

PROTEIN-PROTEIN INTERACTIONS IN FORMING *ADHERENS JUNCTIONS*

IN VITRO AND IN VIVO ANALYSIS OF
PROTEIN-PROTEIN INTERACTIONS INVOLVED IN THE FORMATION OF
EPITHELIAL ADHERENS JUNCTIONS

BY

MICHELLE MELONE, H.B.Sc.

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AUTHOR: Michelle Melone B.Sc. (McMaster University)

SUPERVISOR: Dr. Christian Baron

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ABSTRACT

Adherens junctions are a main cell-cell adhesion structure found in epithelial cells. The stability of *adherens junctions* is attributed to various protein-signaling cascades and importantly the interaction between the transmembrane protein E-cadherin and cytoplasmic p120 catenin. This interaction is critical for cell adhesion and prevention of uncontrolled growth in normal cells. The interaction interface between these two binding partners was previously determined to comprise p120's Armadillo repeat domain (p120Arm) and E-cadherin's cytoplasmic juxtamembrane domain (Ecadc). Based on this information, peptide aptamers were derived from p120Arm and their interaction with Ecadc was tested *in vitro*. We reasoned that those could be expressed *in vivo* to stabilize *adherens junctions* at the cell-cell junction. In this study, we established protein-protein interaction assays to demonstrate p120Arm's ability to bind Ecadc and then used these assays to determine if p120Arm-derived peptides may competitively bind Ecadc. We demonstrated the interaction between p120Arm and Ecadc using assays that were not previously used such as: co-precipitation, analytical gel filtration and the bacterial-2-hybrid assay. However, the p120Arm-derived peptides did not bind to Ecadc or compete its interaction with p120Arm. This may be due to the nature of the assays that may not reflect competitive binding or the aptamers may not adopt the native conformation preventing binding to Ecadc.

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CONTRIBUTIONS BY OTHERS

Vectors with full-length p120 isoform 1A (pBlueScript-p1201A), pGAD-E-cadherin, and pGEX4T1 donated by J. Daniel. Bacterial-2-hybrid plasmids: pUT18C, pKT25, pUT18C-Zip and pKT25-Zip generated by G. Karimova. Bicistron backbone vector (pUT18CFB8a+SG) generated by D. Sivanesan. M. Melone performed all subsequent cloning experiments, vector manipulations and experiments.

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ABBREVIATIONS

Ab	<u>A</u>nti<u>b</u>ody
APC	A denomatous p olyposis c oli
Arm	<u>A</u>rmadillo
BSA	<u>B</u>ovine <u>S</u>erum <u>A</u>lbumin
bp	<u>b</u>ase <u>p</u>air
Carb	<u>C</u>arb<u>e</u>n<u>i</u>cillin
Cm	<u>C</u>hlor<u>a</u>m<u>p</u>henicol
CPP	<u>C</u>ell-<u>P</u>enetrating <u>P</u>eptide
Ecadc	<u>E</u>-<u>c</u>ad<u>h</u>er<u>i</u>n <u>c</u>ytoplasmic domain
IPTG	<u>I</u>sopropyl-β-<u>D</u>-<u>t</u>hiogalactopyranoside
JMD	<u>J</u>uxt<u>a</u>m<u>e</u>mbrane <u>D</u>omain
M.U.	<u>M</u>iller <u>U</u>nits
OD	<u>O</u>ptical <u>D</u>ensity
p120	<u>p</u>120 catenin
PBS	<u>P</u>hosphate <u>B</u>uffered <u>S</u>aline
rpm	<u>r</u>otations <u>p</u>er <u>m</u>inute
SD	<u>S</u>hine <u>D</u>algarno sequence
Strep	<u>S</u>treptavidin tag
TestX	<u>T</u>est <u>E</u>xpression
TnT	<u>T</u>ranscription <u>a</u>nd <u>T</u>ranslation

1. INTRODUCTION

1.1. Overview

An important function of cells is their ability to form complex tissues that are integral to organ and proper metabolic function. When examining the mammalian tissue architecture, it is apparent that one of the critical components involved in tissue formation are cell-cell adhesions, which are essential for cellular communication and cooperation. The cell junctions that stabilize tissues are required firstly for the cell to maintain its structural integrity and secondly for the control of cellular differentiation and death of the tissue (Lutz and Siahaan, 1997). The proteins that are involved in cell-cell adhesions, specifically *adherens junctions*, are well studied and were characterized mainly through *in vivo* studies. The key protein components of *adherens junctions* are the classical cadherins and catenins (Lutz and Siahaan, 1997; Takeichi, 1995).

The focus of my thesis was the *in vitro* characterization of the interaction between the epithelial cadherin (E-cadherin) and two of its cytoplasmic binding partners: p120 catenin (p120) and Hakai. The aim of the interaction studies was to identify the minimum binding sites of E-cadherin, p120 and Hakai *in vitro*. Once the protein interactions are established, peptides could be designed and tested to replace p120 or inhibit Hakai in attempts to stabilize E-cadherin at the cell junctions. The long-term goal of this approach is to use peptide aptamers as cancer therapeutics to prevent metastasis.

1.2. Cell–Cell Adhesion:

In the formation of tissues and complex organs, individual cells must adhere to or detach from their neighbouring cells depending on the developmental stage of the tissue. To facilitate such fluidity, cells possess cell-cell adhesion protein complexes. Such complexes are the tight junctions, the desmosomes and the *adherens junctions*. These complexes are built on a platform of transmembrane and cytoplasmic proteins that further anchor the complexes to the cell's ultrastructure. The variety of cell adhesion molecules (CAMs) is correlated to the stages in development and tissue type. CAMs are glycoproteins that commonly adhere to each other and the extra-cellular matrix (Kerrigan *et al.*, 1998). Over 50 different CAMs have been reported which fit into four main families: immunoglobulin (Ig)-like CAMs, selectins, integrins and cadherins (Okegawa *et al.*, 2004).

1.3. Adherens Junctions (zonula adherens)

Epithelial cells maintain their contact through a structure known as the *adherens junctions*. This complex of proteins is centered on a cadherin as the transmembrane CAM. This protein participates in homophilic dimerization and is bound by intracellular catenin partners to anchor the complex to the actin cytoskeleton. The cadherin-catenin protein complex created is a scaffold for cell signaling, a determinant of cell morphology, and of cell fate such as mesenchymal to epithelial transitions or the reverse as seen in cancer (Takeichi, 1995).

1.4. Cadherins

The cadherin glycoprotein family is a fundamental component of cell-cell adhesions. Their role in maintaining the integrity of a tissue is critical for organ formation and proper function. The classical cadherins, E-cadherin (epithelial), N-cadherin (neural), and P-cadherin (placental) are a subgroup of the cadherin super-family (Lutz and Siahhaan, 1997; Takeichi, 1995). The classical cadherins are further subdivided into type I and type II molecules that differ in their strength of adhesion. The type I molecules (eg. E-cadherin) have been found to participate in stronger cell junctions than the type II molecules (eg. N-cadherin). Both are required for diverse functions of the cell, such as strong adhesion in tissue formation and the loosely associating molecules play roles during initial embryonic development (Takeichi, 1995).

The cadherins, in general, are composed of an extracellular (EC) N-terminal domain with five sub-domains that are involved in the homotypic interactions between cadherins of adjacent cells (Figure 1). These sub-domains, named EC1 to EC5, with EC5 being the closest to the cell membrane, are involved in the initial adjacent cell dimerization of E-cadherin (Shapiro *et al.*, 1995). Specifically, EC1-EC2 are involved in homophilic adhesion “zipper” dimerization with the neighbouring cell in a Ca²⁺-dependent manner and EC1 contains the conserved sequence HAV (His-Ala-Val) that serves as a recognition site between cells (Berx *et al.*, 1995; Lutz and Siahhaan, 1997; Shapiro *et al.*, 1995). The Ca²⁺ binding sites were characterized as DXNDN and LDRE found in all EC domains (Lutz and Siahhaan, 1997). Cadherins also possess a transmembrane domain and a highly conserved cytoplasmic domain containing the

juxtamembrane domain (JMD) that is crucial for catenin interactions (Jou *et al.*, 1995; Thoreson *et al.*, 2000; Yap *et al.*, 1998).

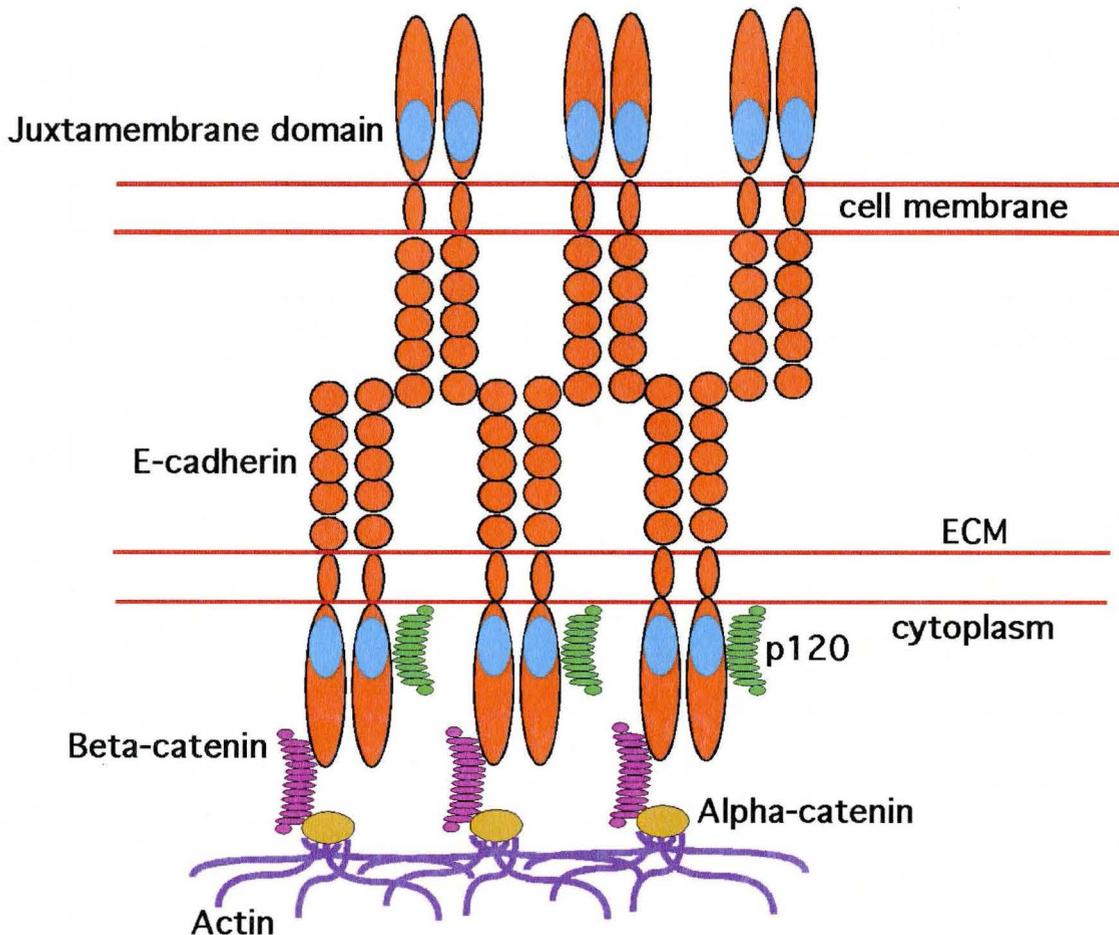


Figure 1: Extracellular cadherin dimer formation between cells to create adherens junctions. Image illustrates the five subdomains of the extracellular domain of cadherins where subunit EC1 is the furthest from the cell membrane (Lutz and Siahaan, 1997; Shapiro *et al.*, 1995). Image also shows the dimer “zipper” formation of cadherins at the cell membrane and how these dimers interact with the same dimers on the neighbouring cell. Within the cytoplasm, E-cadherin, binds p120 catenin, through its juxtamembrane domain, and to Beta-catenin via its C-terminal catenin-binding domain. Beta-catenin anchors E-cadherin to the Actin cytoskeleton via Alpha-catenin while p120 regulates E-cadherin protein stability and turnover through their association (Yap *et al.*, 1997). Modified from: (Takeichi, 1995).

