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A Study of Calponin's Role in Secretion

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A Study of Calponin’s Role in Secretion
The Proposed Model and the Experimental Design

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A Study of Calponin’s Role in Secretion

Abstract

Calponin is an actin-binding protein found in a variety of cells that is well known for its inhibition of actin-activated myosin ATPase. We hypothesize that calponin inhibits cellular secretion by two mechanisms. One mechanism is to prevent the cleavage of actin in the cytoskeleton, a gel-like mesh that acts as a physical barrier to vesicles travelling to the membrane, by gelsolin and other actin-severing proteins that cut paths for vesicles to travel. The other mechanism is to assist in the bundling and cross-linking of actin, creating smaller pores in the mesh that are more difficult for the vesicles to move through. This project conducted necessary background research, designed study and future experiments, and amplified pET and pCMV-HA vectors that will be used in the experiments.

Background

Calponin is a member of the family of actin-binding proteins. It was first identified in chicken gizzard smooth muscle by Takahashi et al. in 1986, and later indicated to inhibit contraction in those cells (Takahashi, Hiwada, & Kokubu, 1986; Takahashi, Hiwada, & Kokubu, 1988). Since then, calponin has been located in a number of cell types exhibiting a number of functions.

Calponin is made up of three major domains. The first is a domain to which calponin has lent its name, the Calponin Homology (CH) domain. This domain is common amongst actin-binding proteins, and is the binding site for most of those proteins. Interestingly, calponin itself does not bind to actin this way, nor is it required for binding (Galkin, Orlova, Fattoum, Walsh, & Egelman, 2006). This is because the single CH domain calponin possesses is not strong enough to bind actin; typical actin-binding proteins may contain several CH domains. Instead, calponin’s primary binding site to actin is located in its second domain, called the “CLIK^{23} repeats” region because it contains a three-tandem repeat of 23 amino acids. Phosphorylation of specific residues in this domain prevents actin binding (Allen & Walsh, 1994).
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The final calponin domain is known as the variable region, located at the very end of the c-terminus (See Figure 2). Variations in this region give rise to different isoforms of calponin; there are three distinct calponin isoforms in all, named h1, h2, and h3-calponin (Strasser, Gimona, Moessler, Herzog, & Small, 1993; Applegate, Feng, Green, & Taubman, 1994). Differences in length and amino acid sequence of the variable region give the isoforms’ distinct isoelectric points: h1-calponin is basic, h2-calponin is neutral, and h3-calponin is acidic. Because the calponin isoforms differ only in this small portion of their sequence, they have nearly identical structures. The structure similarity between them has caused the proposed functions any of the isoforms to be largely considered interchangeable with either of the others.

Calponin’s function in smooth muscle cells has been studied much more extensively than its functions in other cells. In smooth muscle cells,
contraction is completed through what is known as the sliding filament model. Similar to contraction in skeletal muscles, smooth muscle contraction involves the phosphorylation and subsequent binding of a myosin to a high-affinity site on actin (this process is Ca$^{2+}$ dependant). Normally, this process activates the actin-dependent myosin ATPase, which would hydrolyze an ATP to cause myosin to rotate its “head”, forcing actin to slide along the filament and shorten the muscle (i.e. contract).

Calponin’s mechanism of action in smooth muscle, however, is still not fully understood. It has been shown that calponin binds to actin in vitro, and inhibits activation of actin-dependent myosin ATPase (EL-Mezgueldi & Marston, 1996; Winder, Allen, Clément-Chomienne, & Walsh, 1998). Based on these two major factors and others, it is commonly accepted that calponin binds to actin filaments in contractile regions of smooth muscle cells and prevents the activity of myosin’s ATPase by blocking the required ATPase activation site on actin. This inhibition prevents a vital step in the sliding of actin along myosin, thereby preventing muscular contraction.

