites from lysine to glutamine in order to mimic constitutive acetylation, such that the localization of K→Q Cx43 at cell-cell junctions was reduced compared to WT Cx43.
Two other studies tested the effects of prolonged culture in the presence of broad spectrum HDAC inhibitors on GJ formation and found that while Cx43 accumulated in the cytoplasm, HDAC inhibition actually increased the amount of Cx32 in GJs (Vinken, Henkens et al. 2006; Henkens, Vinken et al. 2008). These changes were independent of alterations of transcription of either connexin gene, but were accompanied by an increase in Cx32 and Cx43 protein levels. Neither of these studies addressed whether the connexins were acetylated and if their acetylation levels were changing as a result of the drug treatments.

We found that HDAC6 inhibition increased Cx32 acetylation and the amount of Cx32 at the cell surface. Mutation of C-terminal lysines to glutamine recapitulated the increase in Cx32 at the cell surface and also suppressed the expansion of N2a cell cultures, compared to cells expressing WT Cx32. It is worth noting that we showed that HDAC6 does not seem to regulate Cx43 acetylation, despite a cytoplasmic accumulation of Cx43 that occurs when HDAC6 activity is lost. We concluded that the localization of Cx43 could have been altered indirectly, through increased acetylation of another HDAC6 substrate.

Taken together, our results and the results of the aforementioned studies of HDAC inhibition and connexins reveal that the physiological consequences of acetylation are likely to vary among members of the connexin protein family. It is possible that both Cx32 and Cx43 proteins are stabilized by acetylation through a decrease in ubiquitination that is caused by competition for lysine occupancy. Our work shows that the turnover of WT Cx32 in the presence of HDAC6 inhibitor and the turnover of 5Q(acetylation mimetic) Cx32 are both decreased as compared to the WT control. Cx32 ubiquitination is also decreased in both of these cases. The impact of acetylation on ubiquitination and turnover of Cx43 have not been investigated, but since Henkens et al. (Henkens, Vinken et al. 2008) did show that Cx43 protein accumulated with
HDAC inhibition, it is possible that Cx43 stability is also regulated by acetylation. However, acetylation often modulates protein-protein interactions that are independent of ubiquitination (Spange, Wagner et al. 2009). The putative acetylation sites that have been identified in Cx43 and Cx32 do not share sequence homology, but are all located in cytoplasmic domains. The cytoplasmic location of the residues suggests that they are accessible to interacting proteins and the lack of sequence homology suggests that the interacting proteins are likely to be different for Cx32 versus Cx43. Also, Cx43 and Cx32 do not form heteromeric channels together and are often expressed in different tissues (Elfång, Eckert et al. 1995; Oyamada, Oyamada et al. 2005).

**Questions for Future Investigation**

*How does Cx32 acetylation regulate the growth of cell cultures?*

We observed that transfection with a plasmid encoding 5Q Cx32 resulted in a significantly lower cell count compared to cells transfected with 5R or WT Cx32 or the empty vector. Based upon this result we concluded that 5Q Cx32 was acting as a negative regulator of cell culture growth, but we did not address whether this occurred due to decreased cell division or increased cell death. Future studies should include assays to compare the proliferation rates of cells expressing WT, 5R, or 5Q Cx32 or the empty vector. If cells expressing 5Q Cx32 are dividing at the same rate as WT and 5R Cx32 expressing cells, then apoptosis is likely to be the cause of the reduced cell number and should assayed.

Since the growth regulating function of 5Q Cx32 was associated with increased Cx32 at the cell surface, we hypothesized that increased Cx32 GJ or hemichannel function was regulating
cell division and/or death. 5Q Cx32 GJ function was not altered compared to WT. This was assayed by measuring trans-junctional voltage, which is interpreted as a measure of total GJ activity per cell pair. It is not clear if our system would have registered an increase in trans-junctional voltage compared to the already high WT level, due to high levels of Cx32 expression in all of our transiently transfected cells. Based on this uncertainty, we cannot rule out altered GJ-mediated communication as a regulator of cell division or death, though hemichannel function or altered interactions with cytoplasmic protein partners are other potential explanations.

We could test the contribution of GJ function to the altered cell division or apoptosis that we observed by repeating the cell count experiments while treating cells with a Cx32 specific GJ inhibitor (Patel, Milwid et al. 2012). If increased GJ function is responsible for the reduced cell number after 72 hours of 5Q Cx32 expression, then we would expect that treatment with the Cx32 GJ inhibitor would eliminate the observed difference in cell number caused by 5Q Cx32 expression, compared to WT. This inhibitor may also block Cx32 hemichannels, so if it reverses 5Q Cx32 mediated growth inhibition, we would still need to assay the contribution of hemichannel function.