1.5. Catenins

The catenin proteins are molecular links between the transmembrane cadherins and the cytoplasm. These proteins interact with the cytoplasmic domain of cadherins and transduce their signals downstream to affect various cellular processes, such as those affecting the remodeling of cellular structure (Ozawa *et al.*, 1990).

The catenin family includes α -catenin, β -catenin, plakoglobin and p120 (Daniel and Reynolds, 1995; Ozawa *et al.*, 1990; Reynolds *et al.*, 1994; Reynolds *et al.*, 1996a). Some of these proteins can directly connect to actin bundles of the cell, for instance β -catenin directly binds cadherins as well as α -catenin, which in turn binds actin filaments (Aberle *et al.*, 1994; Ozawa *et al.*, 1990). The cytoskeleton binding partners of catenins have been shown to be actin, myosin, vinculin, α -actinin and radixin (Lutz and Siahaan, 1997). Therefore, when bound to cadherins, many catenins will anchor the cell junctions formed extracellularly to the intracellular foundation of the cell's architecture and participate in protein signaling via "outside-in" and "inside-out" pathways.

p120 was first identified as a substrate of the Src protein tyrosine kinase (Reynolds *et al.*, 1989). The phosphorylation of p120 occurs at the cell membrane since the Src kinase localizes almost exclusively at the cell membrane (Kanner *et al.*, 1991; Reynolds *et al.*, 1989). p120's N-terminal phosphorylation domain is found in all isoforms except 4 and possesses 6 of 8 tyrosines present in p120. This domain is C-terminal to the coiled-coil domain found only in p120 isoform 1 (Figure 2) (Mariner *et al.*, 2001). p120 is also tyrosine phosphorylated by Src in response to external growth stimuli, such as the mitogenic growth factors: epidermal growth factor (EGF) and

platelet-derived growth factor (PDGF) (Downing and Reynolds, 1991). The receptor protein tyrosine phosphatase mu (RPTP μ) was found to dephosphorylate p120 and to specifically recognize the N-terminal domain of p120 (Zondag *et al.*, 2000). Similarly, another RPTP, DEP-1 dephosphorylates p120, however, in a phosphotyrosine-dependent manner (Holsinger *et al.*, 2002). It has been shown that in addition to tyrosine phosphorylation, most p120 is phosphorylated on serine and to a lesser extent on threonine residues (Thoreson *et al.*, 2000).

A hallmark characteristic of p120, similar to its family member β -catenin and contrary to α -catenin (Strumane *et al.*, 2006), is its central Armadillo domain consisting of ten Armadillo repeats (Figure 2). This protein-protein interaction domain mediates the interaction of p120 with a variety of proteins including E-cadherin (Daniel and Reynolds, 1995; Ireton *et al.*, 2002; Kanner *et al.*, 1991). The nomenclature of the Armadillo domain originates from the appearance of a mutant *Drosophila* embryo that had a disrupted segment of the polarity gene *armadillo*. The Armadillo protein encoded by this gene is a homologue to mammalian β -catenin (Nusslein-Volhard and Wieschaus, 1980; Peifer, 1995; Riggleman *et al.*, 1989). Each 42 amino acid Arm repeat forms three alpha helices. When these Arm repeats interact with each other they form a right-handed super-helix that forms the surface for protein interactions (Huber *et al.*, 1997).

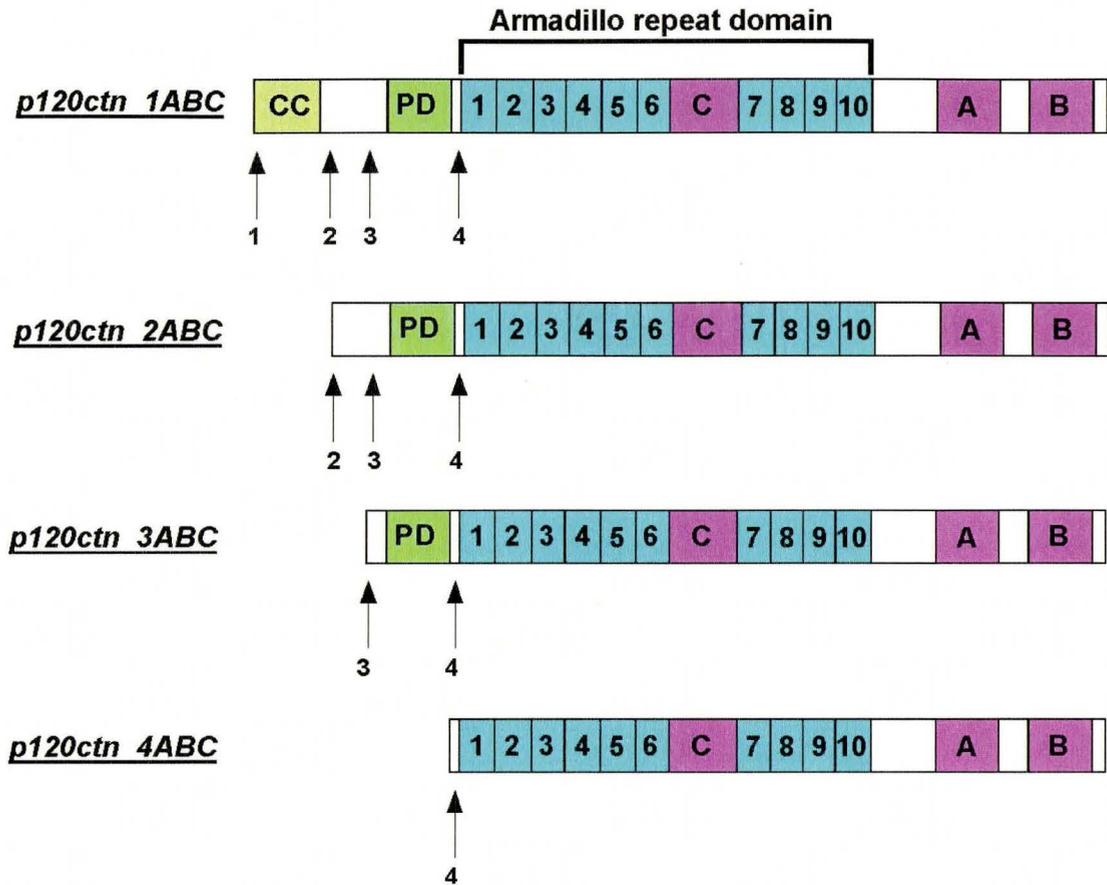


Figure 2: *p120* isoforms 1-4 are splice variants using different ATG start codons of the protein (indicated by arrows 1-4). The exons A-C refer to the individual exon size. The longest *p120* isoform is 1ABC and the shortest is 4ABC (Anastasiadis and Reynolds, 2000). CC- coiled-coil domain found only in isoform 1. PD- Phosphorylation domain found in all isoforms except isoform 4 that has 6 of 8 tyrosines phosphorylated by Src kinase (Mariner *et al.*, 2001). The blue boxes represent the 10 Armadillo repeats (Roczniak-Ferguson and Reynolds, 2003).

1.6. E-cadherin and p120 catenin: Their role in adherens junctions

The E-cadherin (previously known as uvomorulin) gene *CDH1* is located on chromosome 16q22.1 (Berx *et al.*, 1995; Mansouri *et al.*, 1988). The most frequent genetic event in breast cancer is the loss of heterozygosity (LOH). LOH is the loss of function of one allele of a gene by mutation subsequent to the other allele's inactivation.

This mostly occurs along the long arm of chromosome 16 and approximately 50 % of the breast cancer cases show a loss of E-cadherin expression (Cleton-Jansen, 2002).

p120 is a catenin that is encoded by the *p120^{cas}* gene (*CTNND1*) on human chromosome 11q11 close to the centromere (Reynolds *et al.*, 1996b). p120 protein exists as many isoforms (Figure 2) that likely perform unique functions (Daniel and Reynolds, 1995; Keirsebilck *et al.*, 1998).

In the cytoplasm, E-cadherin and p120 have been found to work synergistically in maintaining cell adhesions through the formation of *adherens junctions*. E-cadherin affects the actin cytoskeleton through its interactions with β -catenin, which subsequently interacts with the N-terminal domain of α -catenin that binds actin directly (Figure 1). These protein interactions stabilize and provide structural support for the cell (Jou *et al.*, 1995; Thoreson *et al.*, 2000). The cadherin/catenin complex has also been proposed to regulate cell polarity, particularly during early embryonic development (Lutz and Siahhan, 1997).

A study by Thoreson *et al.*, 2000 (Thoreson *et al.*, 2000) has shown that the core of the JMD, residues D758-L773, is the most significant part of the molecule concerning p120 binding. It is also interesting to note that mutations in the JMD had no effect on the binding of α , β , γ -catenin to E-cadherin (Thoreson *et al.*, 2000; Yap *et al.*, 1998). This is explained by the finding that β -catenin binds the E-cadherin cytoplasmic domain via the carboxyl-terminal catenin-binding domain (residues 815-839) that is located outside the JMD (Jou *et al.*, 1995). The binding of p120 and β -catenin to E-cadherin via two distinct domains is consistent with immunoprecipitation studies demonstrating co-precipitation of

E-cadherin, β -catenin and p120 (Shibamoto *et al.*, 1995). In addition, the Armadillo repeats of p120 required for E-cadherin binding were determined to comprise repeats 1-5 and 7 (Ireton *et al.*, 2002). Interestingly, p120 Armadillo repeats 3 and 5 required for binding E-cadherin are also involved in nuclear trafficking of p120 (Roczniak-Ferguson and Reynolds, 2003).

p120 catenin has many functions postulated to depend on external cell signals or intracellular protein cascades in signaling (Davis *et al.*, 2003). The major role of this catenin is to regulate cell-cell adhesion by binding the cytoplasmic JMD of classical cadherins, such as E-cadherin (Figure 3) (Davis *et al.*, 2003; Ireton *et al.*, 2002; Shibamoto *et al.*, 1995; Thoreson *et al.*, 2000). Once bound, p120 is thought to convert the weak adhesions between cells into strong adhesions by stabilizing the cadherins (Thoreson *et al.*, 2000). As well, p120 has the ability to regulate cadherin turnover at the cell membrane. It was shown that either increasing or decreasing the levels of p120 directly affects the levels of E-cadherin (Davis *et al.*, 2003). This may explain why in tumours a dose-dependent loss of E-cadherin is often observed when the gene for p120 is down regulated (Davis *et al.*, 2003; Ireton *et al.*, 2002).

A major difference between p120 and other Armadillo catenins, such as β -catenin, is that p120 does not bind α -catenin and hence p120 is not involved in anchoring E-cadherin to the actin cytoskeleton. In addition, p120 does not bind Adenomatous Polyposis Coli (APC) protein, a tumour suppressor, further substantiating that the function of p120 is unique (Daniel and Reynolds, 1995).

1.7. Other functions of E-cadherin

Aside from stabilizing *adherens junctions*, E-cadherin appears to indirectly facilitate the assembly of tight junctions made of zona occludin (ZO) proteins (Lutz and Siahaan, 1997). This possibility was raised when the gene for E-cadherin was inactivated *in vivo* and the absence of tight junction formation correlated with E-cadherin loss. However, the molecular basis for this effect is unclear. In contrast, it was shown that E-cadherin has no effect on desmosome formation, a third type of cell-cell adhesion (Tunggal *et al.*, 2005).

1.8. E-cadherin turnover

1.8.1. E-cadherin turnover by Hakai

E-cadherin requires a catenin such as p120 or β -catenin for its stabilization at the cell membrane. Without a catenin, E-cadherin is rapidly degraded. This could possibly occur by ubiquitin-mediated protein degradation that is mediated by a PEST motif found to overlap the β -catenin binding domain on E-cadherin. The protein is targeted to the proteasome or it undergoes lysosomal destruction (Davis *et al.*, 2003; Huber and Weis, 2001). Another binding partner of E-cadherin is Hakai (Japanese for 'destruction'), an E3 ubiquitin ligase that targets E-cadherin at the cell membrane for degradation (Fujita *et al.*, 2002). Hakai binds to tyrosine phosphorylated E-cadherin and targets it for degradation by endocytosis and subsequent proteolysis via the lysosome (Pece and Gutkind, 2002). Hakai recognizes the phosphorylated tyrosines 755 and 756 on the cytoplasmic domain of E-cadherin that are adjacent to the JMD residues starting at 758 to which p120 binds

(Figure 3) (Fujita *et al.*, 2002; Thoreson *et al.*, 2000). Interestingly, these binding residues are only found on E-cadherin, as Hakai was not found to bind to other cadherins (Pece and Gutkind, 2002). Thus, Hakai may displace or compete with p120 binding and thereby favor the loss of cell junctions along with promoting cell motility (Fujita *et al.*, 2002). Hakai's interaction with E-cadherin appears to be antagonistic to p120 function and may also be linked to initiating a metastatic phenotype (Pece and Gutkind, 2002). In fact, all of the above pathways of E-cadherin degradation, if constitutive, may contribute to the onset of cancer.