Calponin localizes not only to actin found in contractile machinery, but also to actin in the cellular cytoskeleton (North, Gimona, & Small, 1994). The actin cytoskeleton’s main role is to provide support and stability to a cell, similar to the way our skeletons help us maintain our shape. It is made up of a tight mesh of actin microfilaments that are bound together by cross-linker proteins. The microfilaments can contract with the help of motor proteins such as myosin. Controlled

![Image](http://www.biolsci.org/v03/p0303/ijsv03p0303g01.jpg)
cytoskeleton contraction and expansion via actin polymerization allows for mobility of specific regions in cells. This regional mobility can be used for a myriad of cellular functions; some examples include cell motility by expanding one end of a cell and contracting the other (See Figure 3), or cytokinesis by contracting a ring of actin microfilaments around the cytoplasm to bisect a dividing cell (See Figure 4).

Calponin has been shown to inhibit actin-myosin mediated activity in the cytoskeleton as well as contractile machinery. Over-expressing h1-calponin in smooth muscle cells decreased their cell proliferation rate (Hossain, Hwang, Huang, Sasaki, & Jin, 2003). Additionally, h1-calponin knockout mice showed early onset of cartilage formation and ossification, and exhibited accelerated bone fracture repair consistent with an increased rate of cell proliferation (Yoshikawa, et al., 1998).

Calponin appears to have a role in stabilizing the actin cytoskeleton. This was demonstrated by an increased resistance of actin to cytochalasin-B, a fungal metabolite that binds to actin and prevents polymerization, in NIH 3T3 fibroblast cells forced-expressing h2-calponin (Hossain, Crish, Eckert, Lin, & Jin, 2005). It has been shown in vitro cross-link and bundle actin filaments (Leinweber, Tang, Stafford, & Chalovich, 1999). This stabilization may be facilitated by another protein, however. A positive correlation has been seen between h2-calponin expression and tropomyosin expression. Tropomyosin is another actin-binding protein that regulates the interaction between actin and myosin in muscle cells. Like calponin, tropomyosin also binds tightly to actin and increases the polymerization rate of actin monomers in vitro (Wen, Kuang, & Rubenstein, 2000). Tropomyosin, however, has also been shown to
increase stability of actin filaments directly (Warren, Lin, Wamboldt, & Lin, 1994; Broschat, Weber, & Burgess, 1989). H2-Calponin and tropomyosin may cooperatively stabilize actin filaments in the cytoskeleton, a relationship that is supported by the simultaneous decrease of endogenous tropomyosin and cell motility in h2-calponin deficient macrophage cells (Huang, Hossain, Wu, Parai, Pope, & Jin, 2008).

**Introduction**

The cytoskeleton has long been assumed to play a significant role in the regulation of vesicle transport and exocytosis during the process of cellular secretion, though little is known about the process. Vesicles that must travel a short distance do so via diffusion. Vesicles required to travel long distances, however, require the assistance of motor proteins such as myosin that transport the vesicle to its destination. The motor protein binds to the cytoskeleton and utilizes it as a track, analogous to a shipping truck carrying cargo along a highway.

Calponin’s inhibition of actin-activated myosin ATPase may play a role inhibiting secretion. It could prevent certain vesicles from being able to reach their destinations by disabling their motor protein. Myosin has not been strongly linked to this type of transport, however, and most motor proteins utilize microtubules, not actin filaments, as tracks to transport vesicles through the cell (Phelps, Foraker, & Swaan, 2003). Calponin cannot bind to tubulin, the main component of microtubules. Therefore, it is very unlikely to affect motor protein-mediated transport of vesicles very strongly.

Regardless of the method of transport, the actin cytoskeleton has been shown to serve as a physical barrier to secretion (Aunis & Bader, 1988). During their journey to the cellular membrane, vesicles must pass through the actin cytoskeleton. The actin cytoskeleton is made up of a tight weave of cross-linked actin microfilaments. We believe that this weave creates pores of varying size that vesicles may have to
navigate through in order to pass through the actin cytoskeleton. This process is similar to the method by which polyacrylamide gels are used to size-separate molecules.

Disruption of the actin cytoskeleton appears to positively affect the rate of secretion in cells. Latrunculin-B is a toxin that binds to actin and prevents polymerization of the actin monomers. Use of latrunculin-B to disrupt the actin cytoskeleton increased the average velocity of large vesicles in endothelial cells (Manneville, Etienne-Manneville, Skehel, Carter, Ogden, & Ferenczi, 2003). The use of the toxin also significantly decreased the overall run length of those vesicle. This suggests that the toxin likely created a cytoskeleton with a looser weave that was easier for the vesicle to navigate.

