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3."Analysis of the p38 isoforms involved in Prolactin induced proliferation of Nb2 lymphoma". Park K.S., Aloor J.J., Hughes J.P., Prentice D.A. (manuscript in preparation)

Grants: Indiana State University Graduate Research Fund, 1998, 2000, 2001, 2002.

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EXPLORING THE STATHMIN AND SCG10 INTERACTOMES

A Dissertation

Presented to

The School of Graduate studies

Department of Life Sciences

Indiana State University

Terre Haute, Indiana

In Partial fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Jim Jose Aloor

May 2007

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DOCTORAL DISSERTATION

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Exploring the Stathmin and SCG10 Interactomes

has been approved by the Examining Committee for the thesis requirement for the

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ABSTRACT

Signal transduction involves a signal at the cell's surface being transmitted internally via a chain of molecules to generate an adequate response. Signaling malfunctions are responsible for many human diseases. Signaling mechanisms are conserved in evolution but complexity is brought about by modifications, regulation, multiple-pathways, cross talk between pathways etc. Signaling molecules conserved through evolution are considered basic and indispensable. The stathmin family (Stathmin, SCG10 etc.) belongs to this class of molecules.

Stathmin (also known as oncoprotein 18, prosolin, leukemia-associated phosphoprotein 18 and others) is a highly conserved, phosphorylated cytosolic protein with apparent ubiquitous expression. Stathmin protein is encoded by a gene present in humans at the locus 1p36.1-p35. Stathmin binds and sequesters tubulin and thus plays a role in microtubule dynamics. Although its other functions are unknown it is a messenger protein that may participate in both general and specific regulatory pathways. Superior cervical ganglion-10 is a membrane-associated protein that shares significant amino acid sequence similarity with stathmin. SCG10 is a neuronal growth-associated protein encoded by a gene present in humans at the locus 8q13.2. Although a few Stathmin/SCG10 interacting molecules (e.g. Tubulin, Hsc70, Tsg101 etc.) have been identified, the mechanism and effect on subsequent signaling pathways remain un-delineated. To delineate this, it is imperative to broaden our database of Stathmin/SCG10-binding proteins.

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To identify binding proteins we used *in vitro* 'GST-Stathmin pull down assays' and i*n vivo* Yeast two hybrid screening. Our *in vitro* study identified Hsp70 as a stathmin-binding protein. We also show that phosphorylation of p38 (α) kinase is involved in Prolactin-induced proliferation of Nb2 cells. Our yeast two hybrid screen identified five novel Stathmin binding proteins, three new SCG10 binding proteins and a putative brain transcription factor that greatly increases yeast mating.

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INTRODUCTION

Signaling

Eukaryotic cells receive extracellular stimuli and transduce them to effector molecules in the nucleus or cytoplasm using an array of signaling molecules. These molecules are classified into pathways based on the signal cascade to which they belong. In a signaling cascade/pathway, each member has one or more upstream and downstream molecules. Interactions between cascade members are dependent upon proper conformation, which is regulated by many covalent and non-covalent modifications. Depending on the roles they play in the cascade, these molecules are classified into various types such as receptors, ligands, enzymes and adaptors. In the early 1980's a new signaling molecule was discovered that does not seem to match any of the above types. Based on its ability to receive and transduce signals in diverse pathways it was called `Stathmin' derived from the Greek word `Stathmos' meaning `relay'.

Stathmin

Stathmin is a ubiquitous, phylogenetically conserved (Koppel et al., 1990), 149-amino-acid, cytoplasmic protein of molecular weight 18 kDa. The stathmin gene in humans maps to 1p36.1-p35. Stathmin was once named LAP18 (Leukemia-associated protein) (Jeha et al., 1996) because stathmin levels were elevated in various types of

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human acute leukemia (Bieche et al., 1998) (Melhem et al., 1991a) (Melhem et al., 1991b) (Roos et al., 1993). Phosphorylation of stathmin correlates with cell growth, as demonstrated by increased phosphorylation when Nb2 cells are induced to proliferate in response to prolactin or 12-O-tetradecanoyl phorbol-13-acetate (TPA), an activator of protein kinase C (Meyer et al., 1992). Other studies also have shown that stathmin expression and phosphorylation are regulated by extracellular signals involved in cell proliferation (Brattsand et al., 1994) (Sobel, 1991) and differentiation (Doye et al., 1992). Antibodies directed against mammalian stathmin recognize stathmin-like proteins in all vertebrate classes and in some plants (Flurkey et al., 1993), indicating that stathmin is an evolutionarily conserved protein. Though its signaling mechanism is yet unclear, stathmin is known to associate with several proteins (as depicted in figure 1 and table 1) via coiled-coil interactions (Larsson *et al.*, 1999a) (Larsson *et al.*, 1999b) (Li and Cohen, 1996) (Maucuer *et al.*, 1995) (Redeker *et al.*, 2000) Proteins found to interact with stathmin include tubulin (Belmont and Mitchison, 1996) (Curmi et al., 1997) (Gradin *et al.*, 1998) (Horwitz *et al.*, 1997) (Jourdain et al., 1997) (Larsson *et al.*, 1999) (Redeker *et al.*, 2000) and the tumor susceptibility protein TSG101 (Li and Cohen, 1996) (Maucuer *et al.*, 1995). Two members of the Hsp 70 family also shown to bind to stathmin are BiP/GRP 78 (Maucuer *et al.*, 1995) and Hsc70 (Manceau *et al.*, 1999). Evidence suggests that Hsc70 preferentially binds to unphosphorylated stathmin (Manceau *et al.*, 1999), as does tubulin (Curmi *et al.*, 1997) (Gradin *et al.*, 1998) (Horwitz *et al.*, 1997) (Jourdain *et al.*, 1997). Table 1 summarizes the cellular localization and tissue expression of different stathmin family members. It also gives an overview of proteins

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that bind stathmin family members and the known significance/function of each interaction.

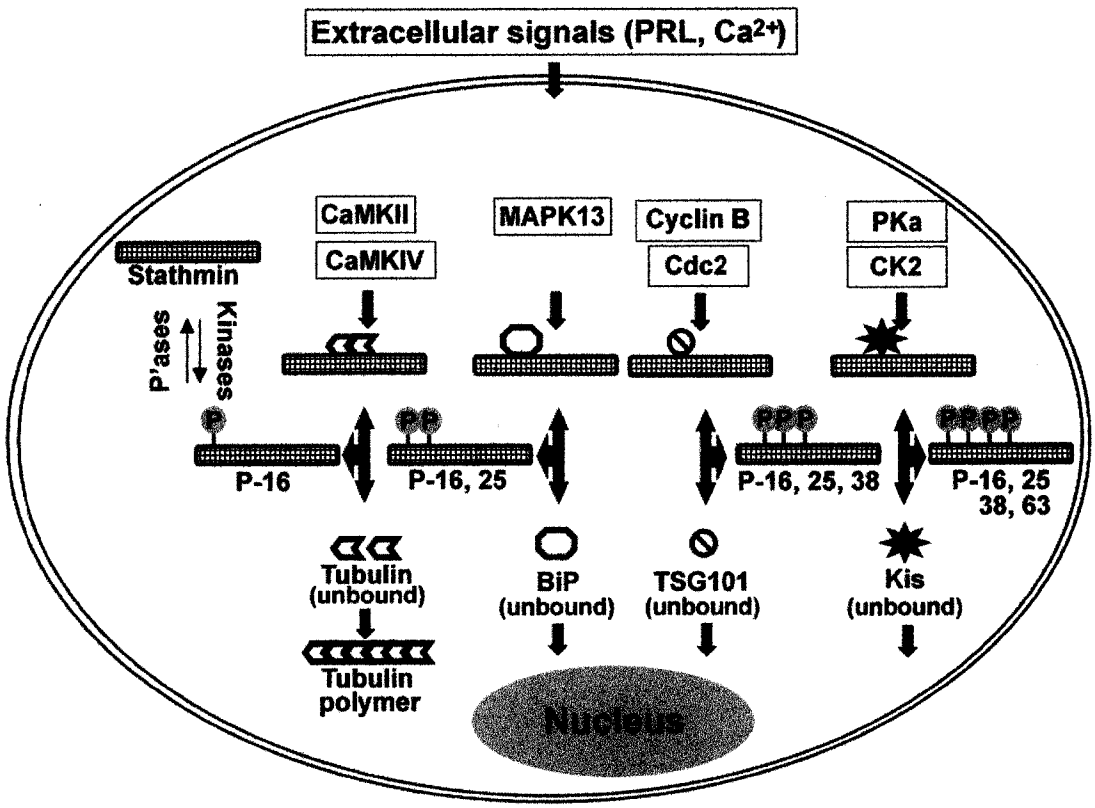

Figure 1: Stathmin regulates polymerization of tubulin and the functions of other effector molecules. Extracellular signals trigger the phosphorylation and dephosphorylation of stathmin by a variety of kinases and phosphatases respectively. The phosphorylation of stathmin at specific serine residues (P-16, 25, 38, 63) modulates its ability to interact with different binding/effector proteins. A specific example of this is the tubulin sequestering activity of stathmin, which inhibits microtubule polymerization and is necessary for the regulation of microtubule dynamics during interphase. Whereas the catastrophe-promoting microtubule de-polymerization activity of stathmin is necessary for the regulation of the mitotic spindle. Abbreviations: CaMKIV, Ca2+/calmodulin-dependent protein kinase IV; CaMKII, Ca2+/calmodulin-dependent protein kinase II; Mapk13, mitogen activated protein kinase 13; cdc2, cell division cycle2; PKa, protein kinase a; CK2, casein kinase 2; BiP, immunoglobulin heavy chain-binding protein; KiS, kinase-interacting stathmin; TSG101, Tumor susceptibility gene 101.

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Table 1. Stathmin family proteins: distribution, location, interactions and functions

STATHMIN FAMILY MEMBER	CELLULAR LOCATION	TISSUE EXPRESSION	BINDING PARTNERS	FUNCTION OF THE MEMBER/BINDING PARTNER/INTERACTION
Stathmin	Cytoplasm	Ubiquitous	1.Tubulin 2. $p27^{Kip1}$ (1) 3. HSC70 4. Hsp70 5. BiP 6. KIS 7. Tsg101 8. PP2A	1. Modulation of tubulin dynamics. 2. Counteracts the tubulin sequestering activity of stathmin and inhibits cell motility. 3-8. Roles not clearly defined.
Superior Cervical Ganglion 10 protein (SCG10)	Membrane associated, golgi	Nervous system, Testes?	1.Tubulin 2.Protocadherin-gamma – b1 (2) 3. TRPC5 (3) 4. RGS6 (4) 5. RGSZ1 (5)	1. Modulation of tubulin dynamics. Neurite outgrowth and neuronal plasticity 2. Modifications of the microtubule cytoskeleton in response to extracellular cues, to aid neurite guidance 3. Regulates neurite extension and growth cone morphology 4. Potentiates the ability of SCG10 to disrupt microtubule organization 5. Blocks SCG10-induced microtubule disassembly
SCG10 like protein (SCLIP)	Cytoplasm, golgi	Ubiquitous; highest levels detected in adult and fetal brain, spinal cord and cerebellum.	1. Tubulin 2. Clusterin (6)	1. Modulation of tubulin dynamics Co-expression with stathmin is postulated to provide some degree of functional redundancy 2. Interrupts the action of SCLIP on microtubule disruption
RB3	Membrane associated	Nervous system	Tubulin	Modulation of tubulin dynamics

(1) Baldassarre, G et al. (2005); (2) Gayet, O. et al. (2004); (3) Greka, A et al. (2003); (4) Liu, Z et al. (2002); (5) Nixon, A. B. et al. (2002); (6) Kang, S. W. et al. (2005).

Stathmin consists of a carboxy-terminal α-helical 'interaction' domain and an amino-terminal 'regulatory' domain. The N-terminal regulatory domain contains the four serine phosphorylation sites (residues 16, 25, 38 and 63) that account for all the phosphoisoforms observed (Sobel 1991). These sites are the targets of multiple kinases (as summarized in figures 1 and 2) regulated both during the cell cycle and by signal

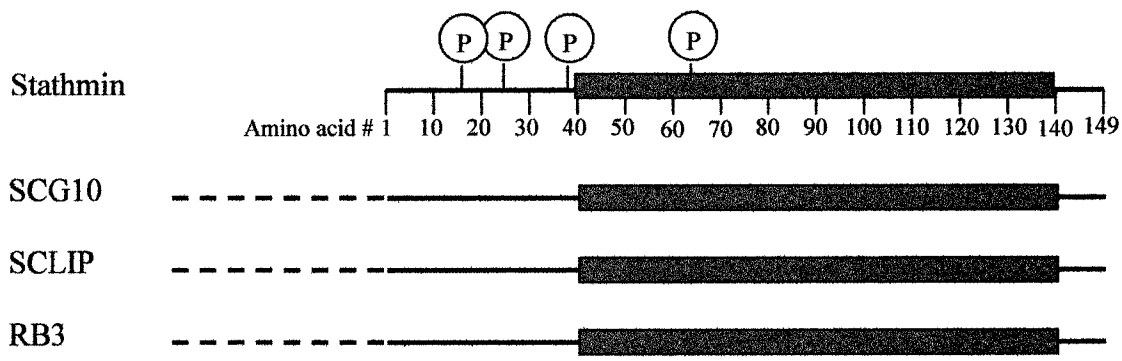

Figure 2: Schematic showing structural/functional similarities among Stathmin family of proteins. The Stathmin like domain (SLD) represented by ■ is the region that binds tubulin. The N-terminal extensions seen in SCG10, SCLIP and RB3 represented by ------ is the region that specifies intracellular membrane association and encodes additional signals for palmitoylation of these protein(s). The serine phosphorylation sites (amino acid 16, 25, 38 and 63) in Stathmin are designated Ⓟ .

transduction cascades (Lawler 1998). Ser25 is a target for mitogen activated protein (MAP) kinase, and both Ser25 and Ser38 are targets for phosphorylation by cyclin-dependent kinases (Cdks). Ser16 and Ser63 can be phosphorylated by protein kinase A (PKA) and Ser16 by the Ca^{2+}/calmodulin-dependent kinase-Gr (CaMK IV/Gr). The terminus folds into a transient α-helical 'interaction' domain and is in rapid equilibrium with a disordered conformation (Steinmetz et al., 2000). This 100-amino-acid region

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which extends from Lys41 to Lys140 undergoes a dramatic 'disorder to order transition' upon binding to tubulin. A 4 A^0 resolution X-ray crystal structure of a tubulin-stathmin-like-domain complex (Gigant et al., 2000) showed an alpha-beta (α-β) heterodimer (aligned head to tail) of tubulin bound to stathmin (T2S complex). The amino-acid sequence from residue 41 to 140 that forms an extended and continuous helix, is amphipathic and has heptad repeats throughout the sequence. This sequence motif is known to mediate alpha-helical, coiled-coil interactions by a hydrophobic seam. From the above described structure of stathmin it is evident that only three of the four phosphorylation sites lie within the N-terminal 'regulatory' domain. Phosphorylation of serine 16 and serine 63 regulate microtubule binding/destabilization and phosphorylation of serine 25 and serine 38 seem to be necessary to allow knases access to the other two sites (Lawler 1998). Interaction of unphosphorylated stathmin with tubulin appears to increase the catastrophe rate of microtubules (Belmont and Mitchison 1996). Stathmin may promote shortening of microtubules by sequestering tubulin or by directly interfering with microtubule polymerization.

Recent discoveries have led to the extension of the stathmin family to include superior cervical ganglion 10 protein (SCG10), rat homologue of Xenopus laevis XB3 protein (RB3) and SCG10 like protein (SCLIP). Figure 2 is a schematic showing structural/functional similarities among Stathmin family of proteins. A multiple aligment of amino acid sequences of proteins from the stathmin family is shown in figure 3. These stathmin family proteins are discussed in brief on the following pages.

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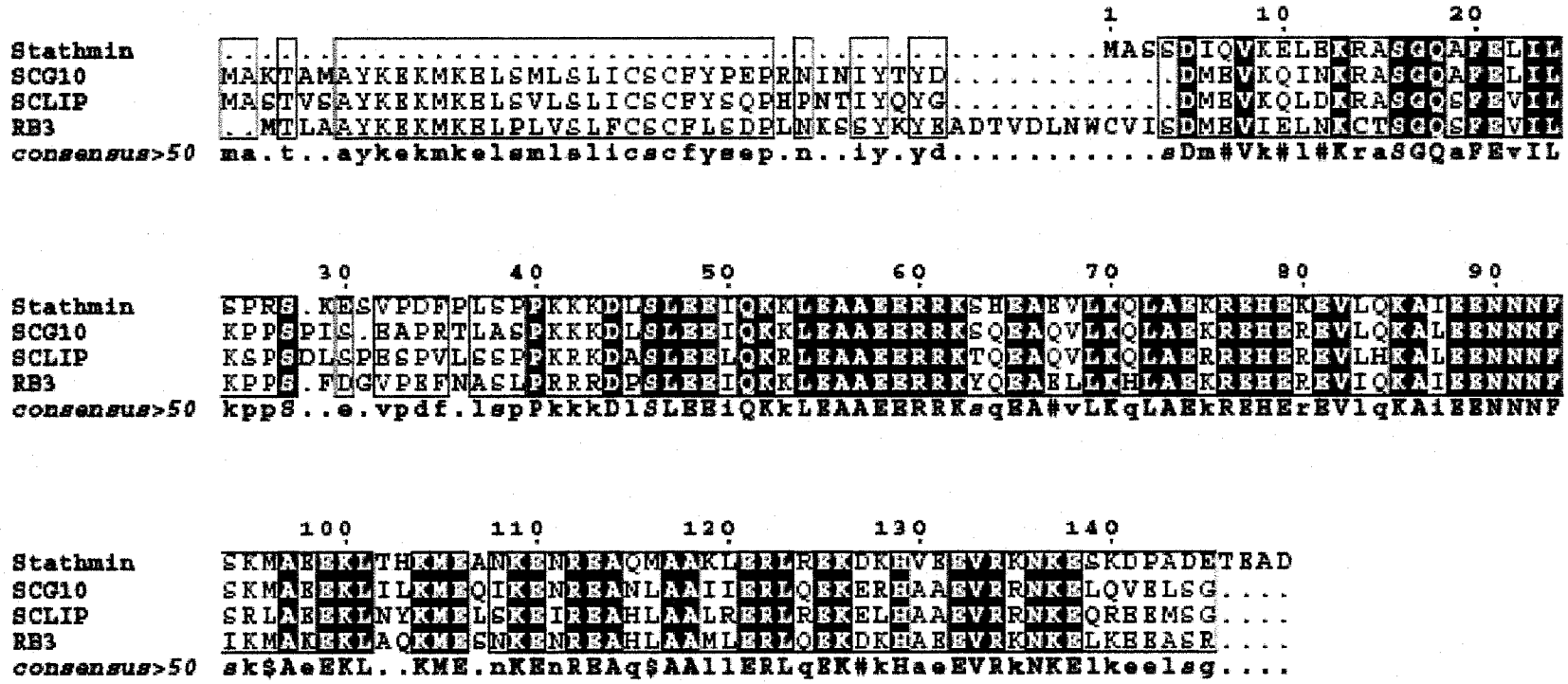

Figure 3: Amino acid identity/homology among Stathmin family of proteins. Multiple aligment of amino acid sequences of proteins from the stathmin family done using the multiple alignment program and ESPript.cgi version 3.06 [Gouet et al. Nucleic Acids Res. 2003 Jul 1;31(13):3320-3]. The alignment shows extensive conservation of amino acid identity or homology. At the molecular level, each possesses a specific "stathmin-like domain", and with the exception of stathmin, N-terminal extensions, that specify association with intracellular membrane compartments. The stathmin-like domain of each family member is capable of binding tubulin. However, they differ with regard to the stabilities of the complexes formed as well as in their interaction kinetics.