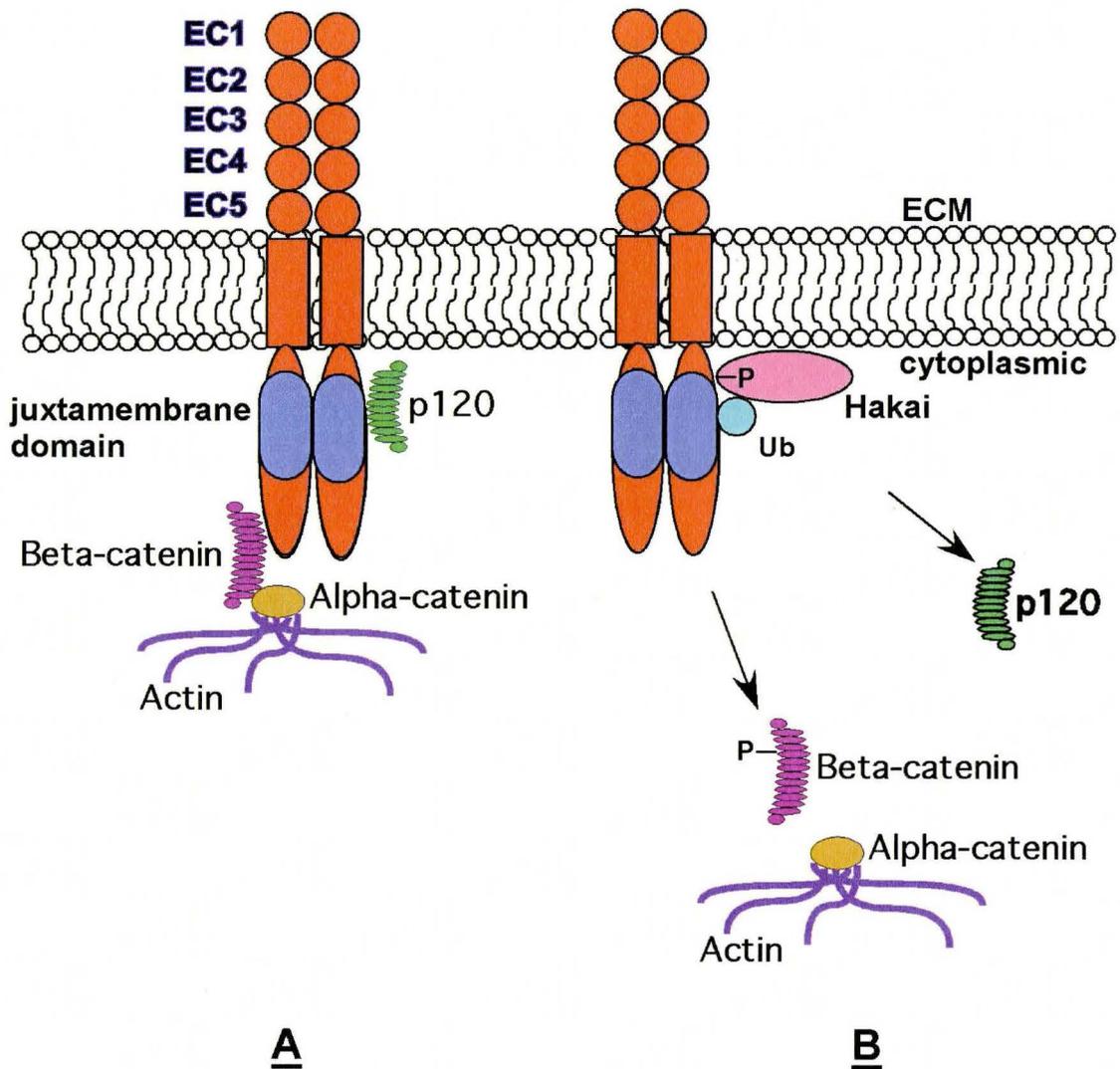


Figure 3: Model of Hakai binding to the cytoplasmic domain of E-cadherin, enhancing cell motility. In (A) p120 and other catenins bind E-cadherin and stabilize the cadherin so that it forms stable cell-cell adhesions as well as anchor it to the cytoskeleton. (B) Hakai binds the cytoplasmic domain of E-cadherin in a tyrosine phosphate-dependent manner and promotes E-cadherin proteolysis that destabilizes cell junctions (Fujita *et al.*, 2002).

1.8.2. E-cadherin turnover by presenilin-1/ γ -secretase

Another mechanism of E-cadherin turnover is determined by the presenilin-1/ γ -secretase system, which can cleave the cytoplasmic domain of E-cadherin and

disassemble the complex with the catenins, thereby destroying the *adherens junctions* (Marambaud *et al.*, 2002). Presenilin-1 (PS1) binds to the cytoplasmic domain of E-cadherin at the JMD, which has also been shown to be involved in p120 binding and as a result, p120 and PS1 may compete for the JMD. PS1, although involved in the cleavage system with γ -secretase, has been found to stimulate E-cadherin/catenin complex formation and associations with the cytoskeleton and like p120 it stabilizes E-cadherin aggregation (Baki *et al.*, 2001). The cleavage by γ -secretase appears to be mediated by PS1 binding and was found to occur between residues Leu731 and Arg732. It was also shown that metalloproteinase (MMP) cleaves at the extracellular face of the plasma membrane after residue Pro700. This fragment of E-cadherin is then further cleaved cytoplasmically by γ -secretase (Marambaud *et al.*, 2002), and this contributes to E-cadherin degradation.

1.9. The correlation of E-cadherin to cancer

The loss of E-cadherin was found to be one of the rate-limiting steps in the progression of cancer in the transition from adenoma to carcinoma. When the levels of E-cadherin are strongly reduced within a cell, a differentiated adenoma can become an invasive carcinoma. It is interesting to note that other cadherins, such as N-cadherin, were not down regulated in carcinogenesis. Thus, E-cadherin is the key cell-cell adhesion molecule required for maintaining a non-invasive phenotype within tumours.

Cadherin switching is a common cellular function during normal development and is usually a late event in cancer development. It is observed in tumour cells and

correlates with aggressive tumours that are able to invade adjacent tissue (Wheelock *et al.*, 2008). Epithelial to mesenchymal transition (EMT) includes a switch from E-cadherin to N-cadherin and it may result in an up-regulation of N-cadherin (Derycke and Bracke, 2004). It was shown that even low levels of N-cadherin in the presence of E-cadherin might contribute to increased motility of cells (Nieman *et al.*, 1999). As the cytoplasmic domain of E-cadherin and N-cadherin bind catenins in a similar fashion, the up-regulation of N-cadherin or of other 'inappropriate' cadherins may create a competition for p120 binding and decrease the available p120 to bind E-cadherin (Maeda *et al.*, 2006). The absence of E-cadherin expression also has the consequence that unbound catenins, such as β -catenin and p120, to enter the nucleus and increase the transcription of their target genes. Consequently, the release of catenins into the cytoplasm could contribute to an oncogenic phenotype (Perl *et al.*, 1998).

Aside from its role in the cytoplasm, it was demonstrated that the cytoplasmic domain of E-cadherin can be translocated to the nucleus via its interaction with p120 (Ferber *et al.*, 2008). Cadherin proteins are cleaved by γ -secretase following a cleavage by metalloproteases, a common method directing proteins to the nucleus (Fortini, 2002). This was previously determined for N-cadherin, whose cytoplasmic domain is released and translocated to the nucleus in neural crest cells (Shoval *et al.*, 2007). Once inside the nucleus, E-cadherin's cytoplasmic domain forms a complex with DNA via p120, thus relieving Kaiso's transcriptional repression of its target genes such as *matrilysin* (Spring *et al.*, 2005). Consequently, the localization of E-cadherin's cytoplasmic domain in the

nucleus has been linked to the suppression of cellular apoptosis, but the mechanism of this effect was not examined (Ferber *et al.*, 2008).

Overall, the versatile functions of E-cadherin within the cell make it an interesting topic for research in developmental and cancer biology.

1.10. The correlation of p120 to cancer

If there is no E-cadherin expression within the cell, p120 will not localize to the cell membrane but will remain in the cytoplasm in an unphosphorylated state (Thoreson *et al.*, 2000). Also, when levels of protein kinase C (PKC) were increased in the cell, p120 was found to localize to the nucleus, thus E-cadherin and PKC levels play a role in directing the subcellular localization of p120 (van Hengel *et al.*, 1999). With the loss of E-cadherin at the cell membrane (a result of p120 uncoupling) β -catenin was found to accumulate in the nucleus and to up-regulate transcription of cell proliferative genes (van Hengel *et al.*, 1999). The p120 nuclear localization signal (NLS) was identified in Arm repeats 6 and 7 at residues 622-629. Interestingly, these residues are also involved in the inhibition of RhoA GTPase, a protein required for cadherin clustering (Anastasiadis and Reynolds, 2000; Kelly *et al.*, 2004; Noren *et al.*, 2000). Once in the nucleus, p120 interacts with the BTB/POZ domain zinc finger transcription repressor Kaiso via Arm (1-7) repeats (Daniel and Reynolds, 1999). p120 inhibits Kaiso transcriptional repression activity by disturbing its DNA binding ability, and as a consequence Kaiso target genes are transcribed (Kelly *et al.*, 2004; Spring *et al.*, 2005; van Hengel *et al.*, 1999). When p120 binds Kaiso and inhibits its DNA binding, this allows β -catenin/TCF to bind to the promoters of Wnt signaling target genes, such as *matrilysin*, to increase their

transcription and promote an oncogenic phenotype (Spring *et al.*, 2005). A nuclear export signal (NES) was discovered in the human p120 exon B, but it is yet to be determined if the NES facilitates the transport of any p120 nuclear binding proteins, such as Kaiso, into the cytoplasm (van Hengel *et al.*, 1999).

The p120 homologues δ -catenin and ARVCF may substitute for p120 and maintain cell junction integrity to some extent. However, these two p120 homologues are poorly expressed in cells as compared to p120 (Davis *et al.*, 2003), so that despite their presence, the loss of p120 leads to deregulation of cell growth and to lung, colon, bladder, prostate, breast and other malignancies (Thoreson and Reynolds, 2002). Collectively these data indicate that p120 is an important protein required for normal cellular function.

1.11. An alternative approach to cancer therapeutics: Peptide engineering

Peptide aptamers are molecules consisting of a protein moiety with affinity for a target protein that is fused to and displayed from a protein scaffold (Hoppe-Seyler *et al.*, 2001). They are relatively easy to design with the knowledge of the protein's three-dimensional structure and may be applied to specifically target proteins to inhibit or enhance their function (Borghouts *et al.*, 2005; Kunz *et al.*, 2006; Lutz and Siahaan, 1997). In addition, peptides can be generated to mimic binding domains to replace protein functions within the cell. It is also recommended that the protein chosen for peptide design have a high-affinity for its partner(s) (Borghouts *et al.*, 2005; Kardinal *et al.*, 2001).

Peptide therapeutics could be administered in combination with other drugs such as small molecules (Borghouts *et al.*, 2005; Kunz *et al.*, 2006). Keeping in mind that cancer is a multifactorial disease involving several genes, targeting more than one mutation or defect in therapy may have advantages in preventing subsequent resistance from exposure to a single drug (Kardinal *et al.*, 2001; Prive and Melnick, 2006). This concept is also referred to as 'multi-focal signal modulation therapy' (MSMT) (McCarty, 2004).

1.12. Peptide aptamer design

Ideally, peptide aptamers should be 10-20 amino acids in length and should span a specific domain of the protein required for its function to be inhibited or mimicked (Borghouts *et al.*, 2005; Prive and Melnick, 2006). An example of mimicking a protein function is peptide aptamers designed from the Arm repeats of p120 that may have potential to replace endogenous p120 and stabilize E-cadherin at the cell membrane.