Treatment with a peptide that specifically inhibits Cx32 hemichannels (De Vuyst, Decrock et al. 2006) could be used to assay the contribution of Cx32 hemichannels to the 5Q Cx32 mediated inhibition of cell culture expansion. Finally, it is possible that neither hemichannel nor GJ activity regulates cell division or death through 5Q Cx32. Instead, altered protein-protein interactions that occur as a result of the mutations could be involved in signaling pathways that regulate cell division or apoptosis. If this is the case, proteomic
comparisons of the interacting factors of WT Cx32 and 5Q Cx32 or WT Cx32 + HDAC6 inhibitor treatment would provide mechanistic insight.

*Does acetylation occur on residues outside of the C-terminus of Cx32?*

We found that both 5R and 5Q Cx32 were still acetylated, though the predicted C-terminal acetylation sites were no longer available for acetylation. As discussed in Chapter 3. of this thesis, it is possible that compensatory acetylation of other residues occurs when the normal acetylation sites are unavailable. Another possibility is that we were detecting other physiologically relevant acetylation sites. In order to address these possibilities, Cx32 acetylation sites could be identified using mass spectroscopy. Use of mass spectroscopy to identify acetylation sites depends on being able to affinity purify large quantities of the target protein in its acetylated state. Identification of the acetyltransferase responsible for Cx32 acetylation could aid in this process because we could either overexpress it in Cx32 expressing cells or use it in an in vitro acetylation assay in order to enrich acetylated Cx32 in the sample. Identification of the normally occurring Cx32 acetylation sites would validate our results and could also provide information about acetylation sites that are not regulated by HDAC6. Additional acetylation may regulate properties of Cx32 that were overlooked by our initial experiments.

*Which acetyltransferases mediate Cx32 acetylation?*

Identifying the enzymes responsible for Cx32 acetylation will provide tools for future experiments that involve the manipulation of Cx32 acetylation and also could provide clues as to the physiological cues that regulate Cx32 acetylation. P300/CBP-associated factor (PCAF) has been shown to acetylate Cx43 (Colussi, Rosati et al. 2011) and thus seems like a good starting point when screening for acetyltransferases that modify Cx32. Treating Cx32 expressing cells
with the PCAF specific activator, SPV106, would be a simple test to see if PCAF acetylates Cx32. If SPV106 fails to enhance Cx32 acetylation, further screening for enhancement of Cx32 acetylation could be conducted via overexpression and pharmacological inhibition of known cytoplasmic acetyltransferases such as alpha-Tat-1, p300, and ELP1.

Recent work has shown that lysine-acetylation can occur within the lumen of the endoplasmic reticulum (ER) as well (Costantini, Ko et al. 2007; Pehar, Lehnus et al. 2012). Acetyl Co-A is transported to the ER and there are least two ER resident acetyltransferases, which have a growing list of substrates (Ko and Puglielli 2009). Perhaps Cx32 acetylation occurs in the ER prior to hemichannel assembly, which takes place in the Golgi (Martin, Blundell et al. 2001). Acetylation within the ER could be involved in the stabilization of monomeric Cx32, which could influence hemichannel formation. Our experiments were carried out with transiently transfected cells that produced a high level of Cx32, so it is not clear if we could have detected reduced, but not entirely defective, hemichannel formation. We did find that Brefeldin-A treatment, which sequesters secretory proteins in the ER, did not prevent Cx32 acetylation. This could have been because acetylated Cx32 was trapped in the ER due to retrograde transport from elsewhere in the secretory system or because nascent Cx32 is acetylated in the ER. The possibility of ER based acetylation has been recognized only recently and has not been investigated as a potential regulator of Cx32 or Cx43. Identifying the enzymes that regulate Cx32 acetylation will provide further insight as to whether there is overlap between the regulators of Cx32 and Cx43 acetylation.
Do deacetylases other than HDAC6 target Cx32?

Co-localization and co-immunoprecipitation studies suggested that HDACs 3, 4, and 5 form a complex that deacetylates Cx43 (Colussi, Rosati et al. 2011). HDAC3 in particular localizes at the plasma membrane (Longworth and Laimins 2006) and thus could be another Cx32 deacetylase. Depending upon the regulatory role of Cx32 deacetylation, it is possible that both cytoplasmic and plasma membrane associated deacetylases could work on Cx32. Deacetylase recruitment to Cx32 could result in altered protein-protein interactions that play different regulatory roles depending on the deacetylase involved. Also, the acetylation status of Cx32 could play different roles depending on the localization of Cx32. Co-immunoprecipitation and confocal microscopy could be used to probe putative interactions between Cx32 and HDAC3, 4, 5 as well.