Similar disruption effects may occur naturally in vivo. Gelsolin, a member of the family of actin-severing proteins, cuts actin with a very high efficiency and appears to be associated with secretion. Knockdown of gelsolin decreased glucose-initiated insulin secretion in MIN6 B1 cells, while over-expressing gelsolin in those cells increased secretion (Tomas, Yermen, Min, Pessin, & Halban, 2006). This suggests that actin-severing activity is a vital component of cellular secretion. It may be required to clear a path for vesicles to travel through in order to reach the membrane.

**Secretion Study – Model, Hypothesis, and Experiments**

From the data above, we have derived a model for vesicle secretion via the actin cytoskeleton. Vesicles arrive at the actin cytoskeleton, by diffusion or molecular proteins depending on the origin and destination of the vesicle. They then must pass through the actin cytoskeleton, navigating through the porous gel of cross-linked actin filaments. The actin-severing protein gelsolin is recruited to assist the vesicle by cleaving specific microfilaments, which provides a clearer path for vesicles to travel through. Once through the cytoskeleton, the vesicle merges with the membrane and expels its contents, thereby completing the process of secretion.
Based on this secretion model, it is our hypothesis that calponin inhibits cellular secretion. We predict that calponin has a two-fold effect in hindering this process, both of which are facilitated by calponin’s stabilizing effect of actin filaments. Calponin binds to actin in the cytoskeleton. There, it shields the actin microfilament from actin-severing proteins such as gelsolin and prevents its cleavage. Calponin also assists in bundling and possibly cross-linking actin microfilaments. This creates a tighter weave of actin with smaller pores. As a result, vesicles have a harder time passing through the actin cytoskeleton with bound calponin, because of the existence of small, uncleaved pores that are difficult to navigate through. This causes a decrease in average vesicle velocity and an increase in total run length because the vesicles must travel around pores that are now too small for them to pass through. Secretion slows as vesicles struggle to escape the cytoskeleton; it is possible even that vesicles become completely trapped by the actin cytoskeleton, preventing secretion all together.

Our hypothesis is supported by a recent study in which Cnn2 (the gene that encodes h2-calponin) knockout RAW264.7 mouse macrophage cells showed a significant increase in phagocytotic activity (Huang, Hossain, Wu, Parai, Pope, & Jin, 2008) (Huang, Hossain, Wu, Parai, Pope, & Jin, 2008). Logically, if a depletion of h2-calponin in macrophages increases uptake of exogenous material, then over-expression of h3-calponin resulting in decreased secretion in neuronal cells is also likely as their mechanisms, though reversed, are largely the same.

We plan to test our hypothesis by monitoring secretion in neuronal cells that are hypo- and hyper-expressing h3-calponin. Over-expression of h3-calponin will be induced by transfection of neural cells with the pCMV-HA vector (See Figure 5) cloned with h3-calponin in the Multiple Cloning Site (MCS). This h3-calponin insert will be under the control of the powerful constitutive cytomegalovirus (CMV) promoter, which will greatly amplify h3-calponin expression in transfected cells. H3-Calponin will also
be labeled with a hemagglutinin (HA) epitope tag. This tag can be detected using HA-TAG Polyclonal Antibodies for proper selection and analysis of transfected cells.

Knockdown of h3-calponin in neural cells will likely be induced by RNA interference. dsRNA with sequence complementary to Cnn3 mRNA will be introduced into neural cells endogenously expressing h3-calponin. This dsRNA will be cleaved by a member of the RNAse III family, resulting in several small interfering RNA (siRNA) fragments. These fragments will be absorbed by the RISC complex, which will locate its complementary mRNA (Cnn3) and induce degradation of the mRNA. A potential problem with this method is the possible existence of sequence homology between genes (such as Cnn2 and Cnn3) that would result in undesired knockdown of other products. One workaround for this issue could be to create the siRNA from cDNA of the variable region of h3-calponin. This would ensure that the inhibition does not suppress the other calponin isoforms; however, homology with another unknown mRNA could still be possible.