The stability of the peptide must also be considered and a means of protection from proteases should be incorporated into peptide design. Peptide stability was increased when they were designed with D-amino acids as compared to the naturally occurring L-amino acids. This made the peptides more resistant to proteolysis and they possessed a higher binding affinity (Hamamoto *et al.*, 2002; Prive and Melnick, 2006). As well, the correct folding of the peptide is required for optimal binding efficiency and function, and this can be enhanced by creating peptides that have both ends attached to a scaffold (constrained peptides) (Roisin *et al.*, 2004).

For therapeutic applications, the peptide must be able to cross cell membranes. One delivery vehicle is lipid encapsulation to form a liposome, which can easily pass the cell membrane. The alternative is fusion of a peptide to a protein transduction domain (PTD), also called cell-penetrating peptide (CPP), allowing it to be taken up by endosomes (Borghouts *et al.*, 2005; Noguchi and Matsumoto, 2006; Prive and Melnick, 2006). An example of a PTD is the HIV Tat peptide, which can be fused to the designed peptide to mediate cellular entry by macropinocytosis (Noguchi and Matsumoto, 2006; Prive and Melnick, 2006). CPPs have been shown to transport proteins larger than 100 kDa (Noguchi and Matsumoto, 2006). The effectiveness of this approach is currently under investigation, however, there is evidence that tumour growth may be inhibited by CPP-fused peptide aptamers *in vitro* (Buerger and Groner, 2003).

The reduction of toxicity or the immunogenic effect of the peptide should also be considered in its design. One would like to minimize the nonspecific binding of the peptide and would like to target only oncogenic cells, although such selectivity is technically challenging. To achieve cellular targeting it may be mediated by surface protein recognition by the peptide such as peptide fusion to localization signals (Hoppe-Seyler *et al.*, 2001). However, target cell specificity can be limiting depending on the type of cancer and cell type involved (Borghouts *et al.*, 2005). Further developments in this promising area will be necessary to increase the efficiency of peptide therapy.

2. PROJECT OBJECTIVE

The main objective of my thesis was to examine the *in vitro* interactions between p120, Hakai and E-cadherin proteins and to determine how these interactions contribute to the stability of *adherens junctions*. The characterization of the association of these proteins is critical for understanding how a tumour may become metastatic. *In vitro* interaction studies were performed to understand the molecular basis of their interaction in the absence of other cellular factors.

2.1. Hypothesis of Proposed Research

The stability of E-cadherin at the cell membrane is enhanced by p120 and inhibited by Hakai. My **hypothesis** is that peptide aptamers spanning the Armadillo domain of p120 will bind E-cadherin *in vitro* and stabilize E-cadherin *in vivo*. Similarly, peptides that span the cytoplasmic domain of E-cadherin will bind Hakai *in vitro* and sequester over-expressed Hakai *in vivo* thus preventing Hakai's association with E-cadherin. If efficient, peptide aptamers may be incorporated into combination therapy for patients suffering from solid tumours to prevent the metastasis of tumourigenic cells.

2.2. Outline of Research

The **specific aims** of the master's thesis were [1] to overproduce and purify critical domains of E-cadherin, Hakai and p120 involved in their interaction [2] study the protein-protein interactions between E-cadherin and its cytoplasmic binding partners p120 catenin (p120) and potentially Hakai *in vitro* [3] to design peptide aptamers to replace the endogenous proteins *in vitro* and eventually *in vivo*.

3. MATERIALS AND METHODS

3.1. Buffers and reagents

Table 1: The buffers and reagents used in the experimental protocols of this work

Strep-Tactin purification:

Strep-Tactin elution buffer	2.5 mM desthiobiotin in phosphate-buffered saline (PBS)
Regeneration buffer	1X HABA (2-[4'-hydroxy-benzeneazo] benzoic acid) (IBA)

Glutathione S-Transferase (GST) purification:

Phosphate Buffered Saline (PBS)	140 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄
GST elution buffer	10 mM glutathione in 50 mM Tris pH 8.0
Regeneration buffers	Buffer 1: 0.1 M Tris pH 8.5, 0.5 M NaCl Buffer 2: 0.1 M sodium acetate pH 4.5, 0.5 M NaCl
Glycerol dialysis buffer	PBS + 0.1 mM DTT, 50% glycerol

Cell lysis:

Phenylmethylsulfonyl Fluoride (PMSF)	100 mM stock dissolved in ethanol, final 0.5 mM (Roche Diagnostics)
Protease inhibitor cocktail tablets (Complete Mini-EDTA free)	1 tablet in 20 mL PBS for pellet re-suspension Proteases= Pancreas extract, Thermolysin (Metalloprotease), Chymotrypsin, Trypsin and Papain (Roche Diagnostics)
Leupeptin	100 mg/mL stock, final 0.1 mg/mL (Roche Diagnostics)
Aprotinin	2 mg/mL stock, final 0.002 mg/mL (Roche Diagnostics)
Lysozyme- Egg White	10 mg/mL stock, final 0.1 mg/mL (EM Science Merck)

DNase I 10 mg/mL stock, final 0.1 mg/mL (Roche Diagnostics)

Sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE):

4X Upper buffer (stacking gel) 0.5 M Tris/Cl pH 6.8, 0.4 % sodium dodecyl sulfate (SDS)

4X Lower buffer (separating gel) 1.5 M Tris/Cl pH 8.8, 0.4 % SDS

Ammonium persulfate (APS) 10 % APS in H₂O

2X Laemmli sample buffer 12.5 % 1 M Tris/Cl pH 6.8, 10 % β-mercaptoethanol, 4 % SDS, 20 % glycerol, ~ 0.1 g bromophenol blue

1X Running buffer 0.3 % Tris, 1.4 % glycine, 0.1 % SDS

Coomassie stain solution 30 % methanol, 10 % acetic acid, 0.1 % Coomassie Blue 250

Destaining solution 20 % methanol, 8 % acetic acid

Amido Black stain solution 45 % methanol, 10 % acetic acid, 0.1 % Amido Black

DNA Electrophoresis:

1X TAE 40 mM Tris pH 7.8, 20 mM acetic acid, 1 mM EDTA

6X Loading dye Purchased from Fermentas®

Western blot:

TBST 20 mM Tris pH 8.0, 137 mM NaCl, 0.1 % Tween-20

Blocking buffer 5 % Carnation powdered milk in TBST

Blotting buffer 1.44 % glycine, 1.51 % Tris pH 8.0, 20 % methanol

Chemoluminescence Reagents Amersham® ECL Detection Reagents 1 & 2, 4 mL of each mixed and used directly after

p120 primary antibody	Donated by Dr. J. Daniel- mouse monoclonal (15O2), used 1:1,000 diluted
E-cadherin primary antibody	Mouse monoclonal (BD Transduction Laboratories), used 1:1,000 diluted
GST-tag primary antibody	GST (B-14) mouse monoclonal (Santa Cruz Biotechnology), used 1:1,000 diluted
Strep-tagII primary antibody	Strep-tagII specific mouse monoclonal (IBA), used 1:1,000 diluted
Goat Anti-mouse secondary antibody	Goat Anti-mouse IgG (H+L)-HRP Conjugate (Bio Rad), used 1:3,000 diluted
Goat Anti-rabbit secondary antibody	Goat Anti-rabbit IgG (H+L)-HRP Conjugate (Bio Rad), used 1:3,000 diluted

β-galactosidase Activity Assay

ortho-Nitrophenyl-β-galactoside	Stock 8 mg/mL in Z-buffer, final 0.008 mg/mL
Z-buffer (J. Sambrook, 1989)	0.06 M Na ₂ HPO ₄ x 7H ₂ O, 0.04 M NaH ₂ PO ₄ x H ₂ O, 0.01 M KCl, 0.001 M MgSO ₄ x 7H ₂ O, pH 7.0
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	Stock 40 mg/mL, final 0.04 mg/mL dissolved in dimethylformamide
ONPG buffer	8 μg/mL ONPG, 0.3 % β-mercaptoethanol, 0.013 % SDS, in Z buffer

In vitro transcription/translation protein pull down

Binding Buffer	25 mM Hepes pH 7.5, 100 mM NaCl, 10 % glycerol, 3 mM β-mercaptoethanol, 0.1% NP-40, 3.4 μg/μl PMSF, 10 μg/mL Leupeptin
Gel drying solution	25 % Isopropanol, 10 % Acetic Acid

Amplification solution Amersham Biosciences NAMP100V Amplify

3.2. Media and antibiotics

Table 2A: Media for bacterial growth

Luria broth (LB) 0.5% yeast extract, 1% tryptone, 1% NaCl (1.5% agar for plates)

Table 2B: Media for yeast growth

Yeast Extract Peptone Dextrose Medium (YEPD) 1 % yeast extract, 2 % peptone, 2 % dextrose (2 % agar for plates)

Media for intracellular expression in yeast

Minimal Glycerol/Methanol Medium Histidine (MGYH or MMH) 1.34 % yeast nitrogenous base (YNB), 1 % glycerol or 0.5 % methanol*, 4 x 10⁻⁵ biotin, 0.004 % histidine

Media for secreted expression in yeast

Buffered Minimal Glycerol/Methanol Histidine 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 1 % glycerol or 0.5 % methanol*, 4 x 10⁻⁵ biotin, 0.004 % histidine

Buffered Glycerol/Methanol complex Medium 1 % yeast extract, 2 % peptone 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 4 x 10⁻⁵ biotin, 1 % glycerol or 0.5 % methanol*

**during induction, methanol must be added daily to a concentration of 0.5 %*

Table 3A: Antibiotics used for selective bacterial growth

<i>Antibiotic:</i>	<i>Stock concentration:</i>	<i>Final concentration:</i>
Carbenicillin (Carb)	100 mg/mL in H ₂ O	0.1 mg/mL
Kanamycin (Kan)	50 mg/mL in H ₂ O	0.05 mg/mL
Chloramphenicol (Cm)	34 mg/mL in ethanol	0.034 mg/mL or 0.015 mg/mL*

*The concentration of Cm is lower in large-scale growth to allow for faster growth since the higher concentration would retard the bacterial growth by several hours.

Table 3B: Antibiotic used for selective yeast growth

<i>Antibiotic:</i>	<i>Stock concentration:</i>	<i>Final concentration:</i>
Zeocin (Zeo)	100 mg/mL in H ₂ O	0.1 mg/mL

3.3. Cloning into expression vectors

3.3.1. List of strains:

Table 4A: Bacterial Strains

<i>Strain name:</i>	<i>Characteristics/Genotype:</i>	<i>Reference/Source:</i>
<i>E. coli</i> JM109	<i>endA1 gyr96 thi hsdR71 supE44 recA1 relA1</i> $\Delta(lac-proAB)$ (<i>F'</i> <i>traD36 proAB⁺ lacI^q</i> <i>lacZ</i> Δ M15)	(Yanisch-Perron <i>et al.</i> , 1985)
<i>E. coli</i> BL21 (DE3)	<i>F' ompT hsd S_B (r_B⁻m_B⁻) gal dcm</i> (DE3)	Lab collection
<i>E. coli</i> Rosetta (DE3) pLysS	<i>F' ompT hsd S_B (r_B⁻m_B⁻) gal dcm</i> (DE3) pLysSRARE (Cm ^R)	(Novagen, 2006)
<i>E. coli</i> BL21 Star (DE3)	<i>F' ompT hsd S_B (r_B⁻m_B⁻) gal dcm rne131</i> (DE3)	(Invitrogen, 2002)
<i>E. coli</i> BTH101	<i>F'</i> , <i>cya-99, araD139, galE15, galK16, rpsL1</i> (Str ^R), <i>hsdR2, mcrA1, mcrB1</i>	(Karimova <i>et al.</i> , 2000)

Table 4B: Yeast Strains

<i>Strain name:</i>	<i>Genotype/Phenotype:</i>	<i>Function</i>	<i>Reference:</i>
X-33	Wildtype/ Mut ⁺	Create new mutants	(Invitrogen, 2007)
GS115	<i>his4/ His⁻, Mut⁺</i>	High expression	(Invitrogen, 2007)
KM71H	<i>Arg4 aox1::ARG4/ Mut^s, Arg⁺</i>	Low expression	(Invitrogen, 2007)
GS115/Albumin	<i>HIS4/ Mut^s</i>	Secreted control	(Invitrogen, 2007)
GS115/pPICZ/lacZ	<i>his4/ His⁻, Mut⁺</i>	Intracellular control	(Invitrogen, 2007)