Does acetylation regulate connexin function in a tissue specific manner?

Reconciling the basic topological and functional redundancy of connexin channels with the existence of at least 20 different connexin genes in mammals raises questions about the unique properties of different connexin proteins. GJ and non-junctional hemichannel composition influence the physiological cues that cells are able to generate and respond to. Each combination of connexins forms channels with specific permeability characteristics (White, Bruzzone et al. 1994; Koval, Geist et al. 1995; Sosinsky 1995; Martinez, Hayrapetyan et al. 2002). Also, each connexin has a specific repertoire of cytoplasmic interacting factors that provide links to signaling pathways that are both dependent upon and independent of cell-cell
Experiments comparing the function of GJ composed of different connexins in a GJ deficient background support the premise that while functional conservation exists, different connexins confer unique, physiologically relevant properties to the GJ that they form. For example, germline deletion of Cx43 results in lethal cardiovascular defects (Ya, Erdtsieck-Ernste et al. 1998). Replacement of the Cx43 gene with either Cx32 or Cx40 rescued viability, but the resulting transgenic mice had various morphological defects (Plum, Hallas et al. 2000). The morphological defects that resulted from the gene replacements were not as severe as the Cx43 deletion, but included mild heart defects and other, defects that were unique depending on the replacing connexin. This study suggests that different connexins have both shared and unique functions within tissues.

Interestingly, overexpression of Cx43 also caused heart and neural tube defects, suggesting that temporal regulation of connexin protein level is important during morphogenesis (Ewart, Cohen et al. 1997). Reduced GJ mediated cell-cell coupling can protect tissues from injury (Frantseva, Kokarovtseva et al. 2002), but enhances tumor cell proliferation (Omori, Zaidan Dagli et al. 2001). Studies of inherited connexin mutations within human populations also provide evidence for the conserved and unique roles of connexins in specific tissues. For example, hereditary Cx32 mutations that cause loss of GJ function result in demyelinating neuropathy (Abrams, Freidin et al. 2001). Similarly, mutations that reduce Cx43GJ function result in developmental defects in the patterning of bones, skin, and eyes (Shibayama, Paznekas et al. 2005). In both of these cases, many tissues remain unaffected since they do not depend upon the mutated connexins for proper cell-cell communication. Taken together, these studies
indicate that the spatial and temporal regulation of GJ activity must be tightly regulated to maintain tissue homeostasis.

Cell type specific GJ and connexin hemichannel composition is dependent upon the transcriptional regulation of connexin genes (Oyamada, Oyamada et al. 2005), as well as later regulatory events that modulate the assembly of connexin hemichannels, hemichannel trafficking to the plasma membrane, and cell surface connexin dynamics. Our results add to a growing body of evidence that post-translational modifications could be involved in regulating the cell type specific composition of connexin hemichannels and GJ by influencing the localization and turnover of connexins. Acetylation seems to act as a negative regulator of Cx43 GJs (Colussi, Rosati et al. 2011), while our data indicate that acetylation is likely to enhance the function of Cx32 at the cell surface. Recent proteomic studies have suggested that acetylation occurs in a tissue specific manner (Spange, Wagner et al. 2009) and can be linked to metabolism (Zhao, Xu et al. 2010). Thus, it is possible that tissue specific activity of the enzymes that mediate lysine acetylation and deacetylation could be involved in regulating the fate of expressed connexin proteins. Connexins that are positively regulated by acetylation could be the major mediators of GJ mediated cell-cell communication in cells that have high levels of the appropriate acetyltransferase activity and/or low levels of the appropriate HDAC activity.

We hypothesize that the maintenance of functional hemichannels or GJ composed of a particular connexin can be regulated through the expression and modulation of acetyltransferase and HDAC activity. Future studies should probe the acetylation of connexins under various physiological conditions in order to determine if there are certain conditions that promote connexin acetylation. Based on our Cx32 results and Cx43 acetylation studies conducted in other laboratories, we predict that altering global acetylation will have connexin and thus tissue
specific effects on GJ-mediated communication. Also, proteomic comparisons of connexin interacting partners under high and low acetylation conditions should provide mechanistic insights into how acetylation regulates connexin localization and stability.
References


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