Superior Cervical Ganglion 10 Protein (SCG10)

Superior cervical ganglion-10 is a 21-kDa protein associated with neuronal growth. It shares significant amino-acid sequence similarity (over its 179 amino acid sequence) with the phosphoprotein stathmin. Stein et al. (Stein et al., 1988) cloned rat SCG10 cDNA and showed that it is a membrane-associated protein that is localized in neuronal growth cones. Okazaki et al. (Okazaki et al., 1993) isolated most of the mouse gene. The predicted amino-acid sequences of SCG10 in the two species differ by a single residue. Okazaki et al. (Okazaki et al., 1993) suggested that the neuron-specific SCG10 gene evolved by duplication and acquired a tissue-specific promoter which limited the expression of SCG10 in comparison to the more broadly expressed stathmin gene. Schoenherr and Anderson (Schoenherr and Anderson 1995) cloned a transcription factor, which they termed neuron-restrictive silencer factor (NRSF), that represses transcription of SCG10. NRSF binds to a 24-bp regulatory element, called the neuron-restrictive silencer element (NRSE), located in the distal 5-prime regulatory region of the gene. Another regulator of SCG10 may be the REST transcription factor. Bahn et al. (Bahn et al., 2002) used indexing-based differential display PCR on neuronal precursor cells to study gene expression in Down's syndrome and found that genes regulated by the REST transcription factor were selectively repressed. One of these genes, SCG10, was almost undetectable. The REST factor itself was also downregulated by 49% compared to controls. In cell culture, the Down-syndrome cells showed a reduction of neurogenesis, as well as decreased neurite length and abnormal changes in neuron morphology. The authors noted that REST-regulated genes play an important part in brain development, plasticity, and synapse formation, and they suggested a link between dysregulation of

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REST and some of the neurologic deficits seen in Down's syndrome. Riederer et al. (Riederer et al., 1997) suggested that SCG10 promotes neurite outgrowth through regulation of microtubule dynamics in growth cones. SCG10 is 69% identical to stathmin in amino-acid sequences, has a conserved stathmin like domain (SLD) capable of binding tubulin, and maintains phosphorylation sites. Di Paolo et al. (Di Paolo et al., 1997) have shown that SCG10 is present in dendrites as well as in growth cones and that the major structural difference between the two proteins is that SCG10 has an N-terminal domain that mediates membrane association. Lutjens et al. (Lutjens et al., 2000) showed that two cysteine palmitoylation (amino acids 22 and 24) sites within this N terminal region specify localization of SCG10 to the trans-face of of Golgi complex. Okazaki et al. (Okazaki et al., 1995) have shown that there are no quantitative changes in total SCG10 mRNA or protein in Alzheimer's Disease brain. However, they found that SCG10 concentration shows a positive correlation with the number of tangles found in AD brains and that SCG10 protein accumulated in the cell bodies in AD-affected regions. The authors suggest that SCG10 compartmentalization and metabolism may be altered in AD possibly due to mechanisms related to tangle formation in this disease.

RB3 Protein (Rat Homologue of Xenopus *laevis* XB3 Protein)

Maucer et al (Maucer et al., 1993) identified and characterized several stathmin-family genes using a Xenopous *laevis* brain cDNA library. The genes identified by Maucer et al. include Xenopus homologues of stathmin, SCG10 and a new protein of the same family that they named XB3. Ozon et al. (Ozon et al., 1997) used a systematic PCR-based approach and identified several novel mammalian sequences of which two

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coded for expressed members of the stathmin family. The translated RB3 sequence shared 88% amino-acid identity with that of XB3 and thus is likely the homologue in the rat; RB3' corresponds to an alternatively spliced product of the same gene, encoding a truncated form. The stathmin-like domain of RB3 contains a conserved alpha-helix, later shown to be responsible for coiled-coil, protein-protein interactions, as well as the two consensus phosphorylation sites seen in stathmin and SCG10. RB3 N-terminal domain has two cysteine-residues. Palmitoylation at cysteine residues allows association with membranes, and RB3 proteins were shown (Ozon et al., 1997) to be associated with the particulate, membrane-containing fraction. RB3 proteins appear as several spots of decreasing pI on two-dimensional immunoblots, suggesting that they are phosphorylated in vivo. As with SCG10, RB3 mRNA is detectable by in situ hybridization only in the nervous system (Ozon et al., 1997). Unlike SCG10, RB3 is expressed at similar levels in the newborn and the adult brain, whereas SCG10 expression decreases in the adult (Ozon et al., 1997). Furthermore, RB3 mRNA is undetectable in PC12 cells, whereas SCG10 mRNA increases after treatment with nerve growth factor induces neuronal differentiation.

SCG10 Like Protein (SCLIP)

Ozon et al. (Ozon et al., 1998) screened EST databases for stathmin-like proteins and identified mouse Stmn3, which they designated SCLIP. The deduced 180-amino-acid protein has a calculated molecular mass of 21 kDa and shares 70% amino-acid identity with SCG10. SCLIP contains an N-terminal 9-amino-acid signature sequence and a putative alpha-helical domain predicted to form coiled-coil interactions with other

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proteins. It has two cysteines that may be palmitoylated and four consensus phosphorylation sites. Ozon et al. (Ozon et al., 1998) also identified a human STMN3 gene, which encodes a deduced 180-amino acid protein. Human STMN3 shares 95% amino acid identity with the mouse protein and has the same structural features, including the two cysteines and predicted phosphorylation sites. Northern blot analysis of rat tissues detected a major 1.2-kb transcript highly expressed in neonatal and adult brain; expression was not detected in other tissues examined. This neuronal-specific expression is believed to be in part through the negative control exerted by the NRSF, a transcriptional repressor expressed in most non-neuronal tissues and neuronal progenitor cells that binds to a conserved element known as the neural NRSE or repressor 1. Bieche et al. (Bieche et al., 2003) used real-time quantitative RT-PCR to amplify SCLIP in human tissues. Highest levels were detected in adult and fetal brain, spinal cord, and cerebellum. Lowest levels were detected in adult and fetal liver and in skeletal muscle while other tissues showed intermediate expression. Thus SCLIP, whose expression was thought to be neural-specific, exhibits a broader tissue distribution. Analyses of the SCLIP gene showed that it contains several NRSE-like elements that display low or no affinity for the cognate binding protein NRSF. The widespread expression of SCLIP, suggest functions for this protein outside the nervous system and raises the possibility that its coexpression with stathmin could provide some degree of functional redundancy. Gavet et al. (Gavet et al., 1998) showed that HeLa cells over-expressing mammalian SCLIP displayed a punctate staining throughout the cytoplasm, with some large dispersed spots that colocalized with vesicles of disorganized Golgi apparatus. When over-

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expressed in interphase cells, SCLIP showed microtubule-depolymerizing activity attributable to its C-terminal stathmin-like domain.

Partially/Non-Overlapping Roles

Thus the Stathmin family of phosphoproteins (stathmin, SCG10, SCLIP, and RB3/RB3'/RB3") are involved in signal transduction and regulation of microtubule dynamics. With the exceptions of stathmin and perhaps SCLIP, they are expressed almost exclusively in the nervous system (table 1), where they display different spatio-temporal and functional regulations and hence play at least partially distinct and possibly complementary roles in relation to the control of development, plasticity, and neuronal activities. At the molecular level, each possesses a specific "stathmin-like domain", and with the exception of stathmin, N-terminal extensions (as shown in figure 1), that specify association with intracellular membrane compartments. Charbaut et al. (Charbaut et al., 2001) showed that the stathmin-like domain of each family member is capable of binding tubulin. All family members sequester alpha/beta tubulin heterodimers as revealed by inhibitory actions on tubulin polymerization and by gel filtration chromatography. However, they differ with regard to the stabilities of the complexes they form with α/β tubulin as well as in their interaction kinetics. Using surface plasmon resonance, Charbaut et al. (Charbaut et al., 2001) showed that the differences in stabilities and interaction kinetics are as follows: strong stability and slow kinetics for RB3; medium stability and intermediate kinetics for SCG10, SCLIP, and stathmin; and weak stability and rapid kinetics for RB3'. This suggests that evolution has favoured replication of the original stathmin gene and fine-tuned the stathmin-like domains of new members of the

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family to allow for specific functional roles of stathmin family proteins in the regulation of microtubule dynamics within the various cell types and subcellular compartments of the developing or mature nervous system.

Rationale for our Experiments

The primary goal of the project was to characterize prolactin (PRL) signaling pathways. Initial studies showed that p38 MAP kinase and stathmin are important components. Consequently, early experiments focused on regulation of p38 by PRL. Attention was then turned to stathmin, because the members of the stathmin family of proteins were emerging as important intracellular signaling agents. Few stathmin binding partners had been identified; therefore, the focus became identification of proteins that interact with stathmin or stathmin family members. It was our belief that a greater knowledge of stathmin binding partners would allow us to understand the proposed "relay" function of the protein.

PRL-Induced Phosphorylation of p38

Prolactin is a hormone secreted by the anterior pituitary. As its name suggests prolactin is involved in growth, development of the mammary gland and lactation, but prolactin has other important functions. Some of its diverse functions include osmoregulation and immunomodulation (Nicoll 1980) (Russell 1989). Gout et al. (Gout et al., 1980) derived T lymphoma cells from an estrogenized male Nb rat. These cells (Nb2) proliferated in response to prolactin. Previous studies (Meyer et al., 1992) have shown that phosphorylation of stathmin correlates with cell growth, and phosphorylation

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is increased substantially by the hormone prolactin as well as by the growth promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA, an activator of protein kinase C). Work in our lab (Park K, et al. 2006 Manuscript in preparation) has shown that in prolactin stimulated Nb2 cells, phosphorylation of p38 (which is an index of its activation) increases, though the total amount of p38 MAP kinase protein does not change substantially. However the specific isoform of p38 MAP kinase involved in the prolactin mediated proliferation response of Nb2 cells was not unidentified. We therefore thought it pertinent to study which specific isoform of p38 was activated in response to prolactin induced proliferation.

Analysis of Stathmin-Hsp70 Interactions

The importance of the interaction of stathmin with members of the Hsp family is less well understood. However, interaction of stathmin with members of the Hsp70 family opens additional pathways for regulation of cell growth and development by stathmin. Hsp70 protein levels increase in Nb2 cells with a time course similar to stathmin phosphorylation (deToledo et al., 1987). Given this fact and knowing that stathmin interacts with other members of the Hsp70 family, we examined whether Hsp70 itself interacts with stathmin using a probe consisting of stathmin coupled to glutathione-S-transferase (GST). The probe was used to identify putative stathmin-binding proteins in the Nb2 lymphoma and PC12 cells.

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Yeast Two Hybrid Screen for Stathmin/SCG10 Binding Proteins

Several investigators studying protein-protein interactions with respect to stathmin, have used the yeast two-hybrid screen. The Yeast two-hybrid screens for stathmin binding proteins, so far have identified four binding partners, they are:

1. BiP a member of the hsp70 heat-shock protein family (Maucuer et al., 1995) (Manceau et al., 1999)
2. HSC70 a member of the heat-shock cognate protein family (Manceau et al., 1999)
3. CC1 which has since been shown to be a sub-domain of the protein encoded by KIS. KIS was a previously unidentified serine/threonine kinase, since shown to also have a RNA recognition motif (Maucuer et al., 1997).
4. CC2 which has since been shown to be a sub-domain of the protein encoded by the tumor susceptibility gene Tsg101 (Maucuer et al., 1995)

Two-hybrid screening (Fields and Song 1989) determines the ability of two molecules to interact physically with sufficient affinity to provide a read-out from two reporter systems. Therefore it is necessary to confirm two-hybrid results using complementary methods, establish physiological occurrence of the interaction and prove relevance using in vivo techniques. The currently known stathmin binding proteins can be classified into two groups:

Novel: these are proteins about which there is almost no or very little knowledge. Proteins such as KIS and TSG101 are examples of this group. This is a difficult group to study because finding the implications of stathmins interactions with them would first require their thorough biochemical/biophysical characterization followed by an

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investigation of their normal cellular roles. This in itself is a time-taking and painstaking process that deviates from our main goals, we wish to minimize time spent studying this group.

Characterized: These are proteins about which there is a wealth of information. This group contains one subgroup of proteins whose roles are difficult to interpret because of their broad range of functions (relative non-specificity) in cells. For example HSC70 though designated a `heat shock protein' is believed to play a role in in response to a variety of other stressors such as osmotic shock, pH change and anoxia. The other subgroup, which consists of proteins with specific cellular functions, includes tubulin. Tubulin has been thoroughly characterized by a number of researchers and so has its interaction with stathmin; hence relatively fewer major insights may be expected by further studying it. We therefore decided to employ a two-pronged approach consisting of GST-pulldowns (Smith and Johnson 1988) and yeast two-hybrid screening (Fields and Song 1989), to discover a larger database of proteins that interact/bind with stathmin and SCG10. Identification of additional stathmin and SCG10 binding proteins will make it possible to characterize important pathways more fully and to build on earlier work. A human brain cDNA library was used for the two-hybrid studies because all stathmin family members appear to play an important role in brain development and function. Also the expression of stathmin and SCG10 in the brain is temporally regulated and they play non-overlapping roles during distinct phases of development.

We reasoned that the two approaches of GST-pulldowns and yeast two hybrid screening would provide us with a large database of interacting proteins of which at least a few were likely to be well characterized proteins (important for reasons mentioned

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earlier) that would generate strong evidence about the roles and specific pathways in which stathmin is involved. Having specific mechanisms and contexts of interaction of stathmin with other binding proteins (other than the well characterized tubulin) would enable us to draw generalizations regarding stathmins structure-function relationships.

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MATERIALS and METHODS

Cell Culture and Prolactin Stimulation

Rat Nb2 lymphoma cells were obtained from Dr. H. G. Friesen (Manitoba, Canada) and maintained in Fischer's medium (Sigma, St. Louis, MO) for leukemic cells of mice supplemented with 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO) and 10% (v/v) horse serum (Sigma, St. Louis, MO), 0.1mM 2-Mercaptoethanol (2-ME), 100U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of CO_2/air (1:19) at 37 °C. Approximately 24 hours before induction with prolactin (PRL), the Nb2 cells were growth-arrested in the early G_1 phase by a pre-incubation in lactogen-free assay medium (Fischer's medium containing 2-ME, antibiotics, 10% lactogen free horse serum). After 24 hours, the cells were collected by centrifugation at 400 x g at 4 °C for 4 minutes and re-suspended in fresh assay medium. The cells were then stimulated with 10ng/ml of PRL, which cause the growth-arrested cells to resume progression through the cell cycle in synchrony.

Preparation of Nb2 Cell Extracts

The prolactin-stimulated cells were collected at 24 hours, rapidly chilled to 4 °C, centrifuged and then resuspended in a lysis buffer containing 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1mM Na_3VO_4, 1 mM

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phenylmethylsulfonylfluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml pepstatin. The cells were incubated in the buffer at 4 °C for 20 minutes and lysed by repeated passage through a 19 gauge needle using a 3 ml syringe. The lysate was centrifuged at 14,000 r.p.m at 4 °C for 10 minutes in a tabletop Eppendorf centrifuge and the supernatant fraction was collected. The protein concentration in the supernatant fraction was estimated using the BCA Protein Assay reagent (Pierce Chemical Co, Rockford, IL).

Analysis of Proteins by SDS-PAGE, Western Blot and Immunostaining

Using the buffer system of Laemmli (Laemmli 1970), cell lysate or protein samples were electrophoresed in Novex pre-cast 10% or 12% SDS-polyacrylamide gels (Invitrogen, Carlsnbad, CA). Proteins were visualized by staining with Commassie blue R250 (Sigma Chemical Co., Saint Louis, MO) or were transferred to nitrocellulose membranes (Hybond) using Novex western transfer apparatus as per the manufacturer's protocol. Membranes were blocked for 2 hours at room temperature in TBST (10mM Tris pH 8.0, 150mM NaCl, 005% Tween20) containing 5% non-fat dry milk (for general use) or containing 1% milk (for phospho tyrosine antibodies). The membranes were then probed for 1 hour at room temperature with the primary antibody diluted in fresh blocking solution. The membranes were subjected to four 5-minute washes in TBST at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies or alkaline phosphatase-conjugated secondary antibodies for 30 minutes at room temperature. After four washes with TBST, the blots were developed per manufacturer's instructions.

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For HRP-conjugated antibodies : the enhanced chemiluminescence (ECL) method (Santa Cruz Biotechnology, Santa Cruz, CA) followed by exposure to Kodak BioMax XL light film (Eastman Kodak, New Haven, CT). OR

For Alkaline phosphatase-conjugated antibodies: alkaline phosphatase was detected using OneStep BCIP/NBT (Pierce Chemical Co., Rockford IL).

Cell Growth and Radioactively Labeled Protein Lysate Preparation for Pull-Down Assay

Nb2 cells, a rat lymphoma, were grown in Fischer's Medium (for leukemic mouse cells) as described above. Proliferating Nb2 cells (approximately 2 x 10^7) were washed and resuspended in 3 ml of a methionine and cysteine-free medium (Minimum Essential Medium Eagle without methionine, cysteine, Sigma, St. Louis, MO) supplemented with prolactin (PRL, 10 ng/ml), 0.1 mM 2-mercaptoethanol, 10% horse serum and a mixture containing ^{35}S-methionine and ^{35}S-cysteine (1 mCi, Express^{35}S^{35}S, NEN Life Science Products, Boston, MA). PRL stimulates growth of Nb2 cells in the absence of FBS; thus, FBS could be eliminated from the labeling medium. Reduction of serum promoted stronger labeling of proteins with the ^{35}S-methionine/^{35}S-cysteine mixture, because FBS contains significant amounts of unlabeled cysteine and methionine. PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum, 5% FBS, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Proliferating PC12 cells (approximately 1 x 10^7) were suspended in 3 ml of the methionine- and cysteine-free medium supplemented with 10% horse serum, 5% FBS, and 1 mCi of the ^{35}S-methionine/^{35}S-cysteine mixture. FBS was added to the labeling incubation to maintain the viability of

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the PC12 cells. All cells were incubated in their respective media for 6 hours at 37 °C in CO_2/air (1:19).

Cells were collected by centrifugation at 400 x g for 4 min at 4 °C, washed twice in Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), and resuspended in Tris-saline containing proteinase inhibitors [pepstatin (10 μg/ml), leupeptin (10 μg/ml), and aprotinin (0.2 TIU/ml)]. Nb2 cells were passed 10 times through a 23-gauge needle and centrifuged at 12,000g for 1 min at 4 °C. The supernatant fraction (cytosol) was separated from the pellet, passed through a 0.45 μm filter, and washed and concentrated in a Centricon 10 (Amicon Inc., Beverly, MA). The crude membrane pellet was suspended in Tris-saline containing 1% Triton X-100, 2mM EDTA and protease inhibitors, passed 10 times through a 23-gauge needle, and centrifuged at 12,000 x g. The supernatant fraction containing solubilized membranes was removed for use in binding studies. Because many of the PC12 cells adhered to the culture dish, they were solubilized at 4 °C in Tris-saline buffer containing 1% Triton X-100 and protease inhibitors. The solubilized cells were centrifuged at 12,000 x g for 1 min and the supernatant fraction was passed through a 0.45 μm filter (Amicon Inc., Beverly, MA). PC12 proteins were washed and concentrated in a Centricon 10 (Amicon Inc., Beverly, MA).

Vectors, cDNAs and Synthesis of Constructs

Human stathmin cDNA sub-cloned into pGEX-5X1 (Amersham/Pharmacia, Uppsala, Sweden) was kindly provided by Neeti Vasi (Indiana State University, Terre Haute, IN). Rat SCG10 in pBS plasmid was a kind gift from Dr. A. Sobel (INSERM

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U153-CNRS URA614, Paris, France). PCR primers with BamH1 and Xho1 ends were used to amplify the SCG10 fragment from pBS-SCG10 template, separated on a 1% agarose gel, cut out, extracted from the agarose using the BIO-101 gel extraction kit and directionally cloned into pGEX-5X1 (Amersham/Pharmacia, Uppsala, Sweden) and pLexA (figure 4A) vectors (pLex A from Clontech, Palo Alto, CA).

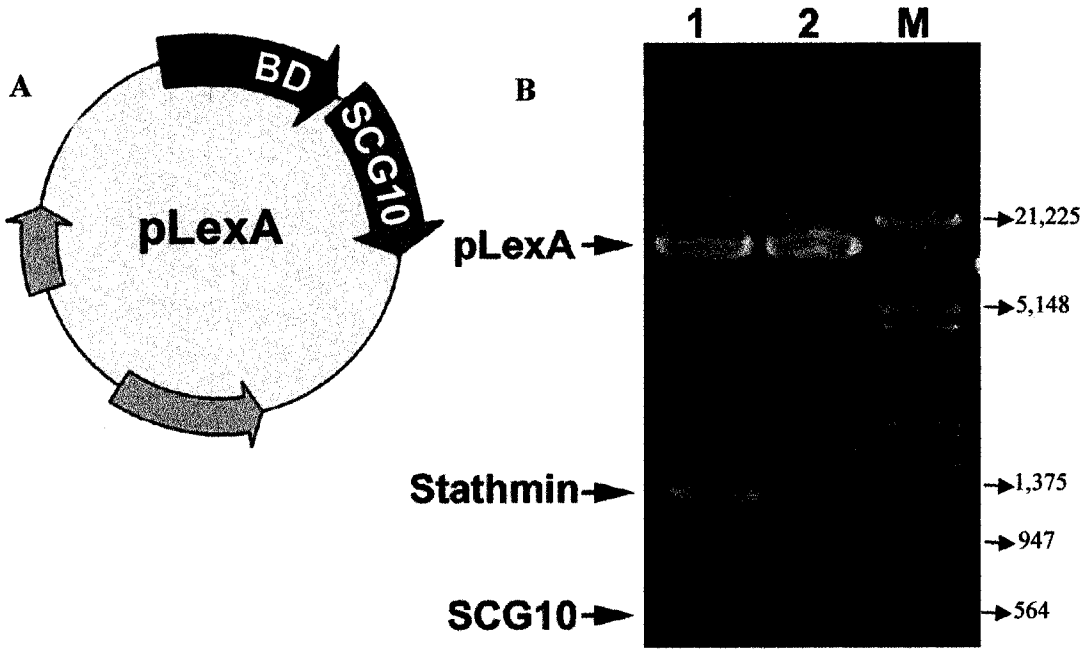

Figure 4: Cloning of Stathmin and SCG10 into pLexA. (A) Stathmin and SCG10 were directionally cloned into pLexA vector. (B) BamH1, Xho1 digests of pLexA-Stathmin (lane 1) and pLexA-SCG10 (lane 2) indicating correct size of the cDNA inserts and the vector/backbone. The DNA markers (M) used as reference (EcoR1 + HindIII digest of Lambda DNA), are indicated by the bands seen, with adjacent numbers representing size in number of base-pairs. Sequencing showed stathmin and SCG10 in-frame with BD of pLexA. Good quality and quantity of DNA was purified for yeast two hybrid experiments. Stathmin or SCG10 expressed from this plasmid as a hybrid protein coupled to a DNA-binding domain (BD) acts as the bait in the yeast two hybrid screen.