3.3.2. List of primers

Table 5: The primers for sub-cloning and sequencing

Primer	Restriction Site (bold underline)
Over-expression in <i>E. coli</i>:	
<i>Human Hakai full length into pT7-7StrepII</i>	
Forward: 5'-GCGCG GGTACCC ATGGATCACACTGACAATGAG	<i>Acc65I(KpnI)</i>
Reverse: 5'-CGAG GTCGACTT CATTGGTAATACGGTCTATATCT	<i>Sall</i>
<i>Internal Sequencing primers for Hakai (murine or human- 80 nt from 5' of gene to check if N-terminal tag is in frame)</i>	
Reverse: 5'-TTGTTTGGAGATGAGCTTTATAGG	
<i>Human p1201ABC into pGEX4T1</i>	
Forward: 5'- CGCGGAG CGGCCG CATATGGACGACTCAGAGGTGGAG	<i>NotI</i>
Reverse: 5'- GGCCAT GCGGCCG CTCAATCTTCTGACTGGAGGGGTA	<i>NotI</i>
<i>Human p1201A isoform into pT7-7StrepII</i>	
Forward: 5'-CCGGC GGTACCC ATGGACGACTCAGAGGTGGAG	<i>KpnI</i>
Reverse: 5'-GCGC GAATTCT AAATCTTCTGCATCAAGGGTGT	<i>EcoRI</i>
<i>Human p120 Armadillo domain into pGEX4T1</i>	
Forward: 5'-GCG GTCGACT CAGCCTGCGCAAAGGAGGG	<i>Sall</i>
Reverse: 5'- CGAT GCGGCCG CTCTTACCAGATTGTCTGTAATACAAGTGC	<i>NotI</i>
<i>Human E-cadherin cytoplasmic domain into pT7-7StrepII</i>	
Forward: 5'- GCGC GGTACCC CGGAGGAGAGCGGTGGTCAAAGAGC	<i>Acc65I(KpnI)</i>
Reverse: 5'-CGGC CTGCAG CTAGTCGTCCTCGCCGCCTCCGTA	<i>PstI</i>
<i>Inverse primers for generation of E-cadherin cytoplasmic domain mutation to yield protein variant Y755E and Y756E</i>	
Forward: 5'- GACACCCGGGACAACGTT GAGGA AATATGATGAAGAAGGAGGC	<i>Mutation bolded</i>
Reverse: 5'- GCCTCCTTCTTCATCATAT TCCTCA ACGTTGTCCCGGGTGTGTC	<i>Mutation bolded</i>
<i>Oligonucleotides of linker sequence to generate pGEX4T1-GST-6XHis</i>	
Forward: 5'- GGCCG CATATCGAAGGTCGTCATCACCATCACCATCACTG AC	<i>Partial NotI/ Partial EagI</i>
Reverse: 5'- GGCCG TCAGTGATGGTGATGGTGATGACGACCTTCGATAT GC	<i>Partial EagI/ Partial NotI</i>
<i>Human Hakai for dual tags in pGEX4T1-GST-6XHis</i>	
Forward: 5'- GCCC GTCGACT CATGGATCACACTGACAATGAG	<i>Sall</i>

Reverse: 5'- CGGCAT <u>GCGGCCG</u> CTCTTGGTAATACGGTCTATATCTTG	<i>NotI</i>
Bacterial-2-hybrid cloning:	
<i>Sequencing primer for pUT18C</i>	
Reverse: 5'-CTTAACTATGCGGCATCAGAGC	
<i>Hakai for pUT18C</i>	
Forward: 5'-CGGCACTCTAGAGATGGATCACACTGACAATGAG	<i>XbaI</i>
Reverse: 5'-GGCCAGGTACCCGTCATTGGTAATACGGTCTATATC	<i>KpnI</i>
<i>p120IA for pUT18C</i>	
Forward: 5'-GCACTCTAGAGATGGACGACTCAGAGGTGGAG	<i>XbaI</i>
Reverse: 5'-GCCGGTACCCGCTAAATCTTCTGCATCAAGGGTGT	<i>KpnI</i>
<i>E-cadherin cytoplasmic domain for pUT18C:</i>	
Forward: 5'-GCACTCTAGAGCGGAGGAGAGCGGTGGTCAAAGAG	<i>XbaI</i>
Reverse: 5'-CGCGGTACCCGCTAGTCGTCTCGCCGCCTCCGTA	<i>KpnI</i>
<i>p120 Armadillo domain for pUT18C</i>	
Forward: 5'-GCACTCTAGAGAGCCTGCGCAAAGGAGGGCCT	<i>XbaI</i>
Reverse: 5'-GCCGGTACCCGTTACCAGATTGTCTGTAATACAAG	<i>KpnI</i>
<i>p120 Armadillo repeats 3-5 for bicistron pUT18C-p120Arm-Shine Dalgarno (SD)</i>	
Forward: 5'-GCCCCATGGATGTTTGGACGTGACCAGGATAAC	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTATGATAAGTTCCGAAGAAGGCA	<i>EcoRI</i>
<i>p120 Armadillo domain repeats 3-5 for pUT18C</i>	
Forward: 5'-GCACTCTAGAGTTTGGACGTGACCAGGATAAC	<i>XbaI</i>
Reverse: 5'-GCCGGTACCCGTTATGATAAGTTCCGAAGAAGGCA	<i>KpnI</i>
Spanning Armadillo domains peptides for BACTH system:	
<i>p120 Armadillo domains 2 to 3 (aa 416-458)</i>	
Forward: 5'-GCCCCATGGATGGGATTGTTAGACCATCCCAA	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTATCGCACAAAGGGCAGGCACACC	<i>EcoRI</i>
<i>p120 Armadillo domains 3 to 4 (aa 459-509)</i>	
Forward: 5'-GCCCCATGGATGTTGCTTCGAAAGGCTCGTGAT	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTACTCCCAACCAGAATGAGGAAT	<i>EcoRI</i>
<i>p120 Armadillo domains 4 to 5 (aa 510-562)</i>	
Forward: 5'-GCCCCATGGATGCGGGAACCTAATGAAGACTGT	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTAAACAATGAAAATGAGGGCATC	<i>EcoRI</i>
<i>p120 Armadillo domains 5 to 6 (aa 563-616)</i>	
Forward: 5'-GCCCCATGGATGCAGGCTGAGATTGGGCAGAAG	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTAGGCAGCATGTGGCCAGTATT	<i>EcoRI</i>
Primers for constrained peptides:	
<i>Creating Thioredoxin A in pUT18C-p120Arm as a bicistron</i>	
Forward: 5'-GCCCCATGGATGAGCGATAAAATTATTCACCTG	<i>NcoI</i>
Reverse: 5'-GCGGAATTCCTTAGGCCAGGTTAGCGTCGAGGAACTC	<i>EcoRI</i>
<i>Constrained p120Arm 4-5sp in bicistron with p120Arm in pUT18C</i>	

Forward: 5'-GCCTGGT <u>GCGGTCCG</u> CGGGAACCTAATGAAGACTGT	<i>RsrII</i>
Reverse: 5'-GCAC <u>GGA</u> CCGCCAACAATGAAAATGAGGGCATC	<i>RsrII</i>
<i>Constrained p120Arm 4-5sp with T18 N-ter fragment</i>	
Forward: 5'-GCCACTCTAGAGATGAGCGATAAAATTATTCACCTG	<i>XbaI</i>
Reverse: 5'- GCCC <u>GGTAC</u> CCGTTAGGCCAGGTTAGCGTCGAGGAACTC	<i>KpnI</i>
<i>Constrained p120Arm 3-4sp in bicistron with p120Arm in pUT18C</i>	
Forward: 5'-GCCTGGT <u>GCGGTCCG</u> TTGCTTCGAAAGGCTCGTGAT	<i>RsrII</i>
Reverse: 5'-GCAC <u>GGA</u> CCGCCCTCCCAACCAGAATGAGGAAT	<i>RsrII</i>
<i>In vitro transcription and translation:</i>	
<i>p120 Armadillo domain with GST tag in T7 vector</i>	
Forward: 5'-GCCGGTACCAGCCTGCGCAAAGGAGGGCCT	<i>Acc65I(KpnI)</i>
Reverse: 5'-CGGG <u>GTCGACT</u> CCTTATCCCCAGATTGTCTGTAATAC	<i>Sall</i>
<i>Hakai with GST tag in T7 vector</i>	
Forward: 5'-GCC <u>GGTACC</u> ATGGATCACACTGACAATGAG	<i>Acc65I(KpnI)</i>
<i>p120 Armadillo domain without tag in T7 vector</i>	
Forward: 5'-GCC <u>GGTACC</u> ATGAGCCTGCGCAAAGGAGGGCCT	<i>Acc65I(KpnI)</i>
<i>Spanning peptide of p120Arm 3-4 with GST tag in pT7-GST</i>	
Forward: 5'-GCC <u>GGTACC</u> TTGCTTCGAAAGGCTCGTGAT	<i>Acc65I(KpnI)</i>
Reverse: 5'-CGGCTC <u>AGCTG</u> GTCCTCCCAACCAGAATGAGGAAT	<i>Sall</i>
<i>Spanning peptide of p120Arm 4-5 with GST tag</i>	
Forward: 5'-GCC <u>GGTACC</u> CGGGAACCTAATGAAGACTGT	<i>Acc65I(KpnI)</i>
Reverse: 5'-CGGCTC <u>AGCTG</u> TTAAACAATGAAAATGAGGGCATC	<i>Sall</i>
<i>Over-expression in P. pastoris:</i>	
<i>Intracellular expression of p120 Armadillo repeats in pPICZA</i>	
Forward: 5'- GCGAGGAATTCACCATAATGTCTAGCCTGCGCAAAGGAGGGCC T	<i>EcoRI</i>
Reverse: 5'- GCCTGG <u>GCGGCCG</u> CCTCCCCAGATTGTCTGTAATACAGGTGC	<i>NotI</i>
<i>Intracellular expression of Hakai in pPICZA</i>	
Forward: 5'- GCG <u>ACCTCGAG</u> CCGCATAATGTCTATGGATCACACTGACAATG AG	<i>XhoI</i>
Reverse: 5'-CGGG <u>GCGGCCG</u> CCTTGTAATACGGTCTATATCT	<i>NotI</i>
<i>Intracellular expression of E-cadherin cytoplasmic domain in pPICZA</i>	
Forward: 5'- GCGAGGAATTCACCATAATGTCTCGGAGGAGAGCGGTGGTCAA AGAG	<i>EcoRI</i>
Reverse: 5'- GCCTGG <u>GCGGCCG</u> CGTCGTCCTCGCCGCTCCGTACAT	<i>NotI</i>

<i>Secreted expression of p120 Armadillo domain in pPICZαC</i>		
Forward: 5' - GCGAT GAATTC AAGCCTGCGCAAAGGAGGG		<i>EcoRI</i>
Reverse: 5' - GCGGG CGGCCG CCAGATTGTCTGTAATACAAGTGC		<i>NotI</i>
<i>Secreted expression of Hakai in pPICZαC</i>		
Forward: 5' - GCGT CGGTACCT TATGGATCACACTGACAATGAG		<i>KpnI</i>
Reverse: 5' - GCGT TCTAGA AATTGGTAATACGGTCTATATCT		<i>XbaI</i>
<i>Secreted expression of E-cadherin cytoplasmic domain in pPICZαC</i>		
Forward: 5' - GCGAT GAATTC ACGGAGGAGAGCGGTGGTCAAAGAG		<i>EcoRI</i>
Reverse: 5' - GCGGG CGGCCG CGTTCCTCGCCGCCTCCGTACAT		<i>NotI</i>