This study will measure secretion rate over time by monitoring levels of Human Growth Hormone (hGH) present in the cell medium across multiple time points. hGH is a protein naturally secreted by the pituitary gland, however we will use neural cells that do not endogenously express hGH. These cells will also be transfected with a vector for controlled hGH expression. One vector of choice would be the pCI-neo Mammalian Expression Vector (Promega) that utilizes an inducible T7 promoter.
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Secreted hGH levels in cell culture media will be measured in an ELISA assay using an anti-hGH antibody. Because the ELISA will be measuring hGH present outside the cell, the process does not require cell lysis and is non-destructive. Therefore, it can be performed repeatedly on a single culture. When the assay is applied continuously over time a rate of secretion can be established.

These rates will be measured in the cells that have varying levels of calponin: cells over-expressing calponin, cells under-expressing calponin, and cells whose endogenous calponin expression is unchanged. Neural cells without transfected hGH, and a separate neural cell line with no endogenous h3-calponin expression would be used as a negative controls. So far, only the vector that will be used to over-express calponin has been created; no cells have yet been transfected. Based on our hypothesis, the rate of secretion will decrease as the presence of calponin increases.

This project worked to design the experiments for this study and another calponin study as part of on-going calponin research at the University of Edinburgh. The scope of these studies is larger than any single project. Therefore, it was the goal of this project to research calponin, design study experiments, propose future studies, and complete as much preliminary lab work as time allowed. The result of the lab work was the amplification of pET and pCMV-HA vectors that will be used in the experiments. The pET vector (See Figure 6) is an E. coli based expression vector for controlled expression of a gene of interest through implementation of a T7 promoter and lacO repressor.

Figure 6 – A simple representational map of the pET vector.  
<http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/California/pET.html>
Materials and Methods

Amplification of pET, pCMV-HA, and GFP-Calponin Vectors

Three vectors were amplified for the secretion experiments during the course of this project. For the calponin secretion study, three h3-calponin-containing vectors were obtained from an associate lab that had modified them for a previous study. The vectors consisted of a pET vector for controlled translation of h3-calponin in *E. coli*, a GFP-Calponin vector for detection of h3-calponin, and a pCMV-HA vector for transfecting and over-expressing h3-calponin in neural cells. The steps of amplification are described below, beginning with the creation of calcium competent cells, the transformation of those cells with the vectors above, and finally preparation of the transformed cultures to extract purified vector.

Creation of DH5α and XL-10 Gold Calcium Competent Cells

Competent cells for later transformative use were created from stocks of DH5α and XL-10 Gold *E. coli* cells obtained from a cell bank. Each stock was grown in a starter culture of 2mL LB broth, and then used to inoculate a 50mL flask of LB broth. The 50mL culture was incubated at 37°C for 1.5 to 2.5 hours to ensure log phase growth of the cells. The cells were transferred to a 50mL Falcon tube and incubated on ice for 10 minutes before being pelleted in a J-68 centrifuge at 6,000g for 15 minutes. The pellet was resuspended in 25mL of 100mM CaCl₂ and incubated on ice for 15 minutes. The cells were pelleted again and then resuspended in 2mL of 100mM CaCl₂. The suspension was stored at 4°C and used for one week before being discarded.

Transformation of Calcium Competent Cells

Vectors were used to transform cells for the purposes of amplification during this project. 1μL of the transforming vector was added to 100μL of competent cells, mixed, and then incubated on ice for 30 minutes. After this time, the cells were heat-shocked in a 42°C heat block for exactly 90 seconds, and then returned to ice for one minute. 500μL of LB broth were added to the cells and then incubated at
37°C for 30 minutes. 20μL or 500μL were streaked onto each of two plates containing the antibiotic resistance conferred by the transforming vector (amp for pCMV-HA and pET, kan for GFP-calponin). Plates were then incubated at 37°C overnight.

**Midi-Prep of Transformed Culture**

Transformed cells containing vectors of interest were midi-prepped to yield pure and concentrated vectors. A single colony was picked from the 20μL selective plate and used to inoculate a 50mL flask of LB broth containing the appropriate antibiotic. The flask was then incubated at 37°C overnight with shaking. The QIAGEN Plasmid Midi Kit was used to extract the plasmid, and the basic procedure presented in the product handbook was followed. The culture cells were pelleted in a J-68 centrifuge at 6,000g for 15 minutes, and then resuspended in 4mL of P1 resuspension buffer (that contained RNAse and was stored at 4°C). 4mL of P2 lysis buffer was added to the cells, mixing by inversion, and then incubated at room temperature for 5 minutes. 4mL of P3 neutralization buffer was added, mixing by inversion, and then incubated on ice for 15 minutes. The solution was centrifuged in a J-10 ultracentrifuge at approximately 20,000g for 30 minutes and then again for 15 minutes, removing the plasmid-containing supernatant after each spin.