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The stathmin fragment obtained from BamH1, Xho1 digest of pGEX-5X1-stathmin was similarly purified and directionally cloned into the pLexA vector (Clontech, Palo Alto, CA). Ligations were desalted using an innovative technique of dialysis through agarose, against a glucose solution (Atrazhev and Elliott 1996). Constructs were transformed into electro-competent cells of E.coli strains HB101 or TG1 for plasmid purifications and into BL21-DE3-plysS for protein expression.

Verification of Authenticity of cDNA/Gene-Plasmid Constructs

All cDNA/gene-plasmid constructs used in the experiments were verified by subjecting them to the appropriate restriction digests followed by analysis by agarose gel electrophoresis (figure 4B). Samples of the constructs also were sent for sequencing (using appropriate primers) to the Sequencing facility of the University of Chicago (Cancer Research Center, University of Chicago, Chicago, IL). Sequences received were analyzed using BLAST (Altschul et al., 1990) and other web-based alignment and analysis tools (NCBI, NIH, Bethesda, MD). Correct orientation, reading frame and lack of mutations in the sequence were similarly verified (example: figure 5).

Expression and Purification of Proteins

Colonies of pGEX-5X1-Stathmin (figure 12A) and pGEX-5X1-SCG10 in either TG1 or Bl21DE3-plysS strain of E.*coli* were grown overnight in Luria Bertani (LB) medium (10 g/L NaCl, 10 g/L Tryptone, 5 g/L Yeast extract) containing 100 μg/ml of Ampicillin (LB-Amp) at 37 °C. The pre-inoculums were diluted 1:100 into fresh LB-

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Amp and grown at 30 °C with shaking until the the absorbance at 600nm (A_{600}) reached 0.5 – 1.0. The cultures were induced for 3 hours by adding Isopropyl B, D, galactosidase (IPTG) to a final concentration of 0.1mM. Cells were sedimented by centrifugation at 8,000 rpm in a Beckman JA20 rotor for 10 minutes at 4 °C. The cell pellet

pLexA-SCG10 sequence

AGATCTTCGTCAGCAGAGCTTCACCATTGAAGGGCTGGCGGTTGGGGTTATTCGCAA
CGGCGACTGGCTGGAATTCCCGG**GGATCC**GT[redacted]GCTAAAACAGCAATGGCCTACA
AGGAAAAAATGAAGGAGCTGTCTATGCTGTCACTGATCTGCTCCTGCTTCTACCCGG
AGCCTCGCAACATCAACATCTACACCTACGATGACATGGAGGTGAAGCAGATCAAC
AAGCGTGCTTCCGGCCAGGCTTTCGAGCTGATCTTAAAGCCCCCGTCTCCCATCTCGG
AAGCTCCACGAACTCTAGCTTCTCCAAAGAAGAAAGACCTGTCTCTGGAGGAGATCC
AGAAAAAGCTGGAGGCTGCAGAGGGGCGAAGGAAGTCTCAGGAGGCCCAGGTGCTG
AAGCAGTTGGCAGAGAAGAGGGAGCACGAGCGAGAAGTCCTCCAGAAGGCTTTGGA
GGAGAATAACAACTTCAGCAAGATGGCGGAGGAGAAGCTGATCCTGAAAATGGAAC
AAATTAAGGAAAACCGTGAGGCTAATCTAGCTGCTATCATTGAACGTCTGCAGGAAA
AGGAGAGGCATGCTGCCGAGGTGCGCAGGAACAAGGAACTGCAGGTTGAACTGTCT
GGC[redacted]**CTCGAG**TCGACCTGCAGCCAAGCTAATTCCGGGCGAATTTCTTATGATTTA
TGATTTTTATTATTAAATAAGTTATAAAAAAAAATAAGTGTATACNAATTT

pLexA-Stathmin sequence

AGATCTTCGTCAGCAGAGCTTCACCATTGAAGGGCTGGCGGTTGGGGTTATTCGCAA
CGGCGACTGGCTGGAATTCCCGGGGATCCCCGAATTCCCGGGTCTTCTATTCACC[redacted]
[redacted]GCTTCTTCTGATATCCAGGTGAAAGAACTGGAGAAGCGTGCCTCAGGCCAGGCTTT
TGAGCTGATTCTCAGCCCTCGGTCAAAAGAATCTGTTCCAGAATTCCCCCTTTCCCCT
CCAAAGAAGAAGGATCTTTCCCTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGA
AGAAAGACGCAAGTCCCATGAAGCTGAGGTCTTGAAGCAGCTGGCTGAGAAACGAG
AGCACGAGAAAGAAGTGCTTCAGAAGGCAATAGAAGAGAACAACAACTTCAGTAAA
ATGGCAGAAGAGAAACTGACCCACAAAATGGAAGCTAATAAAGAGAACCGAGAGG
CACAAATGGCTGCCAAACTGGAACGTTTGCGAGAGAAGGATAAGCACATTGAAGAA
GTGCGGAAGAACAAAGAATCCAAAGACCCTGCTGACGAGACTGAAGCTGAC[redacted]TT
TGTTCTGAGAACTGACTTTCTTCCCCATCCCCTTCCTAAAATATCCAAAGACTGTACT
GGCCAGTGTCATTTTTATTTTTTTTTCCCTTCCTGAACAAATATTTTAGAAGCCTAATT
GTAGGACTTGTTTAGGGTAGATCCAGATTCCAGACTTGTTAAGATGGTTGNTTTTNAG
GGGGCTTAAAAGGGGGAGNAAACTTNAAAANGTGTNTTNTACCTCNTTTTTTCTANA

Figure 5: Successful cloning of the SCG10 and Stathmin genes. Successful cloning of the SCG10 and Stathmin genes in-frame with the BD gene (as N-terminal fusion) of pLexA was determined by both 5' and 3' sequencing. The sequences with the relevant start and stop codons are shown for (A) SCG10 and (B) for Stathmin.

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was resuspended in 50 µl (per ml of culture) of phosphate buffered saline (PBS) containing protease inhibitors and disrupted on ice using four 15-seconds bursts (with 30-seconds cooling intervals between bursts) using a sonicator (Branson Ultrasonic Corp., Danbury, Connecticut) set at 50% output. After partial clearing of the suspension, 20% Triton X-100 was added to a final concentration of 1% and the suspension was mixed gently for 30 minutes to aid solubilization of the fusion protein. Cell debris was pelleted by centrifugation at 12,000 x g for 10 minutes at 4 °C and the supernatant was saved for further purification. A 50% slurry of Glutathione Sepharose 4B (Pierce Chemical, Rockford, IL) was washed with PBS as per the manufacturer's instructions and the sepharose was added to the saved supernatant (2 ml sepharose per 100 ml sonicate). The mixture was incubated at room temperature for 30 minutes with gentle agitation using an Stovall Belly Dancer shaker (Cole Parmer, Vernon Hills, IL). The suspension was centrifuged at 500 x g for 5 minutes to sediment the matrix. The supernatant were discarded, the pellet was resuspended with ten bed volumes of PBS and centrifuged to sediment the matrix. Two more such washes were performed. The sedimented matrix was resuspended in 1 ml (per ml bed volume of matrix) of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). The resuspended mixture was incubated with gentle agitation at room temperature (22 to 26 °C) to elute the fusion protein from the matrix. Eluate was obtained by centrifugation of the mixture at 500 x g at 4 °C for 5 minutes followed by collection of the supernatant. Elution and centrifugation steps were repeated twice and the eluates were pooled. To eliminate the reduced glutathione in the buffer or to change to a buffer suitable for downstream applications, the eluate was subjected to three rounds of centrifugation in a Centricon 30

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(30 kD molecular weight cut-off) ultrafiltration device (Amicon Inc., Beverly, MA). The purity of the purified protein was estimated by subjecting a small sample to SDS-PAGE followed by staining with Commassie blue. The protein yield was estimated by measuring the absorbance at 280 nm and also by comparison to known quantities of BSA on SDS-PAGE (stained with Commassie blue).

'Pull-Down' (Affinity Precipitation) Assay using GST-Fusion Proteins

Proteins extracted from PC12 cells (whole cell) and from Nb2 cells (cytosol or crude membrane) were incubated with 20 nanomoles of the GST-stathmin fusion protein or equivalent amount of GST protein for approximately 18 h at 25 °C. Incubations were performed in a Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl) containing the protease inhibitors. The pH of the buffer was adjusted to 8.0 or 6.8 as noted in figure legends, Triton X-100 was maintained at 0.05-0.1% in solutions containing solubilized membranes, and $MgCl_2$ was added to a final concentration of 5 mM where noted. After incubation, 25 µl of packed glutathione-Sepharose 4B was added to each reaction (figure 13A) and the tubes were shaken gently for 30 minutes at 25°C. The amount of glutathione on the Sepharose was sufficient to bind all the GST (free or associated with the stathmin-GST fusion protein) in the reaction. Therefore, the beads were capable of insolubilizing stathmin-GST fusion protein and proteins associated with the fusion protein. The Sepharose beads were collected by centrifugation, washed three times in Tris-saline buffer, and stathmin-GST fusion protein was eluted from the sepharose using 10 mM reduced glutathione. In each experiment, equal volumes of the glutathione eluates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

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PAGE) as described by Laemmli (Laemmli 1970) on 10% polyacrylamide gels. Electrophoresis was run on a Mini-Protean II vertical gel system at a constant voltage of 125 V for approximately 90 minutes, at which time the dye front reached the bottom of the gel. Proteins in the gels were stained using commassie blue (figure 13B) to visualize total protein pulled down or were electrophoretically transferred (60 volts, 1 h) to nitrocellulose paper (0.22 μm) using a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA) and the buffer system described by Towbin *et al.* (Towbin *et al.*, 1979). Open sites on the nitrocellulose paper were blocked by incubation in binding buffer (100 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 8.0) containing 5% non-fat dry milk. After three 10-min washes in binding buffer, the nitrocellulose was incubated for 18-20 hours in binding buffer containing the primary antibody. Antibodies used were either anti-Hsp70 (sc-1060) at 1:200 dilution or ani-tubulin (Sigma) at 1: 500 dilution. Unbound antibody was removed by three 10-min washes in binding buffer, and the nitrocellulose was incubated for 1 h with a secondary antibody coupled to alkaline phosphatase. Unbound antibody was removed by three 10-min washes in binding buffer, and the alkaline phosphatase was detected using OneStep BCIP/NBT (Pierce Chemical Co., Rockford IL).

Antibodies

All secondary antibodies conjugated to horse radish peroxidase or alkaline phosphatase were from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1:10,000 dilution. The primary antibodies and the dilutions used are as mentioned below. Anti-Hsp70 (sc-1060, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. Anti-tubulin (Sigma, Saint Louis, MO) diluted 1:500. Anti-p38 (sc-7972, Santa Cruz

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Biotechnology, Santa Cruz, CA) diluted 1:200. p38α (sc-535, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. p38β (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. p38γ (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. Anti-p38 phospho-specific (sc-7973, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200.

Brain cDNA Library Titering, Amplification and Plasmid Purification

Human Brain Matchmaker LexA cDNA library (normal whole brain from a 57 year old Caucasian male) pretransformed into E.coli, was purchased from Clontech Laboratories Inc. (Palo Alto, CA) and aliquots of 100 µl stored at –70 °C. One cDNA library aliquot was thawed on ice and 1 µl was serially diluted to obtain dilution A ($1:10^3$) and dilution B ($1:10^6$). 1 µl of dilution A was mixed with 50 µl of LB broth and plated onto a pre-warmed LB-Amp (75 µg/ml ampicillin) plate. Aliquots of 50 µl and 100 µl of dilution B were also plated onto a separate LB-Amp plate. The plates were inverted and incubated at 37 °C overnight. Colonies were counted to determine the titer (colony forming units/ml) using the following formulas:

(a) cfu/ml = colony # in dilution A x 10^3 x 10^3

(b) cfu/ml = (colony # in dilution B/plating volume) x 10^3 x 10^3 x 10^3

Based on the cfu/ml observed, enough pretransformed library cDNA was plated directly onto 150 LB-Amp plates (150 mm size) to obtain 1 x 10^7 independent clones i.e. 3x the number of independent clones (3.5 x 10^6) in the library. The plates were incubated overnight at 37 °C and the colonies were scraped with cell-scrapers and collected in 2 liters of LB-Amp broth. The culture was incubated at 37 °C for 3 hours with shaking at

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200 r.p.m. Cells were pelleted at 8,000 rpm in a Beckman JA20 rotor for 10 minutes at 4 °C and frozen as pellets weighing 2 grams each. Maxiprep procedures using either Bio101 RPM 1G (now MP Biomedicals, Pasadena, CA) or Quiagen 500 (Qiagen, Valencia CA) kits were employed to obtain milligram quantities of good quality plasmid DNA. Figure 6 shows a schematic of the brain cDNA library in the pB42AD plasmid and a restriction digest analysis on the purified library plasmid pool. In some cases additional cesium chloride purification was performed to ensure transfection-quality DNA.

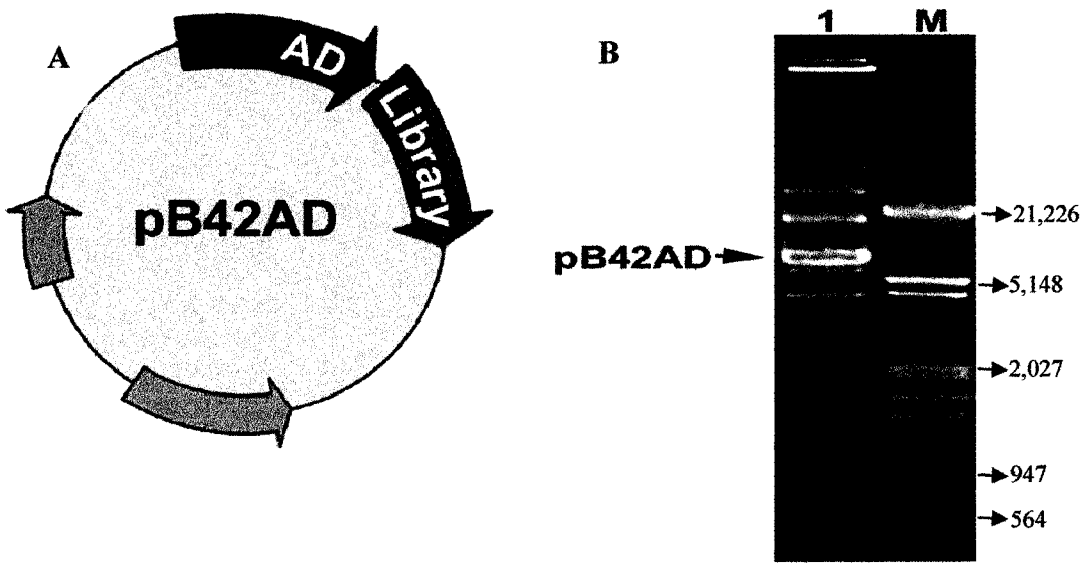

Figure 6: Amplification and titering of a human brain cDNA library. (A) A brain cDNA library (from a 57-year-old Caucasian male) cloned into pB42AD plasmid was obtained from Clontech. The library was amplified by growing and plating e.coli transformed with this library onto ~250 plates of 150mm. Titering of the cDNA library showed that it represented ~3.5 x 10^6 unique clones. (B) Lane 1 shows an incomplete EcoR1, Xho1 digest of the library pool of plasmids. Arrows indicate DNA bands corresponding to the vector and cDNA inserts ranging from 0.9 to 5.5 kb. The DNA markers (M) used as reference (EcoR1+HindIII digest of Lambda DNA), are indicated by the bands seen, with adjacent numbers representing size in number of base-pairs. Good quality and quantity of DNA were obtained for the yeast two hybrid screen. The brain cDNA library genes were expressed from the pB42AD plasmid as hybrid proteins coupled to an activation domain (AD) to act as "Target" proteins for the yeast two hybrid screen.

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Verification of Yeast Strains and Testing of Plasmid Constructs for Expected Phenotype

Yeast strains EGY48 [p8oplacZ] and YM4271 received as glycerol stocks from Clontech (Palo Alto, Ca) were used to make working stock plates and additional glycerol stocks. Each strain was plated as per the supplier's instructions onto the appropriate plate (with/without selection media) and allowed to grow for 4 days at 30 °C. After checking for the correct phenotype, the verified working stock plates were sealed with parafilm and stored at 4 °C. Small-scale transformations were performed with 0.1 μg DNA, using the lithium acetate-polyethylene glycol method, for the following plasmids:

1] pLexA-Stathmin (negative result indicates that bait protein itself does not activate reporter genes)

2] pLexA-SCG10 (negative result indicates that bait protein itself does not activate reporter genes)

3] pLexA-Pos (positive control for B-galactosidase assay)

4] pLexA-53 + pB42AD-T (positive control for interacting proteins)

5] pLexA-Stathmin + pB42AD (negative result indicates that bait protein does not activate reporter genes in the presence of the Activation domain plasmid without a cDNA-library fusion)

6] pLexA-SCG10 + pB42AD (negative result indicates that bait protein does not activate reporter genes in the presence of the Activation domain plasmid without a cDNA-library fusion)

7] pLexA-Stathmin + pB42AD-Library (small scale interaction test)

8] pLexA-SCG10 + pB42AD-Library (small scale interaction test)

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After plating the recommended dilutions on the appropriate media, the plates were incubated at 30 °C for 5 days. The phenotypes of the transformants were recorded and verified and the transformation efficiency calculated (for single transformations as well as co-transformations).

Full-Scale Interaction Screening using Large-Scale, Simultaneous Cotransformation

Transformations were performed as per supplier's instructions using freshly made competent cells. Briefly, a single fresh 2-3 mm diameter colony (from a one-to-three week old plate) of the appropriate yeast strain was used for innoculum into a flask containing 50 ml of the appropriate Synthetic Dropout (SD) medium. The culture was then incubated at 30 °C, 250 r.p.m. (normal condition) for 16 hours, a sufficient quantity of the overnight culture was transferred to a flask containing 300 ml of Yeast-extract Peptone Dextrose (YPD) media to produce a starter culture that had an OD_{600} of 0.3. The incubation was continued under normal conditions until the OD_{600} reached 0.6. Cells collected in 50 ml tubes, were centrifuged at 1000 x g for 5 minutes at 20 °C. Pelleted cells in each tube were re-suspended in 50 ml of distilled water and centrifuged as before. Cells from the six tubes were pooled into one tube with 50 ml of water. The cells were pelleted again and re-suspended in 1.5 ml of freshly prepared, sterile 1x TE-LiAc buffer. DNA-BD vector construct /bait (100 μg) was mixed in a tube containing 2 mg of herring testes carrier DNA and 50 μg of AD vector construct (cDNA library). Previously prepared yeast competent cells (1 ml) were added to this DNA mixture and mixed by vortexing. After addition of 6 ml of sterile PEG-LiAc, the solution was vortexed at high speed and then incubated at 30 °C for 30 minutes with shaking at 200 r.p.m. 700 μl of

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DMSO was added to the culture and mixed by gentle inversion, the culture was then subjected to heat shock at 42 °C for 15 minutes (with swirling at 5 minute intervals) in a water bath. The culture was chilled on ice for 2 minutes followed by centrifugation at 1000 x g for 5 minutes to pellet the cells. The supernatant fraction was discarded, the cell pellet was re-suspended in 1 ml of 1x TE buffer. The transformation suspension was plated at 200 µl per 150 mm plate of SD medium (SD/-His/-Trp/-Ura) and incubated up-side-down at 30 °C for 4 days. Colonies of cotransformants amplified by this step were then harvested by scraping them from the surface of the plate into TE buffer. Glycerol stocks of these cotransformants were made and stored at -80 °C. In order to screen for interacting proteins, glycerol stocks of the cotransformants were plated onto SD/Gal/Raf/-His/-Trp/-Ura/-Leu plates (interaction screening plates) containing 80mg/L of 5-bromo-4-chloro-3indolyl-b-D-galactopyranoside (X-gal) and a solution of 7g/L of $Na_2HPO_4,7H_2O$ and 3g/L of NaH_2PO_4 at pH 7 (BU salts) and incubated at 30 °C for 4 days (in some cases up to 6 days). A schematic of a yeast two hybrid screening is shown in figure 7. Interaction screening plates were marked in sectors to aid scoring of plates, to note day on which positive clones (blue colonies) appeared, the color intensity and colony size. Colonies were classified as small/big and color (faint blue, blue, dark blue, intense blue). Forty-eight colonies from each of the stathmin and SCG10 screenings were picked and cultured in 12 tubes (4 colonies pooled per tube containing 4 ml of medium) at 30 °C, 250 r.p.m. (normal condition) for 16 hours (as per the processing flowchart shown in figure 8). Yeast cultures (2 ml) from each of 3 tubes from a set were pooled (representing 12 unique interactions / colonies) for plasmid preparation and the remaining equivalent amounts were frozen as glycerol stocks. Plasmids were isolated from the

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pooled interactors using either lyticase or glass beads (to break the tough yeast cell wall) followed by routine plasmid isolation using either a BIO101 (now MP Biomedicals, Pasadena, CA) or BioRad (Bio-Rad Laboratories, Hercules, CA) plasmid midi-prep kit.