3.3.3. List of expression plasmids

Table 6A: Bacterial Expression Plasmids

Vector	Inserted gene	Reference
<i>E. coli</i> over-expression		
pGEX4T1	Carb ^R , tac promoter expression vector, for expression of N-terminal GST fusion	Dr. J. Daniel
pT7-7StrepII	Carb ^R , T7 promoter expression vector, for expression of N-terminal Streptavidin (StrepII) fusion	(Balsiger <i>et al.</i> , 2004)
pGEX4T1-GST-Hakai	Human Hakai full-length N-terminal GST tag, contains a 1476bp insert encoding aa 1-492 of the full length <i>Homo sapiens</i> Hakai for expression	This work
pGEX4T1-GST-6XHis	Inserted Factor Xa cleavage upstream newly inserted 6X His tag	This work
pGEX4T1-GST-Hakai-6XHis	Human Hakai full-length N-terminal GST tag C-terminal 6X His tag	This work
pGAD-E-cadherin	Vector used to amplify Human E-cadherin cytoplasmic domain	Dr. J. Daniel
pT7-7StrepII-Ecadc	Human E-cadherin cytoplasmic domain N-terminal StrepII tag, contains a 456 base pair (bp) insert encoding amino acids (aa) R732-D882	This work
pT7-7StrepII-Hakai	Human Hakai full length N-terminal StrepII tag	This work
pT7-7StrepII-EcadcV	Human E-cadherin cytoplasmic domain Variant Y755E, Y756E N-terminal StrepII tag	This work

pBlueScript-p1201A	Vector used to amplify Human p120 isoform 1A and Armadillo domain	Dr. J. Daniel
pGEX4T1-p120Arm	Human p120 Armadillo domain N-terminal GST-tag, contains a 1422bp insert encoding the Armadillo domain aa S352-G825	This work
pT7-7StrepII-p1201A	Human p120 isoform 1A N-terminal StrepII tag	This work
<i>Bacterial-2-hybrid expression</i>		
pUT18C	pUC19 derivative expressing T18 fragment of Cya	(Karimova <i>et al.</i> , 2000)
pKT25	pACYC184 derivative expressing T25 fragment of Cya	(Karimova <i>et al.</i> , 2000)
pUT18C-Zip	Leucine zipper fused to T18 fragment (225-399 aa of CyaA)	(Karimova <i>et al.</i> , 2000)
pKT25-Zip	Leucine zipper fused to T25 fragment (1-224 aa of CyaA)	(Karimova <i>et al.</i> , 2000)
pUT18C-Hakai	Human Hakai full length N-terminal T18 fragment	This work
pKT25-Hakai	Human Hakai full length N-terminal T25 fragment	This work
pUT18C-p1201A	Human p120 1A isoform N-terminal T18 fragment	This work
pKT25-p1201A	Human p120 1A isoform N-terminal T25 fragment	This work
pUT18C-Ecadc	Human E-cadherin cytoplasmic domain N-terminal T18 fragment	This work
pKT25-Ecadc	Human E-cadherin cytoplasmic domain N-terminal T25 fragment	This work
pUT18C-p120Arm	Human p120 Armadillo domain N-terminal T18 fragment	This work
pKT25-p120Arm	Human p120 Armadillo domain N-terminal T25 fragment	This work
pUT18C-EcadcV	Human E-cadherin cytoplasmic domain Variant Y755E, Y756E N-terminal T18 fragment	This work
pKT25-EcadcV	Human E-cadherin cytoplasmic domain Variant Y755E, Y756E N-terminal T25 fragment	This work
pUT18C-mHakai	Murine Hakai N-terminal T18 fragment	This work
pKT25-mHakai	Murine Hakai N-terminal T25 fragment	This work

pUT18CFB8a+SG	Bicistron vector created to express T18 fused protein and a second protein separated by a Shine Dalgarno (SD) sequence	D. Sivanesan
pUT18C-p120Arm-SD-p120Arm3-5	<i>Bicistron</i> : p120 Armadillo domain with N-terminal T18 fragment and Armadillo repeats 3 to 5 separated by SD	This work
pUT18C-p120Arm3-5	p120 Arm repeats 3 to 5 N-terminal T18 fragment	This work
pUT18C-p120Arm-SD-p120Arm2-3sp	Bicistron of T18-p120Arm and Spanning peptide of p120 Armadillo repeats 2-3 (aa416-458)	This work
pUT18C-p120Arm-SD-p120Arm3-4sp	Bicistron of T18-p120Arm and Spanning peptide of p120 Armadillo repeats 3-4 (aa459-509)	This work
pUT18C-p120Arm-SD-p120Arm4-5sp	Bicistron of T18-p120Arm and Spanning peptide of p120 Armadillo repeats 4-5 (aa510-562)	This work
pUT18C-p120Arm-SD-p120Arm5-6sp	Bicistron of T18-p120Arm and Spanning peptide of p120 Armadillo repeats 5-6 (aa563-616)	This work
pT7-H ₆ TrxFus	T7 promoter expression vector, for expression N-terminal H6 TrxA affinity peptide fusions	(Kromayer <i>et al.</i> , 1996)
pUT18C-p120Arm-SD-Trx	Bicistron with T18-p120Arm and Thioredoxin A (TrxA) needed to create constrained peptides	This work
pUT18C-p120Arm-SD-TrxArm3-4sp	Bicistron with T18-p120Arm and Thioredoxin A with spanning peptide of Armadillo repeats 3-4	This work
pUT18C-p120Arm-SD-TrxArm4-5sp	Bicistron with T18-p120Arm and Thioredoxin A with spanning peptide of Armadillo repeats 4-5	This work

Table 6B: Yeast Expression Plasmids

<i>Vector</i>	<i>Inserted gene</i>	<i>Reference</i>
pPICZαC	Zeo ^R , For secreted expression, C-terminal myc and 6XHis	(Invitrogen, 2007)
pPICZA	Zeo ^R , For intracellular expression, C-terminal myc and 6XHis	(Invitrogen, 2007)
pPICZαC-p120Arm	Human p120 Armadillo domain for secreted expression C-terminal myc and 6X His tag	This work
pPICZαC-Hakai	Human Hakai for secreted expression C-terminal myc and 6X His tag	This work

pPICZ α C-Ecadc	Human E-cadherin cytoplasmic domain for secreted expression C-terminal myc and 6X His tag	This work
pPICZA-p120Arm	Human p120 Armadillo domain for intracellular expression C-terminal myc and 6X His tag	This work
pPICZA-Hakai	Human Hakai for intracellular expression C-terminal myc and 6X His tag	This work
pPICZA-Ecadc	Human E-cadherin cytoplasmic domain for intracellular expression C-terminal myc and 6X His tag	This work

Table 6C: *In vitro* transcription and translation plasmids

<i>Vector</i>	<i>Inserted gene</i>	<i>Reference</i>
pT7-GST-p120Arm	T7 promoter with GST tagged p120 Armadillo repeats	This work
pT7-GST-Hakai	T7 promoter with GST tagged Hakai	This work
pT7-GST-p120Arm3-4sp	Spanning peptide of p120 Armadillo repeats 3-4	This work
pT7-GST-p120Arm4-5sp	Spanning peptide of p120 Armadillo repeats 4-5	This work

3.4. Construction of plasmids

3.4.1. Sub-cloning the E-cadherin cytoplasmic domain encoding gene into pT7-7StrepII

The sequence encoding this protein (residues 732-882) in pGAD-E-cadherin was first amplified using polymerase chain reaction (PCR) with primers E-cadherin 5' and E-cadherin 3' (Table 6A) and then sub-cloned into the pT7-7StrepII vector for over-expression. The PCR reaction comprised 95°C for 3mins, [55°C for 1 min, 72°C for 1.5 min, 95°C for 0.5 min], 55°C for 1 min and repeated the enclosed cycle 30 times, followed by incubation at 72°C for 7 min and was held at 4°C. The amplified PCR product and the pT7-7StrepII vector were initially restricted with 5' *Acc65I* (*KpnI*) and 3'

*Pst*I. This was followed by purification using a gel extraction kit (Qiagen) and ligation for 16 h at 14°C. The ligation reaction was subjected to butanol purification, followed by transformation into *Escherichia coli* JM109 and plating on Luria Broth (LB) with carbenicillin (0.1 mg/ml). The following day the DNA from 22 colonies was subjected to a PCR screen, using the E-cadherin primers (Table 5) to detect the presence of the E-cadherin DNA insert. The PCR reaction followed the above cycle except the first step at 95°C was for 5 min in order to lyse the cells used as the template. From this screen, two clones were selected for sequencing (Mobix, McMaster University) in order to ensure that the gene was without mutations.

3.4.2. Sub-cloning the p120 Armadillo repeat domain encoding gene into pGEX4T1

The p120 gene region encoding 10 Armadillo repeats (p120Arm), residues 352-825 (Anastasiadis and Reynolds, 2000), was amplified using primers p120Arm 5' and p120Arm 3' (Table 5) and template pBlueScript-p1201A (Table 6A). The amplified insert was sub-cloned into the pGEX4T1 vector using restrictions 5' *Sall* and 3' *Not*I, downstream of the glutathione-S-transferase (GST) encoding sequence in order to fuse N-terminal GST to the p120 Armadillo repeats (GST-p120Arm) in the expressed protein. The rest of the sub-cloning procedure was conducted as described above except that the ligation of the restricted fragments was conducted at 22 °C for 3 h.

3.4.3. Sub-cloning the Hakai gene into pGEX4T1

The Hakai gene was sub-cloned into the pGEX4T1 vector to enable expression of an N-terminal GST fusion protein. The amplification of the Hakai gene was performed

using primers Hakai 5' and Hakai 3' (Table 5) and cDNA prepared from total RNA of HeLa cells (Firouzchian, 2005). The Hakai insert and pGEX4T1 vector were restricted by 5' *Sall* and 3' *NotI* and purified. The ligation of the restriction fragments was conducted at 22 °C for 3 h and a PCR screen was used to select two clones for sequencing.

Subcloning and amplification of these genes for the bacterial-2-hybrid assays and *in vitro* transcription and translation experiments were performed in a similar manner with restriction sites specific to the primer and vector outlined in Tables 5, 6A, 6B and 6C.

3.4.4. Inverse PCR to create *E-cadherin* variant Y755E, Y756E

Primers were designed to overlap the gene in the region encoding the two tyrosine residues Y755E and Y756E of *E-cadherin* in the 5' and 3' direction. The primers encoded glutamic acid in the place of tyrosine depicted in Table 5. The PCR reaction followed 95 °C for 5mins, [60 °C for 0.5 min, 72 °C for 2.1 min, 95 °C for 0.5 min] repeated enclosed cycle 10 times, [60 °C for 0.5 min, 72 °C for 2.1 min increasing 3 °C every cycle, 95 °C for 0.5 min] repeated enclosed cycle 30 times, 60 °C for 0.5 min, 72 °C for 10 min and was stopped at 4 °C. As the inverse reaction requires replication of the entire plasmid, the plasmid encoding the wildtype *E-cadherin* cytoplasmic domain in pT7StrepII-Ecadc (2992 bp) was amplified, as it is the smallest vector with the Ecadc insert. A *DpnI* treatment was performed to remove the parental vector and the product was purified through gel extraction. The sequence change in pT7StrepII-Ecadc (Y755E, Y756E) was verified by sequence analysis and the gene was then subcloned into a new

pT7StrepII vector. The subcloning was performed because the entire pT7StrepII-Ecadc was not sequenced and through PCR amplification stop codons could have randomly been included on the vector outside of the gene and this could potentially affect protein expression.

3.4.5. Creation of dual tag vector from pGEX4T1 with addition of C-terminal 6X His

A dual tag vector was generated for the purification of expressed proteins. The original pGEX4T1 expresses a protein with an N-terminal GST tag that may be cleaved by Thrombin (site: L, V, P, R, G, S). pGEX4T1 was engineered to express a protein with a C-terminal 6X His tag preceded by a Factor Xa cleavage site (I, E, G, R) for subsequent tag removal if necessary. pGEX4T1 was digested with *NotI* (oligos designed so that the *EagI* restriction site (C/GGCCG) in the 3' end of the linker allows the *NotI* site (GC/GGCCGC) to remain unique). The plasmid was then treated with alkaline phosphatase to remove 5' phosphates and prevent reannealing during ligation (50 μ L reaction, 40 μ L plasmid, 5 μ L 10X CIAP buffer, 2 μ L CIAP (Fermentas®), 3 μ L H₂O at 37 °C for 0.5 h). To create the 6XHis insert, the oligonucleotides were ordered with 5' phosphorylation and diluted to 100 pmol/ μ L. The linker was annealed (1 μ L 6XHis oligo 5', 1 μ L 6X His oligo 3', 5 μ L 120 mM NaCl (sterile) and 3 μ L H₂O), boiled for 10 min, and cooled to room temperature. Ligation was performed as previously stated. The sequence of the clone (pGEX4T1-GST-6XHis) was verified by sequencing.