A QIAGEN-tip 100 was equilibrated by gravity flow with 4mL of buffer QBT. The supernatant from the centrifugation was pulled through the tip by gravity flow, and then the tip washed with two 10mL lots of buffer QC. The plasmid was eluted and captured using buffer QF. The DNA was precipitated with 3.5mL isopropanol and then centrifuged as before, this time discarding the supernatant. The pellet was washed and spun twice with 2mL ethanol, air-dried, and then resuspended in 50μL TE buffer.
Results

Amplification of the pET, pCMV-HA, and GFP-Calponin vectors yielded ample product with which to analyze h3-calponin and over-express it in *E coli* and in mammalian neural cells. Unfortunately, there was not enough time to analyze the products. Thus, no figures have been created to verify these results. Successful transformation can be assumed, however, based on the growth of cells on the antibiotic selection plates and in culture flasks also containing antibiotic.

Because of the lack of analysis, the amplified vectors may contain chemical (i.e.: ethanol), protein, or DNA-based contamination from incomplete purification of the extracted cultures. The products must be separated on an agarose gel to verify successful transformation and purification of the correct vector, to ensure that the vectors were not damaged and that unwanted DNA was not extracted along with the vector. DNA concentrations must be determined via spectrophotometry, both to gauge the relative output of the transformation/purification and to determine whether unwanted proteins are in the products.

Discussion

It has not yet been decided which cell line to use for the experiments. We know that we want to use a neural cell line that endogenously expresses h3-calponin but not human growth hormone. The decision has not been made due to funding issues; we want to determine if we can obtain, as a gift from another lab, neural cells that match our requirements. We will have little choice regarding which line we will be using if this is the case. Thus, it is pointless to choose a specific cell line for the experiments until we are closer to actually conducting them. Many neural cells express h3-calponin endogenously, however, so finding a suitable cell line should not be difficult.
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Much of what is “known” about calponin is based on speculations and assumptions. This can be attributed to two major factors: the difficulty of studying calponin in vivo, and the lack of research regarding calponin’s isoforms. Very little evidence has been gathered about calponin from in vivo studies that can accurately and definitively pinpoint calponin’s roles in the cellular machinery. Additionally, most of what has been discerned about calponin has been from studies of h1-calponin and, to a lesser extent, h2-calponin; very little is known about h3-calponin and its functions. The uncertainty surrounding calponin allows increasingly hypothetical theories to be formulated and tested. The model that we propose is of this type, because so little is known about h3-calponin. We have assumed many aspects of calponin’s involvement in both secretion and the actin cytoskeleton based on studies with only partial or potential relevance to h3-calponin.

Our study attempts to support or reject our hypothesis that h3-calponin inhibits secretion in neural cells that is derived from a model that implicates calponin to have a stabilizing effect on actin. The stabilizing effect that calponin may have on actin has significant implications relating to all cellular functions involving the actin cytoskeleton, including phagocytosis, secretion, cell motility, proliferation, and intracellular transport. We have chosen to study h3-calponin’s impact on the secretion of hGH in neural cells because it can be monitored easily using the hGH ELISA and will provide valuable insight into the validity of our proposed model.

Comparing the rate of hGH secretion in cells that are over, under, or normally expressing calponin will provide us with a correlation coefficient between calponin levels and secretion rate. Our model assumes that there will be a negative correlation between these two factors. That is, as h3-calponin levels increase in neural cells, their rate of hGH secretion will decrease, and vice versa. If the results of our study conclude that the correlation is indeed negative then our hypothesis, and by extension our model, will be supported.
If the RNAi mechanism failed to silence the expression of h3-calponin effectively, the results of the study would indicate a negative correlation between over-expression and endogenous expression of calponin and a no correlation between under-expression and endogenous expression of calponin. Further study to determine the effectiveness of the RNAi experiments would be required. If it turns out to be ineffective, then it can be assumed that the overall correlation between h3-calponin expression and secretion is negative. In this case, the hypothesis will still be supported, although much more weakly.