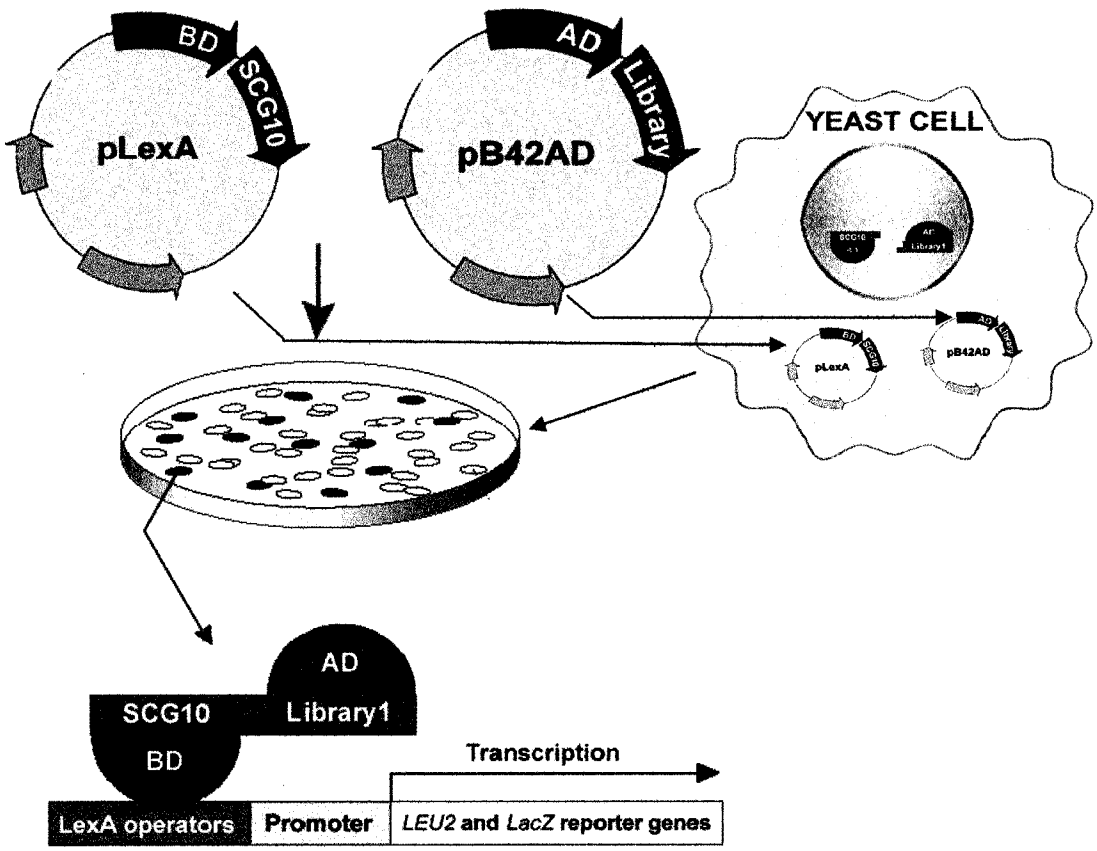

Figure 7: Schematic of a yeast two hybrid screen. Yeast were transformed with pLexA (stathmin or SCG10) and pB42AD (cDNA library) plasmids. Plasmids express hybrid proteins. Hybrid proteins are targeted to the nucleus. Proteins binding to stathmin or SCG10 activate two reporter genes (hence 'dual reporter positive') via AD domain. LEU2 gene allows growth in absence of added leucine; and LacZ expression allows hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in the plate that turns the interaction positive colonies blue (blue colonies appear dark in the figure) in color for easy visualization.

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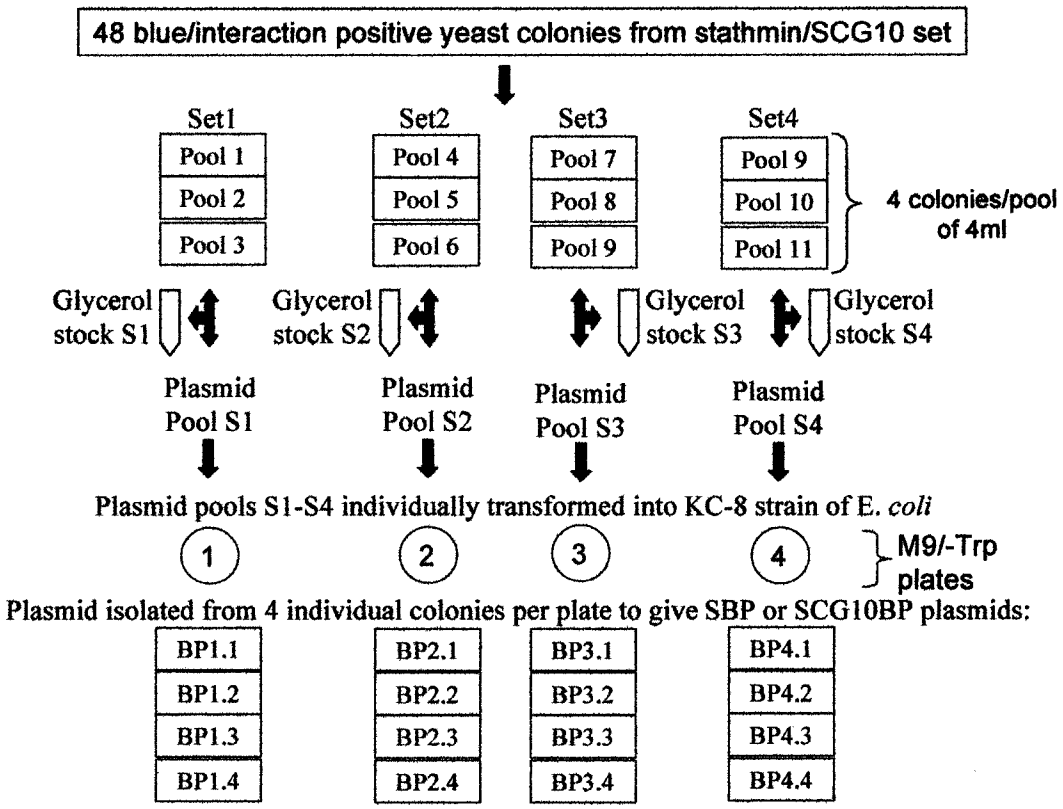

Figure 8: Processing flowchart for interaction positive colonies. Forty-eight colonies from each of the stathmin and SCG10 screenings were picked and cultured in 12 tubes (4 colonies pooled per tube containing 4 ml of medium) at 30 °C, 250 r.p.m. (normal condition) for 16 hours. Yeast cultures (2 ml) from each of 3 tubes from a set were pooled (representing 12 unique interactions / colonies) for plasmid preparation and the remaining equivalent amounts were frozen as glycerol stocks. Plasmids were isolated from the pooled interactors using either lyticase or glass beads (to break the tough yeast cell wall) followed by routine plasmid isolation using either a BIO101 (now MP Biomedicals, Pasadena, CA) or BioRad (Bio-Rad Laboratories, Hercules, CA) plasmid midi-prep kit. Four plasmid pools (representing 12 colonies/interactors) from each screen were individually transformed into electrically competent KC-8 strain of E.coli using electroporation. The resulting transformation suspension was plated onto M9/-Trp medium and incubated at 37 °C, for 24-36 hours. This selects for transformants bearing the AD-library plasmids carrying the TRP1 gene (nutritional selection/rescue via complementation of E.coli mutation) and also causes them to segregate (because unlike yeast, E.coli have plasmid incompatibility systems). Four colonies from each plate/set were picked and grown under normal conditions (in LB-Amp) for plasmid isolation. Plasmid mini-preps were performed using either a BIO101 or Bio-Rad kit with the addition of a phenol:chloroform:isoamyl-alcohol extraction or a alkaline-protease incubation step to remove endonuclease-A activity. Analytical EcoR1, XhoI digests were performed on 1 μg of each plasmid to eliminate obvious duplicates before using the plasmids for sequential transformation and mating in yeast (to verify interactions).

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Segregation of Library Plasmids by Transformation into KC-8 strain of E. *coli*

Four plasmid pools (each representing 12 colonies/interactors) from each screen were individually transformed into electrically competent KC-8 strain of E. *coli* using electroporation. The resulting transformation suspension was plated onto M9/-Trp medium and incubated at 37 °C for 24-36 hours. This selects for transformants bearing the AD-library plasmids carrying the TRP1 gene (nutritional selection/rescue via complementation of E.coli mutation) and also causes them to segregate (because unlike yeast, *E.coli* have plasmid incompatibility systems). A few colonies (4-6) from each plate/set were picked and grown under normal conditions (in LB-Amp) for plasmid isolation. Plasmid mini-preps were performed using either a BIO101 or Bio-Rad kit with the addition of a phenol:chloroform:isoamyl-alcohol extraction or a alkaline-protease incubation step to remove endonuclease-A activity. Analytical EcoR1, XhoI digests were performed on 1 μg of each plasmid to eliminate obvious duplicates before using the plasmids for sequential transformation and mating in yeast (to verify interactions).

Sequential Transformation (small scale)

The small-scale transformation procedure for yeast (described earlier) was used to transform fusion-protein encoding plasmids isolated in the screen (and segregated in KC-8 strain of E.coli) into yeast strain EGY48p8OplacZ and selected by plating on SD/-Trp/-Ura plates. These were then sequentially transformed (using the same small scale transformation procedure) with either pLexA-Stathmin or pLexA-SCG10 to verify the interaction and with pLexA or pLexA-Laminin to verify the specificity of the interaction. However, this process is time intensive and cumbersome and requires additional

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transformations every time a new putative interactor must be verified. Therefore the first step in the sequential transformation (i.e. small scale transformation) was used to generate strains needed for yeast mating. Accordingly small scale transformation of YM4271 strain of yeast with each of the plasmids (independently) pLexA (control), pLexA-Laminin (control), pLexA-Stathmin and pLexA-SCG10 were made to generate yeast strains for mating with the opposite mating type bearing the library fusion plasmids of putative interactors isolated earlier in the screen. Transformants were selected on SD/-His plates. Thus each time a new putative interactor was to be tested, only one new transformation (of the interactor cDNA in pB42AD vector) in EGY48p8OplacZ had to be performed and selected on SD/-Trp/-Ura to test for interaction via yeast mating.

Yeast Mating to Verify Interactions

Yeast containing specific library plasmids (isolated from the preliminary screen) in Mat-a yeast are mated to yeast of type Mat-α containing "bait" (stathmin/SCG10) or control plasmids (as shown in figure 9). One large (2-3 mm) fresh colony (<1 month old plate) of each mating type of yeast was picked and placed into a 1.5 ml microcentrifuge tube containing 0.5 ml of YPD medium. The tube was vortexed to completely resuspend the cells and then incubated at 30 °C, 200 r.p.m. shaking, for 20-24 hours. Aliquots of the mating culture (100 μl) were plated on SD/-His/-Trp/-Ura plates in four sectors as indicated in figure 11 and incubated at 30 °C for 4-6 days for selection of diploids. Diploids were replica plated using a replica plating block and sterile velveteen cloth onto SD/-His/-Trp/-Ura/-Leu +B.U. salts + X-gal plates. The replica plates were incubated at

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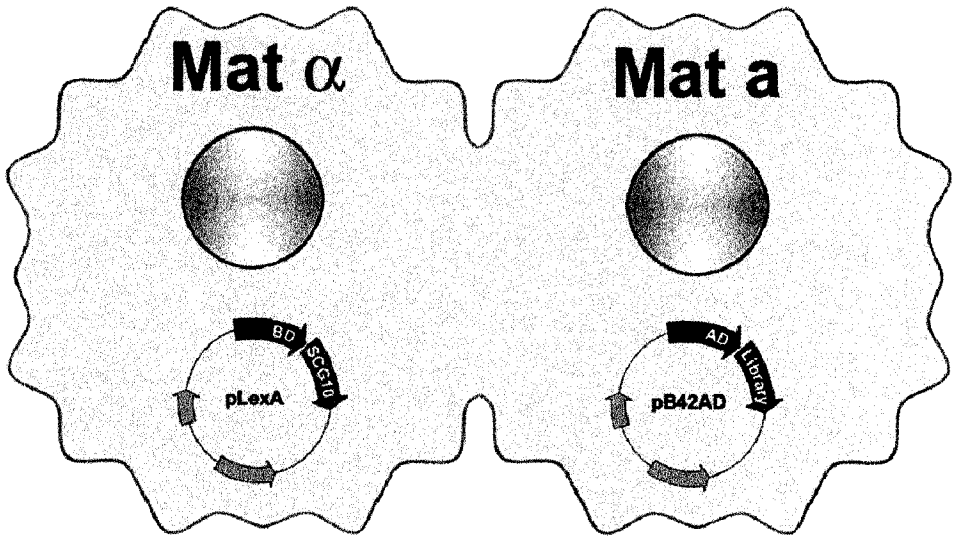

Figure 9: Schematic of yeast mating approach for yeast two hybrid interaction screening. Yeast containing specific library plasmids (isolated from the preliminary screen) in Mat-a yeast are mated to yeast of type Mat-*α* containing "bait" (stathmin/SCG10) or control plasmids.

30 °C for 4-6 days for selection and visualization of true interactors using both Leu and lacZ reporter expression. For each interaction to be tested, the pB42AD-cDNA fusion in EGY48p8OPlacZ strain was mated individually with YM4271 strain bearing one of four independent plasmids (figure 10). The four plasmids were pLexA-Stathmin, pLexA-SCG10 to verify the interaction and with pLexA or pLexA-Lamin to verify the specificity of the interaction.

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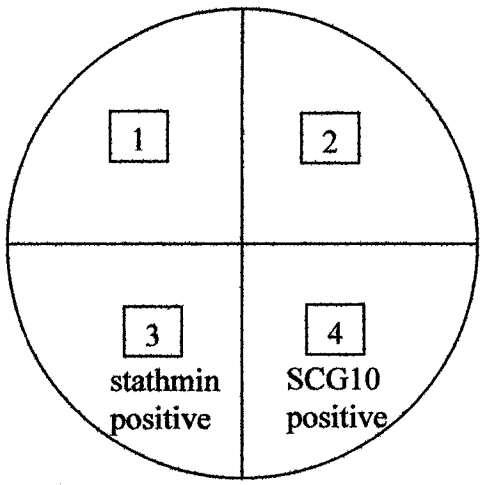

1. pB42AD cDNA`X' + pLexABD
2. pB42AD cDNA`X' + pLexABD-Lamin
3. pB42AD cDNA`X' + pLexABD-Stathmin
4. pB42AD cDNA`X' + pLexABD-SCG10

Figure 10: Schematic of yeast mating approach for yeast two hybrid interaction screening and confirmation. Yeast containing "target" specific library plasmid fused to the Activation Domain (AD) were mated to yeast containing different "bait" for the reasons explained below. To screen for interaction the yeast mating mixtures were plated onto SD/Gal/Raf/-His/-Trp/-Ura/-Leu plates (interaction screening plates) containing X-gal and BU salts and incubated at 30 °C for 4 days. Dual reporter positives are colonies of yeast that can survive on medium lacking Leucine (i.e. they yeast in Leu+) and turn blue in color (LacZ+) on medium containing X-gal.
(1) LexA-BD alone: a dual reporter positive in this quadrant indicates that the library protein has DNA binding ability by itself or through a non-specific interaction with the LexABD and has activation ability by itself or conferred by the AD fused to it.
(2) LexA-BD fused to Lamin protein: a dual reporter positive in this quadrant indicates that the library protein has DNA binding ability through a non-specific interaction with the LexABD-Lamin fusion.
(3) LexA-BD fused to Stathmin: a dual reporter positive in this quadrant indicates that the library protein has DNA binding ability through a specific interaction with the LexABD-Stathmin fusion protein.
(4) LexA-BD fused to SCG10: a dual reporter positive in this quadrant indicates that the library protein has DNA binding ability through a specific interaction with the LexABD-SCG10 fusion protein.

An interaction with the Stathmin fusion and the lack of interaction with the SCG10 fusion product shows that the interaction is specific for Stathmin and not to a close neural specific member (SCG10) of the family (and vice versa).

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Sequencing and Sequence Analysis

Once interactions were verified using yeast mating, the original E.coli (KC-8 strain) plasmid preparation containing the library cDNA coding for the protein responsible for the interaction was sent for sequencing. The kit provided pB42AD sequencing primer (TCCTACCCTTATGATGTGCCA) was used for 5'sequencing and another primer (CGACAACCTTGATTGGAGACT) was designed and custom synthesized (Invitrogen/Life Technologies, Carlsbad, CA) for 3' sequencing. Sequencing was performed by the CRC- sequencing facility (University of Chicago, Chicago, IL). Good quality sequence received was used in a BLAST (Altschul et al., 1990) search (NCBI, NIH, Bethesda, MD) to determine what the gene the cDNA represented and hence what protein it encoded. The amino acid sequence data thus obtained was utilized as input for a coiled-coil prediction program called COILS (Lupas et al., 1991). This enabled prediction of regions (and probability scores) of the binding proteins that had coiled-coil regions, a motif seen in all previously identified Stathmin and SCG10 binding proteins.

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RESULTS

Prolactin Increases the Phosphorylation of p38α in Nb2 Cells

Previous studies (Meyer et al, 1992) have shown that phosphorylation of stathmin correlates with cell growth and is increased substantially by the hormone prolactin as well as by 12-O-tetradecanoylphorbol-13-acetate (TPA, an activator of protein kinase C). Nemeth et al. (Nemeth et al., 1998) have shown that p38 mRNA levels increase 2.5 fold in response to the prolactin stimulation of Nb2 cells for four hours; however no change in protein levels were seen. Work in our lab (Park et al., 2006, Manuscript in preparation) has shown that in response to prolactin stimulation, the total amount of p38 MAP kinase protein in Nb2 cells does not change substantially, though levels of the phosphorylated form of p38 (which is an index of its activation) are increased. However the specific isoform of p38 MAP kinase involved in the prolactin mediated proliferation response of Nb2 cells was not unidentified. Therefore whole cell lysates were prepared from Nb2 cells stimulated with PRL for 24 hours and proteins from the cells were probed with antibodies for p38α, p38β and p38γ. The immunoblot analysis detected a phosphorylated form of p38 (figure 11A), we therefore probed for some p38 isoforms but detected only the p38α isoform (figure 11B). Our results indicate that during the prolactin-stimulated proliferation of Nb2 cells there is a substantial increase in phosphorylated p38α although the total p38 protein levels remain unchanged from 0 to 12 hours. The data indicate that

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neither p38β nor p38γ are detectable under these conditions, and therefore p38α seems to be playing the primary role.

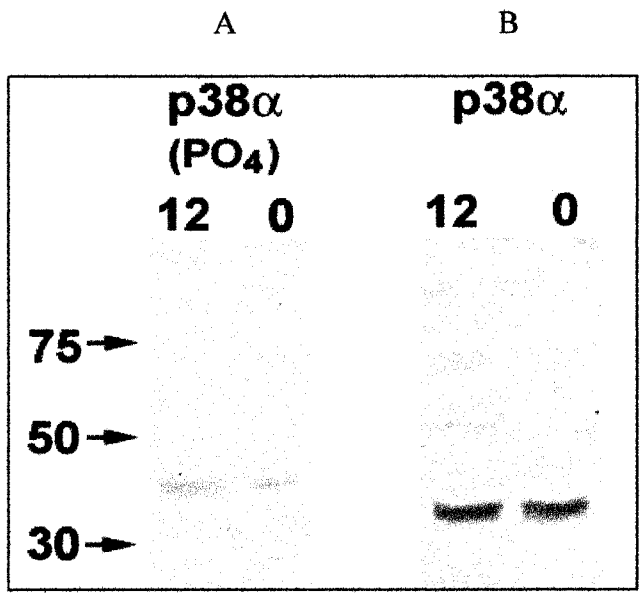

Figure 11: Prolactin stimulates increased p38 alpha phosphorylation. During the 0 to 12 hour window of prolactin-stimulated proliferation of Nb2 cells there is a substantial increase in phosphorylated p38α. This can be seen in the immunostaining of a western blot using phospho-specific p38 antibodies (sc-7973, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 (A). However the total p38 protein levels remain unchanged from 0 to 12 hours (data not shown). Immunostaining of western blots for the 0 and 12 hour time-points shows roughly equivalent staining by a p38α antibody (sc-535, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 (B), but neither p38β nor p38γ are detectable (using corresponding antibodies from Santa Cruz Biotechnology, Santa Cruz, CA) under these conditions (data not shown). The positions of molecular weight markers (M) used as reference, are indicated by the arrows with adjacent numbers representing weight in kilodaltons.