3.5. Transformation, expression experiments and large-scale growth and purification

3.5.1. Transformation of plasmids into *E. coli*

The plasmids were transformed by electroporation into three over-expression strains of *E. coli* BL21 DE3, BL21 Star DE3, and Rosetta DE3 pLysS (genotype in Table 4A, strain characteristics explained in section 4.2.1). In this reaction, 100 μL of competent cells and 1 μL of purified plasmid were mixed, kept on ice for 1 min, and transferred into the electroporating cuvettes on ice. Electrocompetent cells were made in advance as described in the following and stored at $-80\text{ }^{\circ}\text{C}$ until further use. To this end, a 3 mL overnight culture of *E. coli* was grown at $37\text{ }^{\circ}\text{C}$ and used to inoculate 100 mL of LB to OD_{600} 0.05. The cells were grown at $37\text{ }^{\circ}\text{C}$ and 200 rpm for 2-3 h until the OD_{600} reached 0.5-0.7. The culture was then cooled on ice for 30 min and collected by centrifugation at $4\text{ }^{\circ}\text{C}$ for 15 min at 3000 rpm. The pellet was resuspended with 100 mL of ice-cold sterile water and re-spun at the same speed and temperature. This was then repeated with 50 mL of ice-cold sterile water. The pellet was then resuspended in 30 mL of 10 % sterile glycerol and centrifuged similarly. The final pellet was resuspended in 0.5-1 mL of 10 % sterile glycerol and divided into 1.5 mL cryogenic safe snap cap vials in 50-100 μL aliquots. The vials were frozen in liquid nitrogen before storage at $-80\text{ }^{\circ}\text{C}$. Note: for transformation of 1 μL of purified vector, 50 μL of cells is sufficient. For transformation after ligation, the precipitated DNA pellet was first suspended in 10 μL of sterile water and mixed with 100 μL of competent cells. The electroporation setting was 2500 volts using the Eppendorf® 2510 Electroporator. To allow the cells to recuperate

from the transformation, they were inoculated into 1 mL LB in a 15 mL Falcon growth tube and incubated at 37 °C for 1 h. The cells were then plated on LB agar plates with antibiotics. In addition to the antibiotic selection for cells carrying the transformed vector, the Rosetta DE3 strain had to be cultured with 0.034 mg/mL of Cm for selection of the codon plus plasmid pLysSRARE. The plates were incubated at 37 °C overnight and single colonies were used for the following expression experiments.

3.5.2. Bacterial small scale protein expression experiments

The transformed *E. coli* strains were used to inoculate 8 mL LB media with selective antibiotics. The 8 mL *E. coli* culture was incubated at 37 °C and shaken at 200 rpm for 16 h. The following day, 50 mL of LB in a 250 mL autoclaved flask was inoculated with the *E. coli* culture to an OD₆₀₀ of 0.05. The 50 mL *E. coli* culture included 0.1 mg/mL of Carb and, for Rosetta DE3 only, 0.034 mg/mL of Cm. Each strain was inoculated into three flasks of LB and cultivated at 37 °C at 200 rpm until the OD₆₀₀ reached 0.5. At this OD₆₀₀, the bacterial cultures were induced to maximize the protein production during the log phase of bacterial growth. A 1 mL sample of the cells before induction was taken and the cells were sedimented by centrifugation at 13,000 rpm for 2 min at room temperature and stored at 4 °C. The cultures were then transferred to 37 °C, 26 °C, or 15 °C, respectively, and gene expression was induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The expression experiments for Strep-Ecade were performed at 37 °C, 30 °C, or 15 °C as suggested by the literature (Zheng *et al.*, 2004). Samples of 1 mL from the cultures were taken every hour for 3 h to

monitor their protein production and growth and then analyzed by SDS-PAGE. After 3 h the cultures incubated at 37 °C were harvested by centrifugation in 50 mL Falcon tubes on a tabletop centrifuge 4 °C for 15 min at 10,000 rpm. The cultures at 26 °C and 15 °C were incubated for 16 h prior to harvesting. The pellets were stored at 4 °C until lysis. In order to lyse the cells, the pellets were suspended in 0.7 mL of PBS; the suspension was added to 2 mL autoclaved conical screw cap micro centrifuge tubes with 1 g of BioSpec Product Inc. 0.1 mm glass beads. The cell lysis was performed using the BioSpec Products Inc. Mini Beadbeater at the homogenization setting at 4 °C by beating for 1 min, followed by incubation on ice for 1 min, and repeating the above steps three times. The tubes were then centrifuged at 13,000 rpm for 30 min at 4 °C to separate the soluble fraction (supernatant), which was analyzed by SDS-PAGE as we were interested in soluble proteins from the bacterial cellular debris sedimented with the glass beads. The samples taken during the growth were re-suspended in 2X Laemmli Sample Buffer (Laemmli, 1970) and the volume of the buffer was adjusted based on the OD₆₀₀ (OD₆₀₀ of 1 = 0.05 mL of 2X Laemmli sample buffer). The soluble cell lysate was mixed with sample buffer in a 1:1 ratio. All samples were boiled for 5 min and electrophoresed on 7 %, 10 % or 12 % polyacrylamide gels depending on the molecular mass of the proteins to be analyzed.

3.5.3. Bacterial large-scale expression

Based on the results of the small-scale expression experiments that determined the optimal protein yield, the large-scale over-expressions of heterologous protein in *E. coli*

were performed in LB medium. 400 mL of LB in a 2 L flask was inoculated to an OD_{600} of 0.05 with the overnight starter culture. 100 $\mu\text{g}/\text{mL}$ of Carbenicillin and 15 $\mu\text{g}/\text{mL}$ of Cm were added to the culture to select for the over-expression vector and the pLysSRARE plasmid of Rosetta DE3 containing the rare tRNAs respectively. The culture was grown at 37 °C until the OD_{600} reached 0.5. At this point, the culture was shifted to the desired temperature of induction, depending on the test expression results, and 0.5 mM IPTG was added induce exogenous protein over-expression. The cultures were grown for either 3 h at 37 °C or for 16 h at 15 °C. The cells were then sedimented by centrifugation and frozen at -20 °C until cell lysis.

3.5.4. Affinity Chromatography Purification from bacteria

To prepare for lysis, the pellets were re-suspended in 20 mL of PBS buffer that included 1 protease inhibitor tablet (Complete Mini, Roche), 5 $\mu\text{g}/\text{mL}$ Leupeptin (Roche), 2 $\mu\text{g}/\text{mL}$ Aprotinin (Roche), 0.5 mM Phenylmethylsulfonyl Fluoride (PMSF) (Roche), 100 $\mu\text{g}/\text{mL}$ of lysozyme (EM Science) and 100 $\mu\text{g}/\text{mL}$ of DNase I (Roche). Cells were lysed in a French Press (Amico French Pressure Cell) at 18,000 pounds per square inch (p.s.i) (Yuan *et al.*, 2005). The lysate was centrifuged at 10,000 rpm for 30 min and then transferred and re-centrifuged in new 50 mL centrifuge tubes to remove any cellular debris. The resulting soluble fraction of the lysate was loaded onto the affinity columns (GST, StrepTactin or Hexa-histidine depending on the protein fusion tag) of 1 mL bed volume, followed by a washing step with five bed volumes of wash buffer (GST and StrepTactin required PBS, Hexa-histidine required 50 mM NaH_2PO_4 , 300 mM

NaCl 10-20 mM imidazole). The proteins were purified in six elutions of 500 μ L of elution buffer depending on the type of column (GST required 10 mM glutathione and 50 mM Tris pH 8.0 in PBS, Hexa-histidine required 50 mM NaH_2PO_4 , 300 mM NaCl and increasing imidazole (250-500 mM), while Strep-Tactin sepharose required 2.5 mM desthiobiotin in PBS buffer). The eluted fractions were analyzed by SDS-PAGE and concentrated by dialysis in 50 % glycerol buffer dialysis for 16 h. The concentrated proteins were quantified using a Bradford assay with a bovine serum albumin (BSA) standard curve and stored at -20°C .

3.5.5. Size Exclusion Chromatography

The Superdex 75 column in the Amersham[®] Biosciences ÄKTA Purifier Fast Protein Liquid Chromatography (FPLC) was used for the purification of proteins and for the elucidation of molecular masses. The column was equilibrated with 50 mL (approximately two column volumes) of PBS buffer that was filtered using GH Polypro hydrophilic polypropylene membrane filters 47 mm (PALL Life Sciences) to remove particulates. The loading loop was washed three times with PBS buffer between runs. Fractions from previous Affinity Chromatography or Anion Exchange Separations were concentrated using Amicon Ultra- Centrifugal Filter Devices with cutoff values of 10 K or 30 K (depending on the protein size) following manufacturer's instructions. The concentrated protein was mixed with PBS buffer to a final volume of 1 mL and loaded onto the gel filtration column. The size exclusion procedure was programmed at a flow rate of 0.5 mL/min and 0.5 mL fractions were collected. 10 μ L of each fraction was

mixed with 2X Laemmli buffer (1:1) and analyzed by SDS-PAGE and Coomassie staining.

3.5.6. Anion Exchange

Anion Exchange Chromatography by HiTrap™ DEAE FF 1mL column (Amersham Biosciences) was attempted before or after size exclusion chromatography depending on the purification procedure (specified in results section). A pump wash of filtered double distilled (dd) water was performed on the FPLC followed by five column volumes (5 mL) of dd water through the anion column to remove storage ethanol. Another pump wash of PBS (no NaCl) was performed followed by five column volumes of PBS (no NaCl) through the anion column. The column was regenerated before use by running 5 column volumes of PBS (1 M NaCl), followed by 5-10 column volumes of PBS (no NaCl) to remove residual NaCl within the column. The protein was concentrated as described above in case of size exclusion chromatography and it was equilibrated in PBS (no NaCl) and loaded onto the anion column via the 1 mL loop. The anion exchange procedure was programmed on the FPLC with Pump A placed in PBS (no NaCl) and Pump B placed in PBS (1 M NaCl). The program began with five column volumes of PBS (no NaCl) followed by a step gradient of increasing PBS (1 M NaCl) in 0 % (4 mL), 50% (10 mL) and 100 % (2 mL) Pump B intervals. 0.5 mL fractions were collected and analyzed by SDS-PAGE and Coomassie Staining.

3.5.7. Transformation into *P. pastoris*

In order for transcription of the gene of interest, the vector must integrate in the yeast genome, as there is no origin of replication on the vector that is transformed into yeast. The first step is transformation into the yeast cell and this was attempted by two methods: electroporation and Easy Comp chemically competent transformation. The selectable marker for the vector was Zeocin resistance, which can only be acquired if the vector has successfully integrated. Electrocompetent cells were prepared on the day of transformation in the following manner. 5 mL of *P. pastoris* strain was grown overnight at 30 °C in YPD media; 0.5 mL of overnight culture was inoculated into 100 mL of YPD and grown overnight again to an OD₆₀₀ of 1.3-1.5. The culture was centrifuged for 5 min at 4 °C at 3000 rpm and the cells were resuspended with 100 mL of ice-cold sterile water. These steps were repeated with 50 mL of water and then 4 mL of ice-cold 1 M sorbitol. The final pellet was resuspended in 200 µL of 1M sorbitol and 80 µL of cells were used in transformation. Linear DNA (5-10 µg) in 5-10 µL of sterile water was added to the cells and electroporated using 1500 Volts for 5 ms as per manufacturer's recommendations. To recover, cells were mixed with ice-cold 1 M sorbitol and added to a 15 mL tube followed by incubation at 30 °C without shaking for 2 h. The cells were then plated in dilutions on YPDS plates with Zeocin (100 µg/mL). The plates were incubated for 3-10 days at 30 °C. The kit came with three solutions to create chemically competent cells and they were transformed using the manufacturer's instructions (see kit manual) (Invitrogen, 2007).