Another possible result from the secretion study is a positive correlation between h3-calponin levels and hGH secretion. This result would reject our hypothesis outright, and would suggest that our model is fundamentally flawed. The flaw would in turn suggest that assuming the relevance of certain data that is vital to the validity of our model was a mistake. Many aspects of our model were created this way, and error in any of them could create this type of result. Regardless, a new model would have to be created that accounts for the positive correlation.

Finally, the study could result in a lack of correlation, or the inability to determine if a correlation exists. In either of these cases, the results would be largely inconclusive towards the support or rejection of our hypothesis. This could imply that our model is mostly correct except for some small error that serves to skew our results, or that there is be a miss-estimation of the importance of h3-calponin in the proposed model. In this case, secretion could still function largely as described, but may not have an effect as grand as implied.

Alternatively, a lack of correlation would be noticed if calponin was a part of a larger complex in which another member was a limiting factor. In this scenario, increasing calponin’s concentration in the cell would have no effect on the complex’s overall efficiency, and thus no effect on secretion. This would be despite calponin’s involvement in secretion regulation. For example, if calponin must first bind to
another protein before it can bind actin, and this other protein is present in limited quantity, then increasing calponin's concentration would serve no purpose.

Due to the extreme complexity of cellular secretion, it will be difficult to determine all the implications of the results that we will obtain. This is because there is a multitude of explanations for whatever results we might get. Confounding effects, from proteins that are unaccounted for in the proposed model, could skew results significantly from what is expected. These effects could create correlations between calponin and cellular secretion that are not reliable in accepting or rejecting the hypothesis, such as the example described above. Thus, any support or rejection the secretion study results seem to give to our hypothesis will itself need to supported or rejected with further experimentation.

**Future Experiments**

As stated earlier, future experiments will need to be conducted about calponin and secretion no matter what the result of this study is. Obviously, the validity of many future experiments hinges on the results of this one; however, there is merit in certain experiments regardless. The story of cellular secretion is an immense novel, and this study will only reveal a page at best.

Many factors (e.g. cell type, calponin isoform, environmental conditions) will have been highly controlled in order to create as understandable and unconfounded environment as possible. A logical next step will be to create studies which these controlled factors are altered in order to determine the extent of the validity of the proposed model. For example, the study should be repeated in other secretory cell types, such as B cells, or using a different calponin isoform, such as h2-calponin. The results of multiple studies combined will paint a much clearer picture than any single one alone.
In order to affirm the conclusions from this study, a more comprehensive study of h3-calponin and cellular secretion must be created. Establishing a Cre-Lox recombination model for calponin expression would be a good way to do this. In this model, two loxP sites are inserted into two introns that flank an exon of the Cnn3 gene. The addition of Cre recombinase initiates a deletion between and including the two loxP, knocking out expression of a functional h3-calponin protein (See Figure 7). Therefore, the Cre-Lox recombination model allows for completely inducible silencing of h3-calponin, and can be performed in vitro or in vivo.

This model can be used to supplement the results of the proposed study by analyzing the effect of knocking out h3-calponin on hGH secretion in neural cells. Due to the model’s inducible nature, changes in cellular secretion rate can also be monitored before and after knockout. This information would prove to be valuable because statistical analysis of changes in the same population is more sensitive than analysis of changes between different populations. Thus, this model could detect a correlation between calponin and cellular secretion even if its effect was rather small.

Additionally, a Cnn3 knockout mouse can be established using the Cre-Lox recombinase system for analysis of h3-calponin in vivo. A Cnn2 knockout model was created this way Huang et al. (Huang, Hossain, Wu, Parai, Pope, & Jin, 2008). The knockout mice could be examined for symptoms relating to secretion deficiency. This experiment would be more difficult to complete, however its results would be
substantially more useful because they would provide insight into the function of h3-calponin on cellular secretion in the context of an entire organism rather than one cell type. Thus, any conclusions derived from the results would be much more valid than those made from in vitro studies would.

Works Cited


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