Purified GST-Stathmin and GST-SCG10 Proteins

Both GST-Stathmin and GST-SCG10 proteins were purified in milligram quantities for use in in-vitro binding assays. The proteins were approximately 95% pure based on SDS-PAGE followed by staining with Commassie blue (figure 12C).

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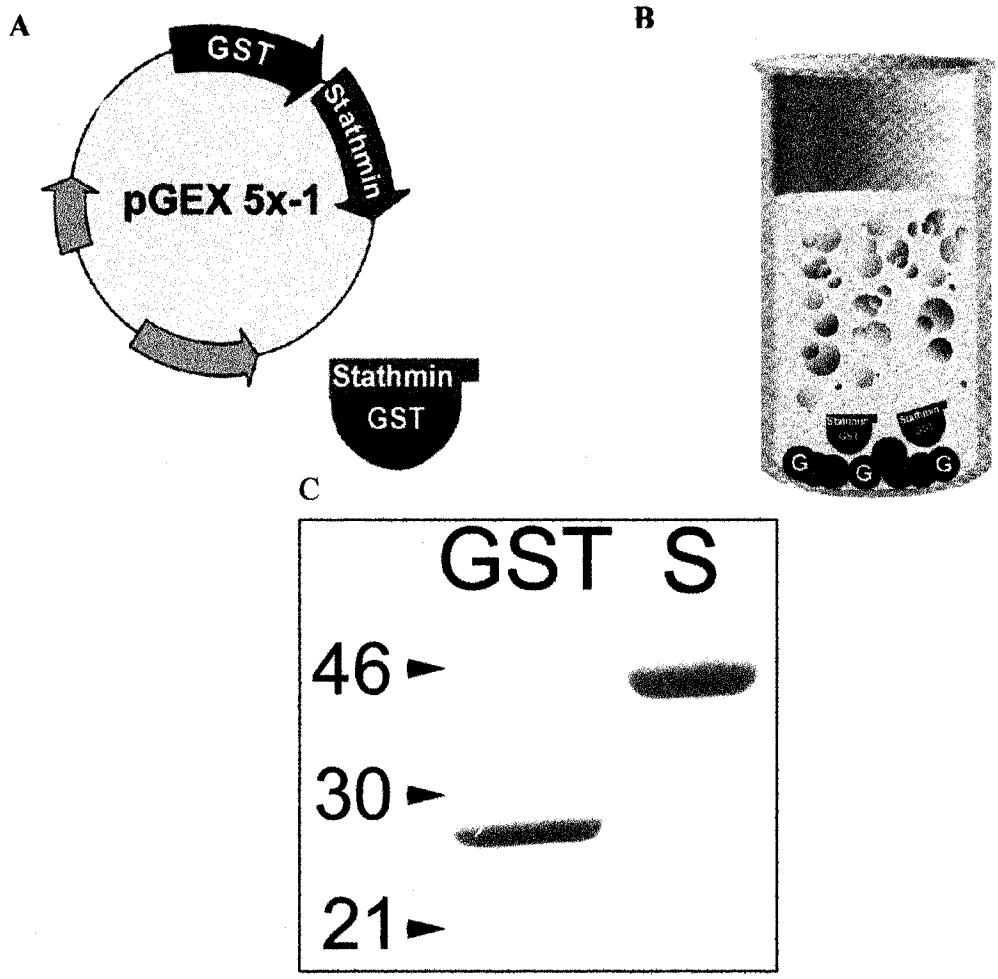

Figure 12: Successful cloning, expression and purification of Stathmin and SCG10 as GST fusion proteins. (A) Stathmin was cloned into vector pGEX5X1 so as to have glutathione-S-transferase (GST) at its N terminal end. Bacterial expression of GST-stathmin (or SCG10) hybrid protein was induced with IPTG. (B) GST-stathmin (or SCG10) was isolated on glutathione coupled agarose (G) beads. (C) Purified GST protein (GST) to be used as a non-specific binding control and GST-coupled stathmin (S) molecular weight 46 kDa seen on a comassie blue stained 12% SDS-PAGE gel. The positions of the molecular weight (in kilodaltons) markers (M), used for comparison are shown on the left. GST-coupled SCG10 was also purified similarly.

The protein yield and concentration were estimated by measuring the absorbance at 280 nm and also by comparison to known quantities of BSA on SDS-PAGE (stained with

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Commassie blue). The protein estimates were used to adjust the relative ratios of the respective proteins to total cell lysate in the GST pull down assay.

GST-Stathmin 'Pull-Down' Assay Identified HSP70 as a Stathmin Binding Protein

The GST-stathmin fusion protein bound several Nb2-cell proteins. Figure 13B shows ^{35}S-labeled proteins isolated on glutathione-Sepharose from cytosol and crude-membrane extracts after addition of no probe (C), GST (T) or GST-stathmin (S) at pH 8.0. Several cytosolic proteins (noted by asterisks on figure 13B) in the 40-92-kDa range were more prominent in the GST-stathmin lane, and thus may represent protein bound to stathmin directly or via another stathmin-binding protein. A prominent 50 kDa-protein preferentially bound by GST-stathmin appeared to be present in both the cytosol and membrane fractions. A protein(s) migrating at approximately 70 kDa appeared to be represented in both cytosol and membrane fractions, but was more prominent in the membrane fraction.

It has been shown (Curmi *et al.*, 1997) that the stathmin-tubulin interaction is stronger at a pH below 7.0 and in the presence of $MgCl_2$. Therefore, binding of stathmin to ^{35}S-labeled proteins extracted from Nb2 cells was examined at pH 6.8 in the presence of 5 mM $MgCl_2$. The pattern of proteins bound by the GST-stathmin probe clearly differed from that obtained at pH 8.0. Several proteins 25-69-kDa in range (noted by asterisks on figure 13B) were present at pH 6.8. A strong protein doublet migrating at approximately 60 kDa was recognized by the GST-stathmin probe in both the cytosol and membrane fractions at pH 6.8. There was no comparable doublet at pH 8.0, though a protein of approximately 60 kDa was identified in experiments performed at pH 8.0.

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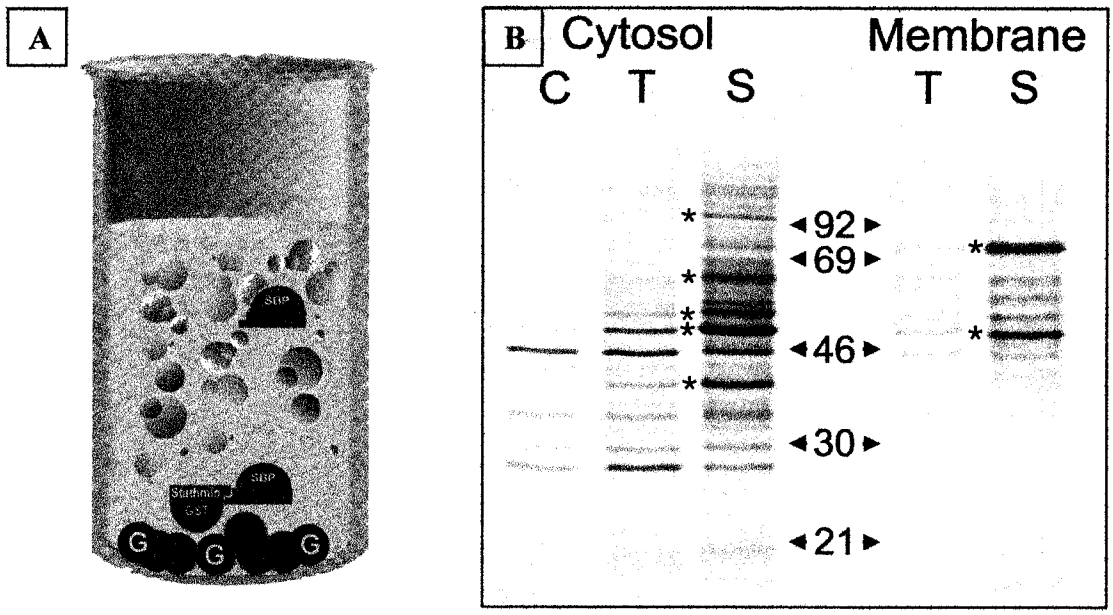

Figure 13: GST-Stathmin "Pull-Down" Assay. (A) GST-stathmin (S) was used to isolate potential binding proteins; Glutathione S-transferase (GST) or Glutathione beads (G) were used as controls for non-specific binding. (B) Potential Stathmin-Binding proteins were isolated from Nb2 cells cytosol or membrane fractions and analyzed by SDS-gel electrophoresis. Asterisks indicated positions of several stathmin-binding proteins that were identified. The positions of molecular weight markers (M) used as reference, are indicated by the arrows with adjacent numbers representing weight in kilodaltons.

The GST-stathmin probe also recognized multiple proteins in the 46-92-kDa range (data not shown) in PC12 cells. Proteins with apparent molecular weight of 46 kDa (found in Nb2 cells) were also identified as GST-stathmin binding protein in PC12 cells, but it is unknown whether the proteins identified in the two cells are related. In PC12 cells, a strong protein doublet migrating with an apparent molecular weight of less than 30 kDa was present in all lanes. This doublet was recognized by anti-GST antibody (data not shown) suggesting that the proteins represent endogenous GST that binds directly to the glutathione-Sepharose beads.

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To determine whether stathmin binds Hsp70, ^{35}S-labeled proteins extracted from Nb2 cells were pulled down at pH 8.0 by GST-Stathmin and probed with anti-Hsp70 antiserum. The antiserum, which recognizes Hsp70 but not Hsc70, identified a protein at 70-kDa (figure 14A) among the proteins pulled down by the GST-stathmin fusion protein (figure 14B). The immunostained protein appeared to match a ^{35}S-labeled protein

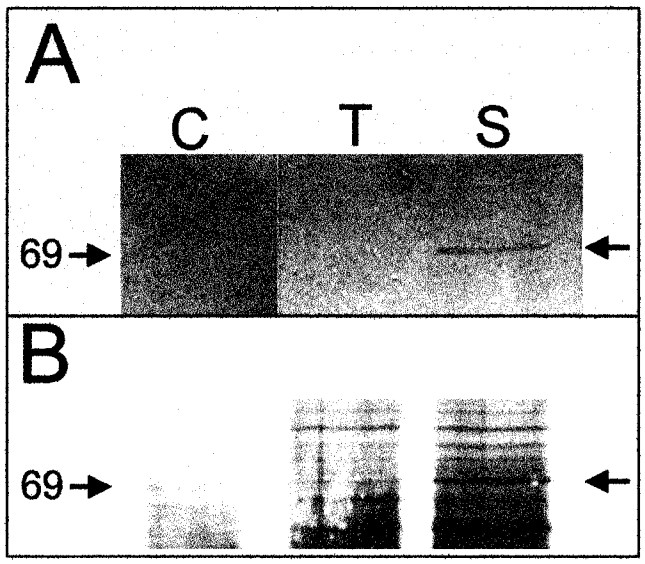

Figure 14: Identification of HSP70 as a Stathmin binding protein. (A) Hsp70 antibody (sc-1060, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 was used to probe the radioactive proteins isolated by affinity chromatography and transferred to nitrocellulose. (B) Radioactive proteins were isolated by affinity chromatography using GST-Stathmin (S) as bait and Glutathion beads (G) and Glutathione S transferase (GST) as controls for non-specific binding. Arrows indicate the position of HSP70 that was identified as a putative stathmin-binding protein. The position of the relevant molecular weight marker (M) used as reference, is indicated by the arrow with adjacent number representing weight in kilodaltons. *"Stathmin binds Hsp70 in the Nb2 lymphoma"*. C.S. King, W. Ko, J.J. Aloor, D.A. Prentice, J.P. Hughes. 112 Proceedings of the Indiana Academy of Science 1-8 (2003)

isolated using the GST-stathmin probe (figure 14B). Thus, the data suggest that stathmin associates with Hsp70 in Nb2 cells.

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Five Novel Stathmin-Binding Proteins Identified by Yeast Two-Hybrid Screening

The Yeast two-hybrid screening for Stathmin binding proteins resulted in over 250 blue colonies (figure 15A) on the dual reporter screening plates. Each of these represented a potential stathmin binding/interacting protein. Initial verification of some of these interactions, were performed via small scale co-transformation. Later, plasmid

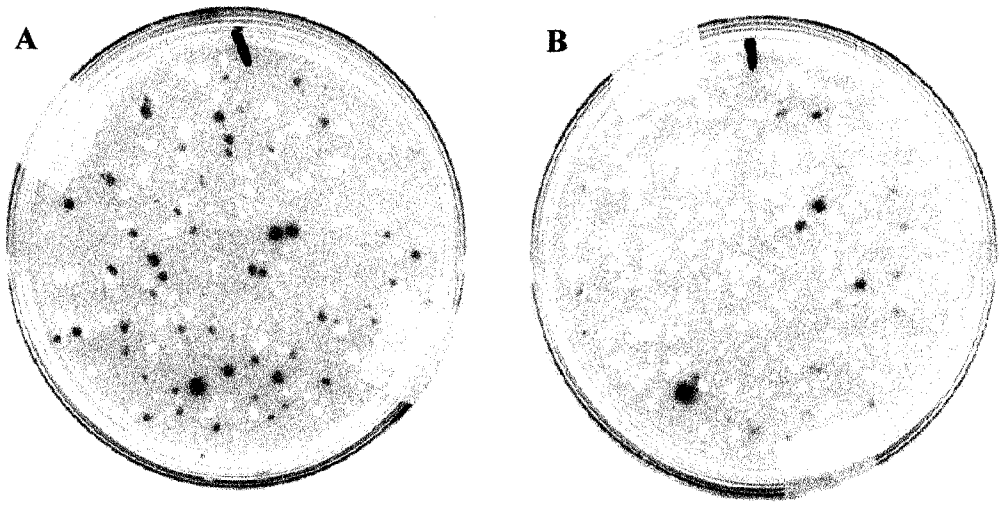

Figure 15: Results of a successful yeast two hybrid screening for Stathmin and SCG10 Binding Proteins. To screen for interacting proteins, glycerol stocks of the cotransformants were plated onto SD/Gal/Raf/-His/-Trp/-Ura/-Leu plates (interaction screening plates) containing X-gal and BU salts and incubated at 30 °C for 4 days. Dual reporter positives are colonies of yeast that can survive on medium lacking Leucine (Leu+) and turn blue in color (LacZ+) on medium containing X-gal. (A) Stathmin + library proteins yielded ~ 250 dual reporter positive/blue colonies and (B) SCG10 + library proteins yielded ~ 200 dual reporter positive/blue colonies.

pools derived from the interaction-positive colonies were transformed into the KC8 strain of E. *coli* to force segregation of individual plasmids containing cDNAs coding for putative stathmin/SCG10-binding proteins. Four colonies from plates representing individual plasmid pools were analysed by restriction enzyme digest and unique cDNAs

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(based on restriction digest pattern) were used to transform yeast with mating type opposite to those containing the bait or test (positive or negative control) vectors. Yeast mating followed by plating on reporter assay plates was then used to verify that the previously observed interaction was a result of that particular cDNA and represented a specific stathmin-interaction. Survival and growth on selective plates and blue coloration on the same plate containing X-gal and 5BU salts are independent tests of an interaction. Interactions could be detected between 24 and 60 hours depending on strength of the interaction. Between 60 – 72 hours light blue color and sluggish growth was often seen in many quadrants/non-interactors. The cDNAs that interacted with stathmin or SCG10 were assigned temporary names and numbers such as Stathmin Binding Protein 2.2 (SBP2.2) and SCG10 Binding Protein 4.1 (S10BP4.1) respectively to indicate binding in the initial small-scale co-transformation screen and to classify them by the plasmid pool they were derived from. The stathmin binding proteins thus identified were as follows. *Secretogranin III.* On the reporter plate, yeast containing cDNA SBP2.2 yielded dark blue colonies/smear (Figure 16) when mated with yeast containing LexABD-stathmin. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA SBP2.2 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-SCG10. These data suggest that the protein coded by SBP2.2 binds to stathmin but not to SCG10, the laminin control protein, or the lexA binding domain. Binding to stathmin but not to SCG10 suggests that the protein coded by SBP2.2 recognizes a specific site(s) not shared by all members of the stathmin family Analysis of the nucleic acid sequence of this Stathmin binding protein showed that it was 100% identical to Secretogranin III (figure 17A) over the 134 base pairs (bp) sequenced.

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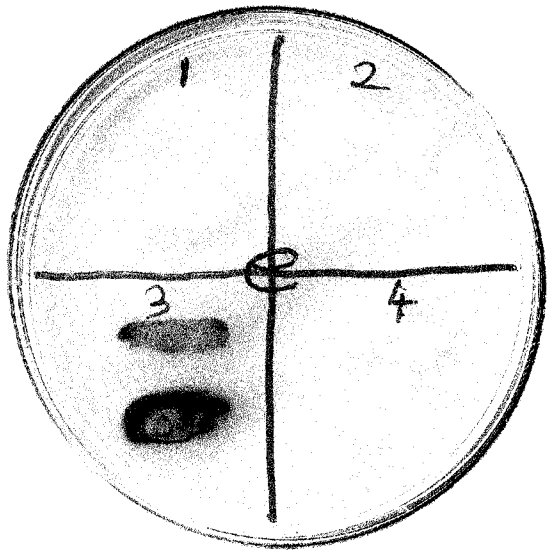

Figure 16: Secretogranin III is a Stathmin binding protein. The reporter plate (refer to figure 10) showed sluggish growth of white yeast clonies/smears in the quadrants 1, 2 and 4, indicating a lack of interaction based on dual reporter criteria. However in quadrant 3, where the mating of cDNA SBP2.2 with LexABD-Stathmin was plated, dark blue yeast colonies/smear could be seen. This indicates a strong specific interaction of stathmin with the protein product of this cDNA; which was found by BLAST search to be Secretogranin III.

All currently known stathmin binding proteins have a coiled-coil domain. The presence of this domain in an amino acid sequence can be predicted by various programs. One such program named COILS (Lupas et. al., 1991) is widely in use and theoretical predictions by this program with a p-value > 0.7 have proven fairly accurate based on experimental observations. We therefore subjected the amino acid sequence of Secretogranin III to this program. The results (figure 18C) showed that four portions of the protein had a distinct (> 0.75) probability of forming a coiled-coil. This indicates that the novel stathmin-binding-protein Secretogranin III follows the conformational characteristics seen in other stathmin-binding proteins.

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A. SBP2.2:
>gi|18265965|gb|AF453583.1| Homo sapiens **secretogranin III** mRNA, complete cds Length = 3371 Score = 262 bits (132), Expect = 1e-67 **Identities = 132/132 (100%)** Strand = Plus / Plus

B.SBP3.1:
>gi|5453604|ref|NM_006430.1| **Homo sapiens chaperonin containing TCP1, subunit 4 (delta) (CCT4)**,mRNA Length = 1883 Score = 168 bits (85), Expect = 1e-39 **Identities = 105/109 (96%)**, Gaps = 2/109 (1%) Strand = Plus / Plus

C.SBP4.1:
>gi|22749106|ref|NM_152530.1| Homo sapiens **hypothetical protein FLJ31331** (FLJ31331), mRNA Length = 1693 Score = 204 bits (103), Expect = 5e-50 **Identities = 181/206 (87%)**, Gaps = 1/206 (0%) Strand = Plus / Plus

D.SBP3.2:
gi|17064186|gb|AC018891.9| Homo sapiens **BAC clone RP11-498M11** from 2, complete sequence Length = 174074 Score = 886 bits (447), Expect = 0.0 **Identities = 463/471 (98%)** Strand = Plus / Plus

E.S10BP2.1:
Homo sapiens **cDNA FLJ14557** fis, clone NT2RM2001896, **weakly similar to CELL DIVISION PROTEIN FTSJ.** Expect= 0.0 **Identities= 484/487 (99%),** Gap= 2/487(0%) Strand = Plus/Plus OR **Homo sapiens cytochrome c oxidase subunit II gene**, mitochondrial gene encoding mitochondrial protein, partial cds.

F.S10BP4.1:
>gi|14017398|gb|AY029066.1| Homo sapiens **Humanin (HN1)** mRNA, complete cds Length = 1567 Score = 385 bits (194), Expect = e-104 **Identities = 194/194 100%)** Strand = Plus / Plus

G.S10BP3.2:
gi|7022893|dbj|AK001564.1| Homo sapiens cDNA FLJ10702 fis, clone NT2RP3000759, weakly similar **to ADP-RIBOSYLATION FACTOR** Length = 2944Score = 470 bits (237), Expect = e-130 **Identities = 237/237 (100%)** Strand = Plus / Plus

H.S10BP4.2:
gi|19310323|gb|AC108021.3| Homo sapiens BAC clone RP11-8N8 from 4, complete sequence Length = 185972 Score = 1043 bits (526), Expect = 0.0 **Identities = 532/534 (99%)** Strand = Plus / Minus

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I.S10BP1.2:

gi|5453546|ref|NM_006334.1| Homo sapiens olfactomedin 1 (OLFM1), transcript variant 2, mRNA. Length = 1009 Score = 470 bits (237), Expect = e-130 **Identities = 243/245 (99%)** Strand = Plus / Plus

J. SBP1.2:

gi|16549289|dbj|AK054693.1| Homo sapiens cDNA FLJ30131 fis,... 515 e-143
gi|21362049|ref|NM_032357.2| Homo sapiens h. protein MGC12981... 515 e-143
Length = 1644 Score = 515 bits (260), Expect = e-143 **Identities = 293/304 (96%),** Gaps = 2/304 (0%) Strand = Plus / Plus

Figure 17: BLAST search results. DNA sequence of some of the newly identified binding partners subjected to the 'BLAST' program, reveal known and hypothetical proteins as novel stathmin/SCG10 binding partners.

Chaperonin Containing TCP1 Subunit 4. On the reporter plate, yeast containing cDNA SBP3.1 yielded dark blue colonies/smear when mated with yeast containing LexABD-stathmin. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA SBP3.1 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-SCG10. These data suggest that the protein coded by SBP3.1 binds to stathmin but not to SCG10, the laminin control protein, or the lexA binding domain. Binding to stathmin but not to SCG10 suggests that the protein coded by SBP3.1 recognizes a specific site(s) not shared by all members of the stathmin family.

Analysis of the nucleic acid sequence of this Stathmin binding protein showed that it was 96% identical to Chaperonin containing TCP1 subunit 4 (figure 17B) over the 105bp sequenced. This protein is one that is required for cyclin E maturation.

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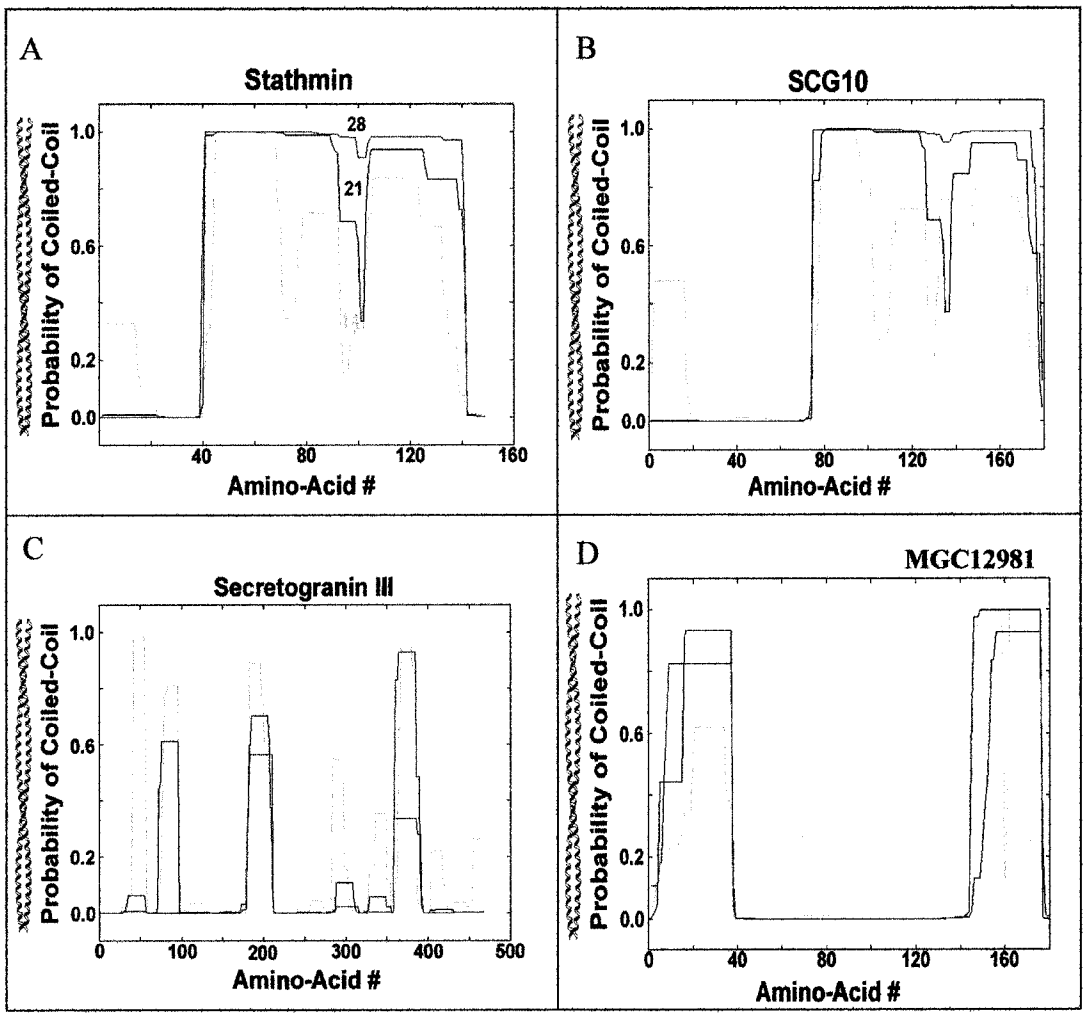

Figure 18: Prediction of coiled-coil regions in Stathmin, SCG10 and some of the newly identified binding partners. Coils prediction using "COILS" a program by Lupas et. al. (1991, Science, 252: 1162-1164) was utilized on stathmin, SCG10 and their putative binding proteins. Figures 18 A-D, shows a sampling of the results indicating that the coiled-coil is a common motif (though not the only one) for stathmin family of proteins to interact with their binding partners.

Hypothetical Protein FLJ31331. On the reporter plate, yeast containing cDNA SBP4.1 yielded dark blue colonies/smear when mated with yeast containing LexABD-stathmin (data not shown). Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA SBP4.1 was mated with yeast containing LexABD,

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LexABD-Lamin and LexABD-SCG10. These data suggest that the protein coded by SBP4.1 binds to stathmin but not to SCG10, the laminin control protein, or the lexA binding domain. Binding to stathmin but not to SCG10 suggests that the protein coded by SBP4.1 recognizes a specific site(s) not shared by all members of the stathmin family.

Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was weakly similar (87% identical) to hypothetical protein FLJ31331 (figure 17C) over the 181bp sequenced.

RP11-498M11 BAC Clone. On the reporter plate, yeast containing cDNA SBP3.2 yielded dark blue colonies/smear when mated with yeast containing LexABD-stathmin. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA SBP3.2 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-SCG10. These data suggest that the protein coded by SBP3.2 binds to stathmin but not to SCG10, the laminin control protein, or the lexA binding domain. Binding to stathmin but not to SCG10 suggests that the protein coded by SBP3.2 recognizes a specific site(s) not shared by all members of the stathmin family.

Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was 98% identical (figure 17D) to Homo sapiens BAC clone RP11-498M11 over the 463bp sequenced. This is likely to be a previously unidentified protein since the library it was derived from is cDNA based and should have no genomic DNA contamination. Though unlikely, it is possible that this indeed is a genomic, non-coding DNA contamination in the cDNA library that is expressed and translated when fused to the pB42AD.

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Hypothetical Protein FLJ14557. On the reporter plate, yeast containing cDNA S10BP2.1 yielded dark blue colonies/smear when mated with yeast containing LexABD-stathmin. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA S10BP2.1 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-SCG10. These data suggest that the protein coded by S10BP2.1 binds to stathmin but not to SCG10, the laminin control protein, or the lexA binding domain. Binding to stathmin but not to SCG10 suggests that the protein coded by S10BP2.1 recognizes a specific site(s) not shared by all members of the stathmin family. This was somewhat surprising since this cDNA was derived from a pool of plasmid that came from colonies selected based on the interaction of their cDNA's protein products with SCG10 in the initial co-transformation screen. The logical explanation for this is that the cDNA encoding this stathmin binding protein was one that was concurrently resident with a cDNA whose protein product was a SCG10 binding protein. The fortuitous discovery of this stathmin binding protein can thus be ascribed to our screening methodology, which tested each of the putative binding proteins for interactions with both stathmin and SCG10.

Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was 99% identical over the 484bp sequenced (figure 17E), to protein encoded by the cDNA FLJ1457 fis. This is a recently discovered protein that is weakly similar to cell division protein FTSJ and mitochondrial cytochrome c oxidase subunit II.

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Three Novel SCG10 Binding Proteins Identified by Yeast Two Hybrid Screening

The Yeast two-hybrid screening for SCG10 binding proteins resulted in over 200 blue colonies (figure 15B) on the dual reporter screening plates. Each of these represented a putative SCG10 binding/interacting protein. Initial verification of some of these interactions, were performed via small scale co-transformation. Later plasmid pools derived from the interaction-positive colonies were transformed into KC8 strain of E.coli to force segregation of different cDNAs. Four colonies from plates representing individual plasmid pools were analysed by restriction enzyme digest and unique cDNAs (based on restriction digest pattern) were used to transform yeast of the opposite mating type to those containing the bait or test (positive or negative control) vectors. Yeast mating followed by plating on reporter assay plates were then used to verify whether the previously seen interaction was a result of that particular cDNA or another concurrently resident cDNA. The SCG10 binding proteins thus identified are as follows.

Humanin. On the reporter plate, yeast containing cDNA S10BP4.1 yielded blue colonies/smear (figure 19) when mated with yeast containing LexABD-SCG10. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA S10BP4.1 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-stathmin. These data suggest that the protein coded by S10BP4.1 binds to SCG10 but not to stathmin, the laminin control protein, or the lexA binding domain. Binding to SCG10 but not to stathmin suggests that the protein coded by S10BP4.1 recognizes a specific site(s) not shared by all members of the stathmin family.

Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 100% identical (figure 17F) to Humanin over the 194bp sequenced.

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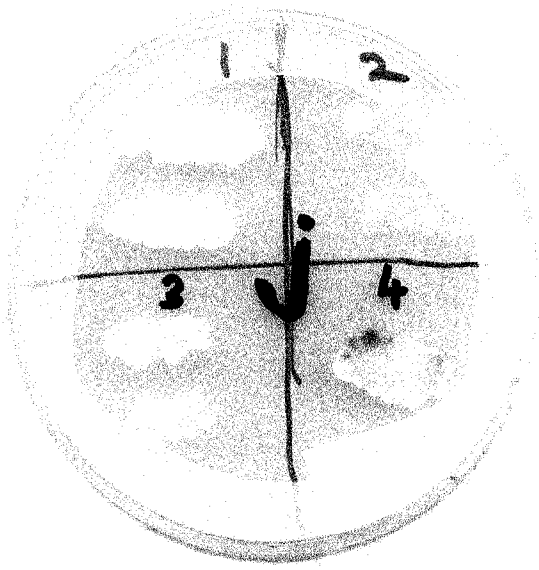

Figure 19: Humanin is a SCG10 binding protein. The reporter plate (refer to figure 10) showed sluggish growth of white yeast clonies/smears in the quadrants 1, 2 and 3, indicating a lack of interaction based on dual reporter criteria. However in quadrant 4, where the mating of cDNA S10BP4.1 with LexABD-SCG10 was plated, blue yeast colonies/smear could be seen. This indicates a strong specific interaction of SCG10 with the protein product of this cDNA; which was found by BLAST search to be Humanin.

Hypothetical Protein FLJ10702. On the reporter plate, yeast containing cDNA S10BP3.2 yielded dark blue colonies/smear when mated with yeast containing LexABD-SCG10. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA S10BP3.2 was mated with yeast containing LexABD, LexABD-Lamin and LexABD-stathmin. These data suggest that the protein coded by S10BP3.2 binds to SCG10 but not to stathmin, the laminin control protein, or the lexA binding domain. Binding to SCG10 but not to stathmin suggests that the protein coded by S10BP3.2 recognizes a specific site(s) not shared by all members of the stathmin family.

Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 100% identical over the 237bp sequenced (figure 17G) to the protein encoded by

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the cDNA FLJ10702 fis. This is a hypothetical protein similar to ADP ribosylation factor and hence is called ADP ribosylation factor-like 10C. It is of great interest to us because since our discovery of this protein as a SCG10 binding protein it has been shown by Okai et al. (Okai et al., 2004) to be an expressed protein belonging to a novel small GTPase subfamily capable of associating with tubulin and required for chromosome segregation. Okai et al have therefore renamed it- novel small G-protein indispensable for equal chromosome segregation 1 (GIE1).

RP11-8N8. On the reporter plate, yeast containing cDNA S10BP4.2 yielded dark blue colonies/smear when mated with yeast containing LexABD-SCG10 and light blue colonies with LexABD-stathmin. Only sluggish growth of white yeast clonies/smears was observed when yeast containing cDNA S10BP4.2 was mated with yeast containing LexABD and LexABD-Lamin. These data suggest that the protein coded by S10BP4.2 binds strongly to SCG10, weakly to stathmin but not to the laminin control protein, or the lexA binding domain. Binding to SCG10 and weakly to stathmin suggests that the protein coded by S10BP4.2 recognizes site(s) shared by both these members of the stathmin family.

Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 99% identical in plus/minus orientation (over the 532bp sequenced) to BAC clone RP11-8N8 (figure 17H). This is likely to be a previously unidentified protein since the library it was derived from is cDNA based and should have no genomic DNA contamination. However it is also (remotely) possible that this indeed is a genomic, non-coding DNA contamination in the cDNA library that is expressed and translated when fused to the pB42AD.

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Additional Interesting Clones

Hypothetical Protein MGC12981. The reporter plate showed strong growth of dark blue yeast clonies/smears (figure 20) in the quadrants in which this cDNA (SBP1.2) in yeast was mated with yeast containing LexABD, LexABD-Lamin, LexABD-SCG10 and LexABD-stathmin. This indicates a strong non-specific interaction or independent action on the promoters of both reporter genes. This cDNA fusion with the activation domain interacts with the binding domain by itself or with it fused to Lamin-protein (Lamin fusion) or with stathmin fusion or with SCG10 fusion protein. The interaction of the cDNAs protein product with the LexA binding domain by itself or with its fusions with Lamin/SCG10/stathmin shows that the protein encoded by this cDNA non-specifically binds all proteins or is/has a DNA binding domain. Analysis of the nucleic acid sequence of this protein showed that it was 96% identical to the hypothetical protein MGC12981 (figure 17J) over the 293bp sequenced. Analysis of the amino acid sequence of this protein using the program COILS showed that both the N-terminal and C-terminal of the protein had stretches of 28 amino acids that had a distinct (> 0.75) probability of forming coiled-coils (figure 18D). This protein has no experimentally proven function. The non specificity of hypothetical protein MGC12981's interaction in the screen, made it not worth pursuing as a stathmin/SCG10 binding protein. However two observations caused us to retain and further study this clone, namely (1) given that it is capable of activating both reporters in the assay and was derived from a brain cDNA library it is a putative brain transcription factor (2) transfection of this cDNA into yeast greatly increased the yeast mating efficiency (figure 21). When BD-fusions (eg. pLexA-Lamin/stathmin) were transformed into YM4271, grown and re-plated on induction plates, contatining X-gal

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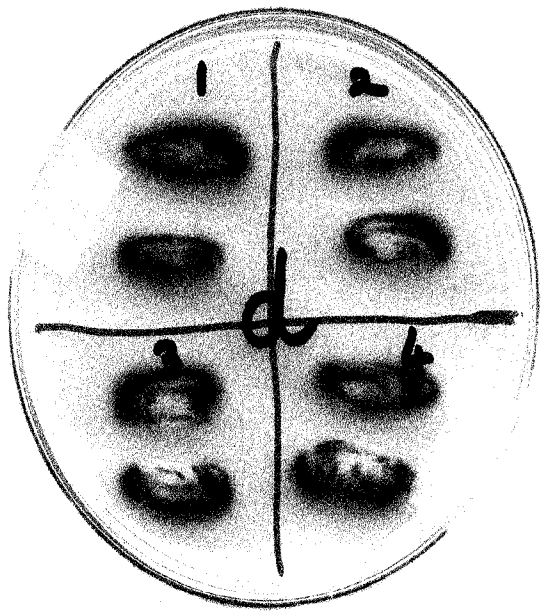

Figure 20: Hypothetical protein MGC12981 shows binding to stathmin, SCG10 and the negative control proteins. The reporter plate (refer to figure 10) showed blue clonies/smears in all four quadrants. This indicates a non specific interaction. A BLAST search using the sequence showed that the cDNA encoded a newly discovered hypothetical protein MGC12981. The interaction of the cDNAs protein product with the LexA binding domain by itself or with its fusions with Lamin/SCG10/Stathmin shows that the protein encoded by this cDNA non-specifically binds all proteins or is/has a DNA binding domain.

and 5BU salts the yeast died between 48 and 72 hours. Similarly when the AD-fusion (eg. pB42AD-T i.e. T-antigen) was transformed into EGY48p8OPlacZ, grown and re-plated on induction plates containing X-gal & 5BU salts, the yeast died within 48 hours. However when EGY48p8OPlacZ yeast were transformed with the activation domain (AD) fused with this cDNA by itself, good growth and blue colonies/smear were seen on the selection plates containing X-gal & 5BU salts (figure 22). This shows that this cDNA is capable of performing the function of a DNA binding domain (BD) and hence is likely to be a transcription factor.

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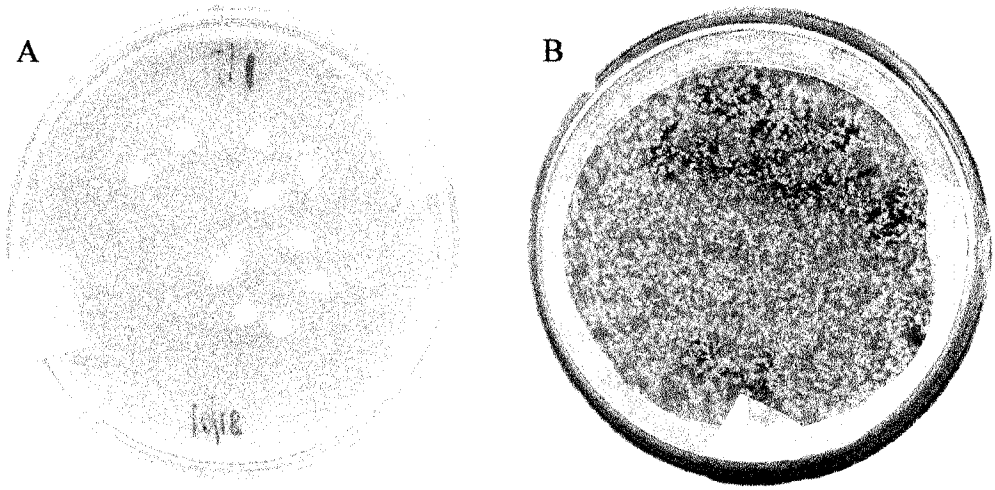

Figure 21: Hypothetical protein MGC12981 enhances yeast mating efficiency. (A) When pB42AD-T containing yeast of type alpha are mated with yeast of the opposite mating i.e. type-a containing pLexABD plasmid and plated on dual selection plates (refer to figure 10) the number of colonies obtained is small. (B) However when pB42AD-T plasmid is substituted by pB42AD- MGC12981 plasmid, the plating shows a huge increase in mating efficiency.

Thyroid Hormone Receptor Interactor 3. On the reporter plate, yeast containing this cDNA yielded dark blue colonies/smear (figure 23) when mated with yeast containing LexABD-stathmin and LexABD-Lamin. Relatively lighter blue yeast colonies/smears were seen in the quadrant where the mating with LexABD was plated. Only sluggish growth of white yeast clonies/smears was observed when yeast containing this cDNA was mated with yeast containing LexABD-SCG10. These data suggest that the protein coded by this cDNA binds strongly to stathmin, laminin and weakly to the lexA binding domain. However it does not bind to SCG10 a close neural specific member of the stathmin family. The lack of interaction with the LexABD-SCG10 shows that the protein product of this cDNA is not non- specifically binding all other proteins. Nucleic acid

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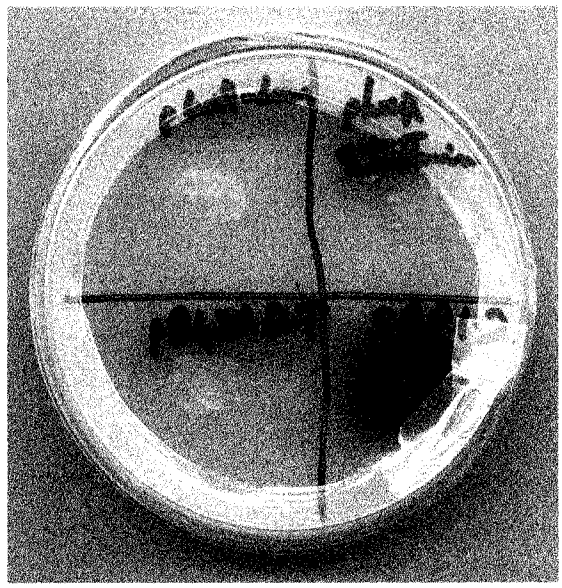

Figure 22: Hypothetical protein MGC12981 shows DNA binding ability. When pLexABD, pLexA-stathmin or pB42AD-T are transformed into yeast containing the reporter plasmid and plated on selection plates containing X-gal and 5BU, they are unable to grow or produce blue colonies. This is because of their inability to bind the reporter gene and activate transcription. However when yeast containing the reporter plasmid were transformed with the activation domain (AD) fused with MGC12981 cDNA, good growth and blue colonies/smear were seen on the selection plates containing X-gal & 5BU salts. Since this growth and reporter activation is despite the absence of the pLexA binding domain it shows that MGC12981 protein is capable of performing the function of a DNA binding domain (BD) and hence is likely to be a transcription factor.

sequence analysis of this stathmin binding protein showed that it was identical to Thyroid Hormone Receptor Interactor 3 (THRI3) a known transcription factor. Given this fact and results showing the interaction with the stathmin fusion, Lamin fusion and to a lesser extent LexABD, there are two possibilities: (1) That THRI3 does not interact with stathmin or Lamin per se but interacts with the DNA Binding Domain (BD) of LexA as seen in three mating screens (LexABD, LexABD-stathmin and LexABD-Lamin) and the reason for its lack of interaction with LexABD-SCG10 is a conformation change induced

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in the LexABD by that particular fusion. (2) That THRI3 does interact specifically with stathmin and Lamin but not with SCG10 and the interaction with LexABD is a nonspecific, lower strength one (since the colonies/smear is a lighter blue in color) brought about by its being a non full-length/ partial cDNA i.e. representing only certain domains of the complete protein.

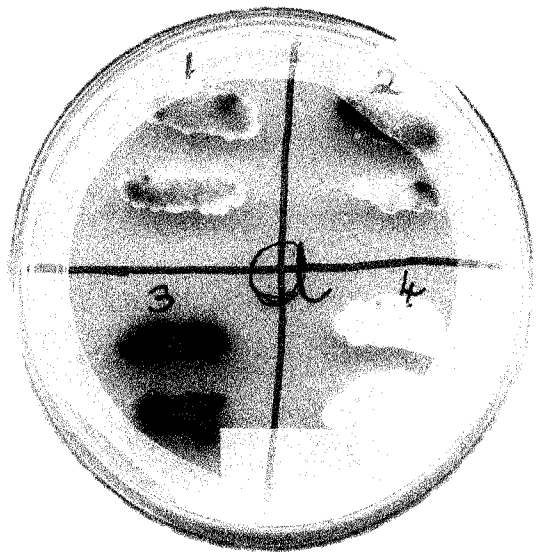

Figure 23: Thyroid hormone receptor interactor 3 is a transcription factor that shows non specific binding to stathmin. The reporter plate (refer to figure 10) showed pale blue to blue clonies/smears in the quadrants 1, 2, 4. This indicates a interaction based on the dual reporter criteria. In quadrant 3, where the mating with LexABD-stathmin was plated, dark blue yeast colonies/smears could be seen and this indicates a strong interaction of stathmin with the protein product of this cDNA. A BLAST search using the sequence showed that the cDNA encodes a transcription factor named Thyroid hormone receptor interactor 3. However since the protein product of this cDNA interacts with the negative controls (i.e. controls LexABD and LexAB-Lamin for non specific interactions) it was not further studied.

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Example of a False Positive/Negative Result

Olfactomedin. During the initial co-transformation experiments to identify both stathmin and SCG10 binding proteins plasmid pools were isolated from blue colonies that grew on the selection plates. Of these one of the plasmids selected as a putative SCG10 binding protein for further verification (using the criteria adapted to prevent duplications and test putative new and interesting interactions) was one that had a cDNA insert that showed 99% identity (figure 17I) over the 243bp sequenced, to the gene encoding the protein Olfactomedin. Mating of yeast bearing this cDNA (as a fusion of the pBB42AD construct) to yeast bearing the pLexA or its fusions with Laminin, stathmin, or SCG10 and performing the dual reporter screen showed no positive result. Although growth was seen in all four quadrants, the yeast colonies/smears were not blue (figure 24). This is because only one of the two reporters of the assay (growth on –Leu) is activated while the lacZ reporter is not activated and this is indicative of a false positive result.

This protein initially was very interesting because a database search revealed that it localized to the golgi, and showed neural-tissue-specific expression (similar to SCG10). Also the protein sequence analysis revealed the possibility (>75%) of a coiled-coil domain (data not shown); a previously noted characteristic of binding proteins of the stathmin family.

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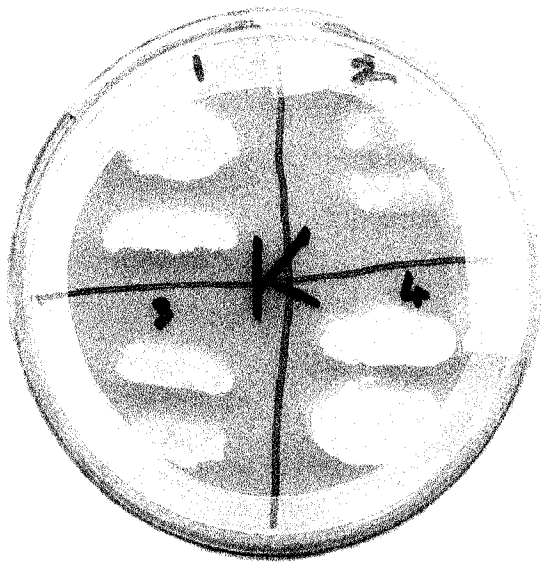

Figure 24: Olfactomedin shows no binding to stathmin or SCG10. The reporter plate (refer to figure 10) showed white clonies/smears in all four quadrants. A BLAST search using the sequence showed that the cDNA encoded Olfactomedin, a protein with numerous characteristics that made it a likely candidate for a stathmin or SCG10 binding protein. Since the protein product of this cDNA does not show an interaction with stathmin or SCG10, using the dual reporter criteria; it was postulated that its initial isolation as a SCG10 interacting protein was a false positive and need not be pursued.

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DISCUSSION

Prolactin Increases the Phosphorylation of p38α in Nb2 Cells

Four isoforms of p38, p38α/CSBP/SAPK2α, p38β/SAPK2β, p38γ/SAPK3/ERK6, and p38δ/SAPK4, have been identified to date. SB203580 inhibits p38α and p38β through competition with ATP for the same binding siteon p38 kinase (Wilson et al.,1997) (Young et al.,1997). All p38 isoforms are known to play important roles in cellular responses to environmental stresses and proinflammatory cytokines (Lee et al.,1994) (Ono and Hahn 2000). In addition to such roles as stress-activated kinases, p38 is believed to take part in mitogenic signal transduction, cell fate specification and control of cellular function in various types of cells.

Our results indicate that during the prolactin-stimulated proliferation of Nb2 cells there is a substantial increase in phosphorylated p38α although the total p38 protein levels remain unchanged for up to 12 hours. Neither p38β nor p38γ are detectable under these conditions. It would appear, therefore, that 38α is playing the primary role. However, a role for p38δ could not be excluded due to lack of an appropriate antibody.

After our studies were completed Parker et al. (1998) reported that p38δ has significantly higher stathmin phosphorylating ability than other p38 isoforms. Stathmin was phosphorylated at serine residues 25 and 38 by p38δ, in in-vitro as well as cellular assays. Studies by Meyer et al. (1992) and others have shown that phosphorylation of

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stathmin correlates with cell growth and is increased substantially by the hormone prolactin as well as by 12-O-tetradecanoylphorbol-13-acetate (TPA). Nemeth et al. (Nemeth et al., 1998) have shown that p38 mRNA levels increase 2.5 fold in response to the prolactin stimulation of Nb2 cells for four hours; but the investigators observed no change in protein levels. Also work in our laboratory (Park et al., 2006 Manuscript in preparation) has shown that in response to prolactin stimulation, the total amount of p38 MAP kinase protein in Nb2 cells does not change substantially, though levels of the phosphorylated form of p38 (which is an index of its activation) are increased. SB203580 is a selective inhibitor of p38 mitogen-activated protein (MAP) kinase (alpha and beta isoforms), which acts by competitively inhibiting ATP binding. Work in our laboratory (Park et al., 2006 Manuscript in preparation) has shown that SB203580 suppressed PRL-induced proliferation of Nb2 cells in a dose dependent manner (57% decrease in cell number at 40μM concentration). Also SB203580 does not inhibit p38γ or p38δ (Lechner et al., 1996) (Goedert et al., 1997).

The above facts taken together with our results indicate that the prolactin stimulated proliferation of Nb2 cells involves p38α phosphorylation and perhaps subsequent stathmin phosphorylation. More experiments will be required, however, to establish whether phosphorylated p38α plays a role in stathmin phosphorylation.

Stathmin Appears to Bind Hsp70

Stathmin is believed to interact with several cellular proteins, including the putative kinase KIS (Maucuer *et al.*, 1995), product of the CC1 gene fragment (Maucuer *et al.*, 1995), Tsg101/CC2 (Li and Cohen, 1996) (Maucuer *et al.*, 1995), tubulin (Belmont

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and Mitchison, 1996) (Curmi *et al.*, 1997) (Gradin *et al.*, 1998) (Horwitz *et al.*, 1997) (Jourdain *et al.*, 1997) (Larsson *et al.*, 1999) (Redeker et al., 2000) and members of the Hsp70 family (Manceau *et al.*, 1999) (Maucuer *et al.*, 1995). Although only two Hsp70-related proteins, BiP/Grp78 (Maucuer *et al.*, 1995) and Hsc70 (Manceau *et al.*, 1999), have been identified as stathmin-binding proteins, it seemed possible that other members of the Hsp70 family may also associate with stathmin.

The stathmin-GST fusion protein, which consists primarily of unphosphorylated stathmin, isolated a protein that reacts with an antibody specific for Hsp70. Hence, unphosphorylated stathmin appears to bind Hsp70. Moreover, Hsp70 would be expected to be elevated in proliferating Nb2 cells, because Hsp70 expression in Nb2 cells is induced by PRL and other mitogens (deToledo *et al*, 1987). Induction of Hsp70 appears to be closely associated with Nb2-cell proliferation and occurs at a time when stathmin phosphorylation increases, but the exact role of Hsp70 is unclear. It is interesting that PRL and phorbol esters induce Hsp70 and stimulate phosphorylation of stathmin in Nb2 cells, because Manceau and colleagues (Manceau *et al.,* 1999) have suggested that phosphorylated forms of stathmin have reduced affinity for Hsp70-like proteins. Perhaps stathmin sequesters Hsp70, and stathmin phosphorylation releases Hsp70 so it can participate in mitogenesis or the response to heat shock. This type of regulation would be consistent with the stathmin-tubulin interaction where phosphorylation of stathmin decreases its ability to sequester tubulin (Curmi *et al.*, 1997) (Gradin *et al.*, 1998) (Horwitz *et al.*, 1997) (Jourdain *et al.*, 1997). If unphosphorylated stathmin does sequester Hsp70, then phosphorylation of stathmin might be an important consequence of heat shock as has been shown in HeLa cells (Beretta *et al.*, 1995). In any case, these data

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suggest, for the first time, that stathmin binds Hsp70. It is therefore possible, that stathmin-Hsp70 interaction participates in signaling in Nb2 cells.

Stathmin also associated with ^{35}S-labeled proteins with molecular weights (55 kDa) approximating those of α and β tubulin. A ^{35}S-labeled protein doublet that might represent α and β tubulin was observed when binding to the stathmin fusion protein was performed at pH 6.8, but not when binding was performed at pH 8.0. Similarly, the anti-tubulin antiserum recognized a doublet only when binding to stathmin was performed at pH 6.8 in the presence of $MgCl_2$, consistent with earlier studies (Curmi *et al.*, 1997). Better binding at a lower pH (i.e., 6.8) has not been shown for other stathmin-binding proteins. In fact, experiments in this study suggest that stathmin may preferentially bind to a different set of proteins at higher pH (i.e., 8.0 vs. 6.8). The dependence on pH could reflect many factors, including inherent differences among proteins, requirements for cofactors, or protein modifications such as phosphorylation. The weakness of the ^{35}S-labeled bands and the tubulin immunostaining may reflect the low affinity of the stathmin-tubulin interaction (Curmi *et al.*, 1997) combined with the extensive washing employed in these studies.

Several other putative stathmin-binding proteins were isolated using the GST-stathmin fusion protein to probe Nb2- and PC12-cell extracts. Additional studies will be required to establish the identities of these proteins and their relationships to previously identified stathmin-binding proteins. The two prominent PC12 proteins migrating just below 30 kDa probably represent two or more isozymes of GST, because the proteins were isolated using glutathione-Sepharose and were recognized by an anti-GST antibody.

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The PC12 GST, which may have been induced by mitogens in the serum (Nur *et al.*, 2000), approximated the amount of GST/GST-stathmin used to probe the extracts.

Overall, the results show that stathmin binds Hsp70 as well as BiP/Grp78 (Maucuer *et al.*, 1995) and Hsc70 (Manceau *et al.*, 1999). Thus, some of the growth-promoting and developmental actions linked to stathmin may involve interaction with Hsp70. Furthermore, the GST-stathmin fusion protein, which proved to be a good probe for stathmin-binding proteins, identified additional potential binding proteins. It seems likely that stathmin associates with multiple binding partners; consistent with the proposal that stathmin acts as an integrative, relay protein (Sobel, 1991).

Stathmin Binds Secretogranin III

Ottinger HP et al. discovered Secretogranin III mRNA and found that it was specifically expressed in neuroendocrine cells of rat (Ottiger et al., 1990). Human secretogranin III (SgIII) cDNA was identified and cloned by Rong et al. (Rong et al., 2002). It encodes a protein of 468 amino acid residues. The human SgIII protein possesses an N-terminal signal peptide, seven dibasic sites, and the repeated DSTK sequences. These structural characteristics are similar to other members of secretogranin family. Human SgIII has homologous proteins in mouse, rat, and in Xenopus laevis. Subcelluar localization and immunoblotting indicate that SgIII is secreted out of the cell through trans-Golgi network (TGN). SgIII may play a role in the biogenesis of secretory granules as a helper protein and be involved in the production or release of peptide hormones in the regulated secretory pathway. Hosaka M et al. (Hosaka et al., 2002) used a yeast two hybrid screen to identify binding partners for chromogranin-A, and found that

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it bound Secretogranin III. They went on to show that this interaction was specific both in vitro and in endocrine cells. Domain deletion mutants of chromogranin-A, defective in binding secretogranin III caused mis-sorting of chromogranin-A to the constitutive pathway, whereas similar mutants of secretogranin III sorted normally. They therefore suggested that chromogranin-A localizes with Secretogranin III by specific binding in secretory granules, in SgIII-expressing pituitary and pancreatic endocrine cells. They suggest that other mechanisms are likely to be responsible for chromogranin-A localization, in secretory granules of secretogranin III-lacking adrenal chromaffin cells and PC12 cells. Hosaka M et al. went on to show that Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A (Hosaka et al., 2004).

Our yeast two hybrid screen for Stathmin binding proteins showed that stathmin specifically bound the cDNA SBP2.2. Analysis of this cDNA showed that it was 100% identical to Secretogranin III over the 134 bp sequenced. The amino acid sequence of Secretogranin III on analysis with the program COILS (Lupas, 1991) showed that four portions of the protein had a distinct (> 0.75) probability of forming a coiled-coil. This indicates that Secretogranin III is possibly a novel stathmin binding protein that conforms to the conformational characteristics seen in previously characterized stathmin binding proteins. Given the known functions of secretogranin III and stathmin, one scenario is that in an un-stimulated cell stathmin sequesters secretogranin III and prevents its association with chromogranin-A thus causing chromogranin-A to mis-sort to the constitutive pathway. In response to certain stimuli stathmin is phosphorylated, this would cause abrogation of the stathmin-secretogranin III interaction and free

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secretogranin III from sequestration. Secretogranin III is thus free to bind cholesterol in the secretory granule membrane and act as an adapter for chromogranin A and aid its appropriate sorting via secretory granules to the exterior of the cell. Stathmin is well documented to be purely cytosolic (while SCG10 is present in golgi bodies) and literature so far suggests that secretogranin III is not. It is possible that some secretogranin III exerts actions outside the Golgi or that stathmin can enter the Golgi system. Alternatively, SBP2.2 may represent part of a cytosolic protein that contains secretogranin III-like domains, or the binding observed in the two-hybrid system could be an in-vitro artifact.

Stathmin Binds Chaperonin Containing T-Complex Polypeptide 1, Subunit 4

The protein product of the cDNA SBP3.1 showed a strong interaction with stathmin in our yeast two hybrid screen. Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was 96% identical to Chaperonin Containing T-Complex Polypeptide 1, Subunit 4 (CCT4) over the portion sequenced.

The chaperonin containing TCP1 complex (CCT), also called the TCP1 ring complex, consists of 2 back-to-back rings, each containing 8 unique but homologous subunits (e.g., CCT3). CCT assists the folding of newly translated polypeptide substrates through multiple rounds of ATP-driven release and rebinding of partially folded intermediate forms. Substrates of CCT include the cytoskeletal proteins actin and tubulin, as well as alpha-transducin. Wu-Baer et al. (Wu-Baer et al., 1996) isolated a cDNA encoding CCT4, which they called Stimulator of TAR RNA-binding proteins (SRB). Sequence analysis predicted that the deduced 539-amino acid CCT4 protein shares

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homology with a number of chaperonin family proteins. Western blot analysis showed that CCT4 is expressed as a 62-kD protein. Binding analysis confirmed that CCT4 enhances the binding of TARBP1 and RNA polymerase II to TAR.

Cyclin E (CCNE1), a partner of the cyclin-dependent kinase CDK2 is implicated in the positive control of the G1/S phase transition. CCNE1 degradation is regulated by ubiquitination and proteasomal action, which occur upon autophosphorylation and activation of the CCNE1-CDK2 complex. Using a yeast-based screen to identify proteins that interact with CCNE1, Won et al. (Won et al., 1998) obtained a cDNA encoding CCT4, which they called CCT-delta. Mutational analysis indicated that CCT is essential for Cyclin E maturation and accumulation.

Given the above facts about CCT4, the interaction with stathmin could be a physiologically significant one that mediates some of the the growth promoting actions of stathmin. It is interesting that stathmin-binding proteins consistently involves links to chaperone proteins (e.g., Hsps), tubulin, and proliferation control. If so, it highlights an underemphasized mode of regulation in signal transduction namely 'sequestration'. Stathmin's role in tubulin binding is to sequester it and prevents microtubule formation. Given that it binds CCT4, a protein that binds tubulin, actin, alpha-transducin and cyclin E it is conceivable that stathmin plays a greater role in cell division by sequestering key players or regulating their activation, until specific signals lead to stathmin phosphorylation. This in turn could lead to disruption of stathmin-tubulin or stathmin-cyclin E interactions which would leave tubulin and cyclin E free to play their roles in the cell cycle.

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Stathmin Binds Hypothetical Protein FLJ31331

Our yeast two hybrid screen showed that hypothetical protein FLJ31331 interacted strongly with stathmin but only weakly/non specifically with SCG10. Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was weakly similar (87% identical) to hypothetical protein FLJ31331 (Isogai et al., 2001. NEDO human cDNA sequencing project, Unpublished data/Direct Submission) over the 181bp sequenced. The amino acid sequence of FLJ31331on analysis with the program COILS (Lupas,1991) showed that two portions of the protein had a distinct (> 0.75) probability of forming a coiled-coil. This indicates that a FLJ31331-like protein is possibly a novel stathmin binding protein that conforms to the conformational characteristics seen in previously characterized stathmin binding proteins. Search for other motifs/domains present in hypothetical protein FLJ31331 using various prediction programs including Conserved Domain Search (Marchler-Bauer and Bryant, 2004) yielded no results. It is therefore likely that the protein either has no additional domains (besides the earlier postulated coiled-coils) or does have additional domains that are novel and hence not represented in the current databases used for comparison in the Conserved Domain Search. Postulating a role for interaction of a FLJ31331-like protein with stathmin at this point would be premature at this point and therefore will have to await detailed characterization of the protein and its interacting domains.

Stathmin Binds Protein Product of RP11-498M11 BAC clone

The protein product of the cDNA SBP3.2 interacted strongly with stathmin. Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was

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98% identical (fig. 17D) to Homo sapiens BAC clone RP11-498M11 (Waterston 1999. Direct Submission) (Abbott and Waligorski, 2001. The sequence of Homo sapiens BAC clone RP11-498M11.Unpublished/Direct Submission) over the 463bp sequenced. RP11-498M11 is likely to code for a previously unidentified protein since the library it was derived from is cDNA based and should have no genomic DNA contamination. Data available in the public domain shows that the 649 amino acid sequence of this protein is derived by conceptual translataion of the nucleotide sequence. The protein shows two domains based on a Conserved Domain Search (Marchler-Bauer and Bryant, 2004) : (1) PH_Apbb1ip, Amyloid beta A4 Precursor protein-Binding, family B, member 1 Interacting Protein (Apbb1ip) pleckstrin homology (PH) domain. Apbb1ip consists of a Ras-associated domain and a PH domain. PH domains share little sequence conservation, but all have a common fold, which is electrostatically polarized. PH domains also have diverse functions. They are often involved in targeting proteins to the plasma membrane, but few display strong specificity in lipid binding. Any specificity is usually determined by loop regions or insertions in the N-terminus of the domain, which are not conserved across all PH domains. (2) GRB7_RA, the RAS-associated like (RA) domain of Grb7. Grb7 is an adaptor molecule that mediates signal transduction from multiple cell surface receptors to various downstream signaling pathways. Grb7 and its related family members Grb10 and Grb14 share a conserved domain architecture that includes an amino-terminal proline-rich region, a central segment termed the GM region (for Grb and Mig) which includes the RA, PIR, and PH domains, and a carboxyl-terminal SH2 domain. Grb7/10/14 family proteins are phosphorylated on serine/threonine as well as tyrosine residues and are mainly localized to the cytoplasm. Analysis of the amino acid

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sequence of RP11-498M11 with the program COILS (Lupas, 1991) showed that four portions of the protein had a distinct (> 0.75) probability of forming coiled-coils.

It is difficult to speculate on the effect of interaction of stathmin with the protein coded by RP11-498M11. The latter protein appears to be a linker protein with possible connections to many growth-promoting pathways. Binding to stathmin might block the activities of the protein, facilitate its actions, or simply direct it to a specific pathway.

Stathmin Binds Hypothetical Protein FLJ14557

The cDNA S10BP2.1 showed a specific interaction with stathmin in our yeast two hybrid screen. Analysis of the nucleic acid sequence of this stathmin binding protein showed that it was 99% identical over the 484bp sequenced, to protein encoded by the cDNA FLJ14557 fis (Isogai et al., 2001, Unpublished results, NEDO human cDNA sequencing project). This is a recently discovered protein that is weakly similar to cell division protein FTSJ and mitochondrial cytochrome c oxidase subunit II (Ota et al., 2004). Katz and colleagues. (Katz et al. 2003) developed a program called "sensitized matrices for scoring methyltransferases" to automate the identification of putative methyltransferases from genomic Open Reading Frames. They compiled a dataset of proteins highly like to be methyltransferases from yeast, human, mouse, Drosophila *melanogaster*, Caenorhabditis *elegans*, Arabidopsis *thaliana*, and Escherichia *coli* genomes. One of the proteins identified in the dataset as a methyltransferase is FLJ14557fis. S-Adenosylmethionine (AdoMet)-dependent methylation is a broad class of biological processes in which a methyl group is transferred from AdoMet yielding S-adenosylhomocysteine (AdoHcy) and a methylated target molecule. Methylation is

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similar to other protein modifications like acetylation, prenylation, sumoylation, ubiquitination and phosphoryltation; because it acts as a modulator/regulator of protein function. Many of these reactions are so basic to the proper functioning of life that the lack of the gene product that performs these reactions is sufficient to severely, if not completely, abrogate the normal functioning of an organism.

SCG10 Binds Humanin

Hashimoto et al. (Hashimoto et al., 2001b) used the death-trap screening method to screen a cDNA library from the occipital lobe of the brain of a patient who had died of Alzheimer's disease. They identified a long cDNA that encoded a deduced 24-amino acid polypeptide, which they called humanin (HN). They found that the transfected humanin cDNA peptide suppressed neuronal cell death induced by 3 Familial Alzheimer's Disease (FAD) genes: amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2). The peptide also abolished death caused by A-beta amyloid, but had no effect on death by expanded polyglutamine repeats (Q79) or superoxide dismutase-1 (SOD1) mutants. Detailed structural analysis by Hashimoto et al. (Hashimoto et al. 2001a) of the HN protein showed the essential roles of cys8, ser14, and the domain from pro3 to pro19 in the rescue action. The humanin cDNA was initially found to be 99% identical to mitochondrial 16S rRNA with a poly(A) tail, which would make it unlikely that the peptide encoded by the cDNA is naturally produced. However, the cDNA is also 99% identical to some nuclear-encoded human cDNAs, indicating humanin cDNA represents a nuclear transcribed mRNA and that the humanin peptide is a natural product. They determined that humanin is secreted into the extracellular medium and acts on the outside

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of cells, and they suggested that there was a specific binding site on the cell surface. Tajima et al. (Tajima et al., 2002) generated an anti-humanin antibody and found that long cDNAs containing the ORF of humanin produced the humanin peptide in mammalian cells, dependent on the presence of the full-length humanin-ORF. Immunoblot analysis detected a 3 kDa protein with humanin immunoreactivity in mouse testes and colon. The findings suggested that the humanin peptide can be produced in vivo. Niikura et al. (Niikura et al., 2003) identified TRIM11 as an humanin-interacting protein using a yeast 2-hybrid screening assay. TRIM11 is a member of a protein family containing a tripartite motif, including a RING finger (RFP) domain that acts as a putative E3 ubiquitin ligase. The coiled-coil domain, and the C-terminal RFP like domain of TRIM11, were necessary for interaction with humanin. Co-expression of humanin and TRIM11 diminished intracellular levels of humanin, suggesting that intracellular humanin levels are regulated by TRIM11-linked ubiquitin-mediated protein degradation pathways. Guo et al. (Guo et al., 2003) found that Bax coimmunoprecipitated with humanin. They also showed that humanin rescued rat hippocampal neurons from Bax-induced lethality. Humanin prevented the translocation of Bax from the cytosol to the mitochondria and suppressed cytochrome c release. Bax is an apoptosis-inducing protein that undergoes conformational changes that result in its translocation to mitochondrial membranes where Bax inserts and causes release of cytochrome c and other apoptotic proteins. Guo et al. (Guo et al., 2003) also noted that the predicted humanin peptides from the nuclear-encoded peptide and the mitochondrial-encoded peptide (which is most likely not expressed in mitochondria) were both able to bind Bax and prevent apoptosis. The authors suggested that the humanin gene arose from mitochondria and transferred to

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the nuclear genome, providing a protective mechanism from Bax. Insulin-like growth factor-binding protein-3 (IGFBP-3) is a protein whose normal role is to regulate IFG bioactivity and to independently modulate cell growth, survival and apoptosis. Ikonen et al. (Ikonen et al., 2003) using a yeast two hybrid screen to identify interactors for IGFBP-3, found that it interacts strongly and specifically with Humanin. IGFBP-3 appears to be elevated in AD brains and has been detected in senile plaques and neurofibrillary tangles suggesting a role in AD-related cell death. Therefore the Humanin-IGFBP-3 interaction and the potentiation of Humanin's protective effect (against A- β-induced cell toxicity) in primary neuronal cells seen by Ikonen et al. is of great significance.

Our yeast two hybrid screen for SCG10 binding proteins showed that the cDNA S10BP4.1 specifically bound SCG10 but not stathmin (a close non-neural specific member of the family). Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 100% identical to Humanin over the portion sequenced. Okazaki et al. (Okazaki et al., 1995) have shown that there are no quantitative changes in total SCG10 mRNA or protein in AD brain. However they found that SCG10 concentration shows a positive correlation with number of tangles found in AD brains and this was because SCG10 protein accumulated in the cell bodies in AD affected regions. The authors suggest that SCG10 compartmentalization and metabolism may be altered in AD possibly due to mechanisms related to tangle formation in this disease. These facts about SCG10 taken together with the facts about Humanin's interactions (and protective actions thereof) with TRIM11, Bax and IGFBP-3 lead us to believe that the novel SCG10-Humanin interaction we have identified could have similar mechanistic

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basis and a similar protective role. This postulated role is buttressed by the following facts:

1. SCG10 too has a coiled-coil domain (similar to TRIM11) capable of interacting with Humanin and
2. Humanin is seen to be expressed in surviving and unaffected areas of AD brain, while SCG10 is seen to accumulate in cell bodies in AD affected regions; probably pointing to a loss of the balance (that perhaps ensures appropriate compartmentalization and metabolism of SCG10) between the two proteins which might be necessary to protect cells from A β-induced cell toxicity.

SCG10 Binds FLJ10702fis/Gie1/ARL-10C

Our yeast two hybrid screen for SCG10 binding proteins showed that the cDNA S10BP3.2 specifically bound SCG10 but not stathmin (a close non-neural specific member of the family). Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 100% identical to FLJ10702fis over the 237bp sequenced. This was a hypothetical protein that was similar to ADP ribosylation factor and hence called ADP ribosylation factor-like 10C. It is of great interest to us, because since our discovery of this protein as a SCG10 binding protein, it has been shown by Okai et al. (Okai et al., 2004) to be an expressed protein belonging to a novel small GTPase subfamily capable of associating with tubulin and required for chromosome segregation.. Okai et al have therefore renamed it `novel small G-protein indispensable for equal chromosome segregation 1' (Gie1).

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The Gie subfamily of GTPases was discovered during a database search by Okai et al. (Okai et al., 2004) for novel GTPases. Gie1 and Gie2 were initially cloned from a human brain cDNA library. Gie1 and Gie2 have been shown to be ubiquitously expressed with highest expression of Gie1 seen in human brain, heart, skeletal muscle, kidney and placenta. Gie2 shows 91% identity and 98% similarity in amino acid sequence to Gie1. The authors' search in the ESTs and protein databases showed that Gie homologs exist in mouse, rat, Drosophila melanogaster and Caenorhabditis elegans. The authors infer that their inability to find homologs in yeast indicates that the protein is conserved in multicellular organisms. Okai et al. (Okai et al., 2004) showed that expression of dominant-negative Gie mutants in human HeLa cells or knockdown of Gie transcripts by RNAi in Drosophila S2 cells induced abnormal morphology in the chromosome segregation. Elegant in vitro and in vivo studies by the authors show that both the GDP and GTP bound forms of Gie are capable of binding microtubules. They conclude that Gie might play an indispensable role in the equal segregation of chromosomes, probably through its association with microtubules.

SCG10 has already been shown to play an important role in microtubule dynamics through the following specific binding interactions:

1. Modulation of tubulin dynamics, neurite outgrowth and neuronal plasticity through its interaction with tubulin
2. Modifications of the microtubule cytoskeleton in response to extracellular cues, to aid neurite guidance, via its interaction with protocadherin-gamma – b1
3. Binding of SCG10 to TRPC5 regulates neurite extension and growth cone morphology
4. RGS6 binding potentiates the ability of SCG10 to disrupt microtubule organization

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5. RGSZ1 binding blocks SCG10-induced microtubule disassembly

Our discovery that SCG10 binds to Gie1 is very interesting in this context, because regulation of microtubule dynamics by SCG10 through different binding proteins is an indication of the diversity of different cellular processes (and hence different signals/pathways) where tight regulation of microtubule dynamics is vital and where SCG10 plays a role as master regulator/relay.

SCG10 Binds Protein Product of RP11-8N8

SCG10 showed a strong interaction with the protein product of S10BP4.2 cDNA in the yeast two hybrid screen. This cDNA fusion with the activation domain does not interact with the binding domain by itself nor with it fused to Lamin protein (Lamin fusion); but it does interact weakly with stathmin fusion. The interaction with the SCG10 fusion and the weak interaction with the stathmin fusion product indicates that the interaction is specific and high-affinity for SCG10 and low-affinity/weak/non-specific for a close ubiquitous member (stathmin) of the family. Analysis of the nucleic acid sequence of this SCG10 binding protein showed that it was 99% identical in plus/minus orientation (over the 532bp sequenced) to BAC clone RP11-8N8 (Meyer et al., 2001. The sequence of Homo sapiens BAC clone RP11-8N8. Direct Submission/Unpublished). This is likely to be a previously unidentified protein since the library it was derived from is cDNA based and should have no genomic DNA contamination. However it is also (remotely) possible that this indeed is a genomic, non-coding DNA contamination in the cDNA library that is expressed and translated when fused to the pB42AD. Portions of the RP11-8N8 DNA are thought to code for WD repeat and FYVE domain containing 3

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(WDFY3), transcript variant 1. A Conserved Domains Search for these reveals the following domain characteristics. The WD40 domain is found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. WD40 typically contains a GH dipeptide (11-24 residues) from its N-terminus and the WD dipeptide at its C-terminus and is 40 residues long, hence the name WD40. Between GH and WD lies a conserved core which serves as a stable propeller-like platform to which proteins can bind either stably or reversibly. This forms a propeller-like structure with several blades where each blade is composed of a four-stranded anti-parallel β-sheet. Instances with few detectable copies are hypothesized to form larger structures by dimerization. Each WD40 sequence repeat forms the first three strands of one blade and the last strand in the next blade; the last C-terminal WD40 repeat completes the blade structure of the first WD40 repeat to create the closed ring propeller-structure. Residues on the top and bottom surface of the propeller are proposed to coordinate interactions with other proteins and/or small ligands. The FYVE domain stands for 'Protein present in Fab1, YOTB, Vac1, and EEA1'. It is a zinc-binding domain, possibly involved in endosomal targetting. Recent data indicates that FYVE domains bind PtdIns(3)P. Whether these domains are present in the protein product of the cDNA we isolated as a SCG10 binding protein remains to be confirmed. If confirmed in vivo, this interaction would have immense functional significance given that the two domains (WD40 and FYVE) are capable of numerous interactions that are of vital importance in a variety of signal cascades.

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Novel Functions and Uses for Hypothetical Protein MGC12981?

As described in the results, the yeast two hybrid screen of the cDNA SBP1.2 with LexABD, LexABD-Lamin, LexABD-SCG10 and LexABD-stathmin showed strong, dual reporter positive interactions. The interaction of the cDNAs protein product with the LexA binding domain by itself or with its fusions with Lamin/SCG10/stathmin shows that the protein encoded by this cDNA non-specifically binds all proteins or is/has a DNA binding domain. Analysis of the nucleic acid sequence of this protein showed that it was 96% identical to the hypothetical protein MGC12981 (Strausberg et al., 2002) (Ota et al., 2004) over the 293bp sequenced. This protein has no experimentally proven function. The non specificity of hypothetical protein MGC12981's interaction in the screen, made it not worth pursuing as a stathmin/SCG10 binding protein. However we further analyzed MGC12981 because it was capable of: (1) activating both reporters in the assay, was derived from a brain cDNA library and so is a putative brain transcription factor (2) greatly increasing the yeast mating efficiency.

Analysis of the amino acid sequence of this protein using the program COILS showed that both the N-terminal and C-terminal of the protein had stretches of 28 amino acids that had a distinct (> 0.75) probability of forming coiled-coils (figure 17D). Motif-scan (Falquet et al., 2002) of the amino acid sequence revealed that the region between the N and C terminals, besides having potential phosphorylation (casein kinase II, protein kinase C) and N-myristoylation sites also had (E. value equal to or greater than 0.0015) a protein prenyltransferase, alpha subunit repeat profile and a cell division control protein 15/Fes/ Cdc42-interacting protein 4 (Cdc15/Fes/CIP4) domain. The protein prenyltransferase, alpha subunit repeat profile in MGC12981 is a single tetratricopeptide

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(TPR) repeat domain. Proteins such as farnesyltransferase (FT), geranylgeranyltransferase 1 (GGT1) and the unrelated $p67^{phox}$ that use the TPR motif for binding other proteins typically have 7 TPR motifs. Therefore the role (if any) of this TPR motif in MGC12981s function is unclear. The Cdc15/Fes/CIP4 domain that is present in MGC12981 has been shown to have different functions in a variety of proteins. Kessels and Qualmann (Kessels and Qualmann, 2006) recently showed that Syndapin forms oligomers via its Cdc15/Fes/CIP4 domain and the oligomers interconnect the machineries for endocytic vesicle formation and actin polymerization. Similarly Laurent et al. (Laurent et al., 2004) showed that in the human c-Fes tyrosine kinase protein, the Cdc15/Fes/CIP4 domain binds soluble unpolymerized tubulin and regulates microtubule assembly. Studies by Takahashi et al. (Takahashi et al., 2003) using microtubule regeneration assays, revealed that Fes (Fes/Fps tyrosine kinase) aggregates colocalized with gamma-tubulin at microtubule nucleation sites in a Fes/CIP4 homology (FCH) domain-dependent manner and that expression of FCH domain-deleted Fes mutants blocked normal centrosome formation. They therefore concluded that the Fes/Fps tyrosine kinase regulates microtubule nucleation through is Fes/CIP4 homology domain.

Given this information about the domains that are present in MGC12981 and the effect of this protein in the yeast two hybrid screen, it is tempting to postulate that the protein is either a non canonical transcription factor or a transcriptional complex organizer. The mechanism of the increased yeast mating efficiency brought about by MGC12981 is unclear, given that a homologous yeast protein was not seen in our BLAST search. However the value of the use of this protein in increasing yeast mating efficiency is obvious in applications such as yeast genetic (forward/backward) screens

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and in yeast two/three hybrid and loss of function screens, where the efficiency of mating and producing daughter cells determines the power of the screen.

Interaction of Stathmin with Thyroid Hormone Receptor Interactor 3

The reporter plate showed lack of interaction of this cDNA product with SCG10. However it did show a strong interaction with stathmin, Lamin and relatively weaker interaction with LexABD alone. Nucleic acid sequence analysis of this stathmin binding protein showed that it was identical to Thyroid Hormone Receptor Interactor 3 (TRIP3). TRIP3 is a protein whose amino acid residues 7 to 36 are predicted by motif prediction programs to form a zinc finger motif which is a common structural feature of transcription factors. Lee et al. (Lee et al., 1995) used the yeast interaction trap, to identify proteins that specifically interact with the ligand-binding domain of rat Thyroid hormone Receptor-beta (TR). They isolated HeLa cell cDNAs encoding several different TR-interacting proteins (TRIPs), including TRIP3. TRIP3 interacted with rat ThRb only in the presence of thyroid hormone. A region of TRIP3 that includes a number of negatively charged residues shows similarity to several short regions of the Drosophila CUT protein, a homeodomain-containing transcription factor. Northern blot analysis detected a 1.1-kb TRIP3 transcript in all tissues examined and so it is not surprising that the human cDNA library we used contained TRIP3.

The lack of interaction with the LexABD-SCG10 showed that the protein product of this cDNA is not non-specifically binding all other proteins. But the interaction with a non-specific control (Lamin) and with the LexABD alone, prompts us to limit and be

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cautious of the interpretation of this result. Furthermore, a protein that binds to an activated receptor may also associate with a number of other transcription factors.

Olfactomedin

During the initial co-transformation experiments to identify stathmin and SCG10 binding proteins, a cDNA that was a putative SCG10 binding protein was isolated. The cDNA sequence of this protein showed 99% identity (fig. 17I) over the 243bp sequenced, to the gene encoding the protein Olfactomedin (also called Neuroblastoma protein or AMY or Noelin). This protein initially was very interesting because Yokoyama et al. (Yokoyama et al., 1996) showed that it was expressed in 4 neuroblastoma cell lines and in adult brain (similar to SCG10). Also the protein sequence analysis revealed the possibility (>75%) of a coiled-coil domain (data not shown); a previously noted characteristic of binding proteins of the stathmin family. The olfactomedin gene family in humans, has numerous variants whose roles are still poorly understood. Molecular evolutionary genetic analysis of the human olfactomedin gene family by Karavanich and Anholt (Karavanich and Anholt, 1998) revealed an accelerated rate of nucleotide substitutions in the mammalian lineage. However, the evolutionary rate at the protein level, was constant, indicating that evolution of olfactomedin is constrained by structural limitations. The authors (Karavanich and Anholt, 1998) infer that the apparent evolutionary pressure toward conservation of primary structure supports the notion that olfactomedin has an important function in the mammalian nervous system.

Based on these observations we performed two hybrid/interaction screening to confirm/reject the earlier seen results. Mating of yeast bearing this cDNA (as a fusion of

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the pBB42AD construct) to yeast bearing the pLexA or its fusions with Laminin, stathmin, or SCG10 and performing the dual reporter screen showed no positive result. Although growth was seen in all four quadrants, the yeast colonies/smears were not blue. This is because only one of the two reporters of the assay (growth on –Leu) is activated while the lacZ reporter is not activated and this is indicative of a false positive result. Therefore the earlier blue smears/colonies that prompted this follow-up, can be inferred to have arisen from concurrent residence of multiple cDNA containing plasmids during a co-transformation based yeast two hybrid assay. Further studies of the protein product of this cDNA were therefore discontinued.

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CONCLUSIONS

Our studies on prolactin induced proliferation of Nb2 cells show that proliferation is associated with isoform specific phosphorylation of p38α. Combined with prior data (from other labs and ours) this ties stathmin and p38α as effector molecules in the signaling cascade that leads to Nb2 cell proliferation in response to certain mitogenic signals.

The affinity pull-down methodology led to our identification of Hsp70 as a potential physiological binding partner of stathmin. Our successful yeast two hybrid screen led to the identification of Secretogranin III; Chaperonin Containing T-Complex Polypeptide 1, Subunit 4; Hypothetical protein FLJ31331; protein product of RP11-498M11 BAC clone; and Hypothetical protein FLJ14557 as stathmin binding proteins. It also led to the discovery of Humanin; FLJ10702fis/Gie1/ARL-10C and protein product of RP11-8N8 as SCG10 binding proteins. The detailed characterization of these novel binding proteins and elucidation of the functional significance of these interactions will help us better understand the diverse roles and mechanisms of action of the stathmin family of proteins.

A fortuitous discovery during the yeast two hybrid screening has been the discovery that the Hypothetical Protein MGC12981 might function as a transcriptional factor/activator. And also that MGC12981's ability to increase yeast mating frequency,

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might be exploited to increase the efficiency of numerous genetic and interaction -screens that utilize the yeast system.

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