3.5.8. Yeast small-scale protein expression

Test expression in *P. pastoris* involves six possible media. A single colony of the selected yeast clone possessing the gene of interest was grown overnight at 30 °C in 25 mL of media containing glycerol to prevent any expression of the *aox1* gene whose promoter also drives the expression of heterologous protein: BMGH (buffered minimal media with glycerol and histidine), BMGY (buffered rich media with glycerol), and MGYH (minimal media with glycerol and histidine). The next day the cells were sedimented by centrifugation and re-suspended in 100 mL of BMMH, BMGH, or MMH (respective to media with glycerol, however, possessing methanol to 0.5 % to induce expression). The cultures were grown for 4 to 6 days depending on the strain GS115 or KM71H respectively. A 1 mL sample of each condition was taken daily for further analysis. Methanol was added to the cultures daily (final concentration of 0.5 % daily as recommended by the manufacturer) (Invitrogen, 2007). As verification that the clone retained the gene of interest after test expression, the daily sample pellets were screened by colony PCR. The samples were then analyzed for protein expression by SDS-PAGE and Coomassie staining. Western blot analysis was also used with protein-specific antibodies.

3.6. Protein-protein interaction studies

3.6.1. Bacterial-2-hybrid (BACTH)

The experimental procedure was optimized from the original procedure (Karimova *et al.*, 2000). Briefly, 2 mL of LB with 100 µg/mL carbenicillin, 50 µg/mL

kanamycin and 1 mM IPTG was inoculated in a 15 mL culture tube with the *E. coli* BTH101 previously transformed with both pUT18C and pKT25 vectors possessing various inserts of genes encoding p120Arm, Ecadc and Hakai. Each pair of pUT18C and pKT25 vectors with gene inserts creating T18 and T25 fusion proteins to be tested for interaction were inoculated into 3 culture tubes and incubated at 37, 30 or 26 °C overnight by shaking at 200 rpm. 16 h later the cultures were tested for β -galactosidase activity in 96 well microtiter plates. Each tube of cells that expressed potentially interacting T18 and T25 fusion proteins was measured in triplicate. A positive control was the interaction between T18-Zip and T25-Zip (expressed by pUT18C-Zip and pKT25-Zip respectively). The negative control was a strain carrying pUT18C and pKT25 vectors expressing T18 and T25 fragments only. Another negative control comprised a vector expressing a potential interacting protein and the second vector did not contain any insert (ie. pUT18C-p120Arm and pKT25). For enzyme assays, 20 μ L of culture was added to each well, followed by 80 μ L of ONPG buffer (Cowie *et al.*, 2006). The reaction was timed to 30 min and terminated by addition of 100 μ L of 1 M sodium carbonate. The plate was read using the SpectramaxPlus³⁸⁴ spectrophotometer (Molecular Devices) at OD₄₂₀ and OD₅₅₀ and 100 μ L of culture was equally measured at OD₆₀₀. The measurements were averaged and the β -galactosidase activity in 0.1 mL was determined by the equation, Miller units = 5000 x ((OD₄₂₀ - 1.75 x OD₅₅₀)/(OD₆₀₀ x time of reaction)) (procedure modified from (J. Sambrook, 1989)).

3.6.2. Co-precipitation (pull-down) assay

A master mix of each protein was prepared with 150 pmol of protein in a 10 μ L aliquot. The proteins (GST-p120Arm/Strep-Ecadc in ratios 1:1, 2:1 and 4:1) and PBS buffer were mixed in 1.5 ml microfuge tubes with 50 μ L of 50 % slurry of Strep-Tactin sepharose equilibrated in PBS (Table 7 for set up) and rotated end over end at 22 °C for 1 h. 100 μ L of PBS was added to the mixture to wash the beads and to remove non-binding proteins. This was followed by washing three times with 200 μ L of PBS buffer. The bound proteins were eluted using 18 μ L 2X Laemmli sample buffer and boiled for 5 min before loading onto a 10 % polyacrylamide gel. The gels were further analyzed by western blotting with specific antisera (modified procedure from refereneces (Harlow, 1988; Yuan *et al.*, 2005)).

Table 7: Pull-down experimental set up for the pair-wise mixture of proteins

Tubes	Strep-Tactin beads						
	1	2	3	4	5	6	7
GST-p120Arm (μ L)	10	10	20	20	40	40	
Strep-Ecadc (μ L)		10	10			10	10
PBS buffer (μ L)	90	80	70	80	60	50	90

3.6.3. Co-precipitation Pull down using radiolabeled protein

In vitro transcription and translation (TnT) was performed as described by the manufacturer (Promega, 2008). The TnT reaction occurred for 90 min at 30 °C. The pull down reaction was performed with 50 μ L of 50 % Strep-Tactin slurry equilibrated in binding buffer. Strep-tagged protein (50 pmol per reaction), radiolabeled protein (10 μ L

of TnT reaction (Cowell and Hurst, 1996)) was incubated with increasing amounts of GST-fused peptide and beads to a final volume of 100 μ L of binding buffer. The mixture was rotated end over end at 4 °C for 2 h. 300 μ L of binding buffer was added to the mixture and the beads were collected by centrifugation at room temperature. The beads were then washed three times with 400 μ L of binding buffer and the proteins eluted from the beads by addition of 18 μ L of 2X Laemmli sample buffer (Cowell and Hurst, 1996). The beads were boiled for 5 min and centrifuged. The supernatant was loaded onto a 10 % polyacrylamide gel. To detect the radiolabeled proteins, the gel was further prepared for drying by shaking incubation with drying buffer for 30 min followed by shaking incubation with amplification solution. The gel was then placed on Whatmann filter paper and dried in the gel dryer (BioRad Gel Dryer Model 583) for 90 min. The gel was exposed to XAR film (Kodak) at -80 °C for various times before developing. The AlphaImager™ 2200 from Alpha Innotech was used to image the blots for densitometry readings. The program Alpha Imager v5.5- SpotDenso measured the decrease in ³⁵S Met-GST-p120Arm on the autoradiograph.

3.6.4. Analytical Gel filtration

The Superdex 200 column in the Amersham® Biosciences ÄKTA Purifier Fast Protein Liquid Chromatography (FPLC) was used for the protein-protein interaction studies (Yuan *et al.*, 2005). The column was equilibrated with 50 mL of PBS buffer that was filtered using GH Polypro hydrophilic polypropylene membrane filters 47 mm (PALL Life Sciences) to remove particulates. The loading loop was washed three times

with PBS between runs. 100-400 pmol of protein was mixed with PBS buffer to a final volume of 1 mL and loaded into the column through a 1 mL injection loop. Each protein was separated individually and the pair-wise mixtures were separated at a flow rate of 0.5 mL/min and 0.5 mL fractions were collected. 50 mL of PBS buffer at a flow rate of 0.5 mL/min was used in between the runs to clean and equilibrate the column. 10 μ L of each fraction was mixed with 2X Laemmli buffer (1:1) and analyzed by western blotting with specific antibodies.

4. RESULTS

In order to examine the ability of peptide aptamers to bind their target protein *in vitro*, we established protein-protein interaction assays to test the capability of the peptides to out compete their full-length protein counterpart in binding the target protein. This was accomplished by cloning the genes encoding the proteins and the peptides we wished to study into over-expression vectors. The expressed proteins or peptides were fused to affinity tags to facilitate their purification. Expression and purification were optimized to find the optimal production temperature and host strain. The proteins were then used to develop assays to test protein-protein interactions that demonstrated the binding between protein targets. These assays were further applied to test the effects of linear and constrained peptides on the interactions and the target binding of the peptides.

4.1. Sub-cloning of genes into expression vectors

4.1.1. Bacterial over-expression vectors

Analysis of the interactions between p120, p120 Arm and Ecadc were previously performed using proteins translated from *Mus musculus* sequences (Melone, 2006). We here changed to the *Homo sapiens* genes, as the long-term goal of peptide aptamers in cancer therapy would be specific to human protein targets. The two expression vectors, pGEX and pT7-7 and modified related vectors were chosen to drive protein expression. The pGEX vectors vary in their multiple cloning and cleavage sites between the upstream glutathione-S-transferase (GST) sequence and the start site of the inserted gene of interest. For controlled expression, the LacI^q repressor encoding gene is also present on

the vector to prevent the *E. coli* host from inducing untimely transcription of the gene of interest. The vector possesses a *tac* promoter, a hybrid between the *trp* and *lacUV5* promoter creating a more efficient promoter than each of the parental promoters (de Boer *et al.*, 1983). These vectors were shown to be very effective at mammalian protein expression in a bacterial host cell, such as the expression of human RhoGPI (Sheffield *et al.*, 1999) and FOXP3 (Hu *et al.*, 2007). Specifically, the pGEX4T1 vector was used to clone downstream of GST the genes of p120 catenin and Hakai. Additionally, the gene encoding the human Armadillo domain of p120 was sub-cloned from the p1201A sequence into the pGEX4T1 vector (Figure 4A) creating an N-terminal GST tag in the protein (GST-p120Arm). To facilitate dual tag purification, the pGEX4T1MM vector with an N-terminal GST and C-terminal 6X His was created including a Factor Xa cleavage N-terminal to the 6X His tag to facilitate selective tag cleavage. This was previously demonstrated to be an efficient strategy in purification seen with the human peripheral cannabinoid receptor (CB2) (Yeliseev *et al.*, 2007).

pT7-7 is a high expression vector that is commonly used to express bacterial proteins in *E. coli*. This vector was shown to be successful at controlled transcription through its T7 promoter specifically recognized by T7 RNA polymerase once its expression is induced by IPTG (Studier and Moffatt, 1986). In planning the experimental approach, we determined that for co-precipitation assays the bait (Ecadc) and prey (GST tagged p120Arm or Hakai) proteins should possess different tags so that either combination of affinity sepharose beads could be attempted. In accordance with this, the gene encoding the E-cadherin cytoplasmic domain was amplified from the pGAD-E-

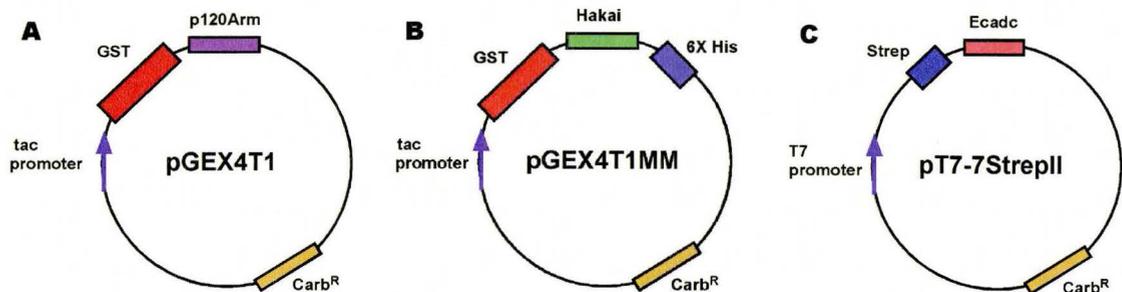
cadherin vector (Table 6A) encoding the full-length human E-cadherin and ligated into the pT7-7StrepII vector creating an N-terminal Strep tag to the protein (Strep-Ecadc) (Figure 4C). Initially, the gene for p1201A was amplified from the pBlueScript-p1201A (Table 6A) and cloned into the pT7-7StrepII vector encoding an N-terminal Streptavidin (Strep) tag (Table 6A). The Strep tag was chosen because it is a small tag (WSHPQFEK) and less likely to sterically hinder the folding of the 108 kDa p1201A than GST. Previously, the murine protein had been cloned into the pGEX4T1 vector creating an N-terminal glutathione-S-transferase (GST) fusion tag in the protein (GST-p120). This protein was difficult to over-produce and the GST tag was easily degraded during purification (data not shown). Also, we hoped that the shorter Strep tag would facilitate over-production and that purification would be more efficient than in the case of the GST fusion.

4.1.2. Yeast over-expression vectors

For the yeast expression, two methods of protein production were attempted requiring different expression vectors. pPICZA is an intracellular expression vector (Figure 4D) used in *P. pastoris*. The genes encoding p120Arm, Hakai and Ecadc were cloned into this vector creating a C-terminal fusion to myc and hexa-histidine tags to the protein of interest. pPICZ α C is a vector for secreted expression (Figure 4E), which will create an N-terminal α -secretion signal fusion and C-terminal myc and hexa-histidine tags for detection and purification. The genes encoding all proteins and their domains

were cloned into pPICZ α C as well and test expression was attempted (Table 6B summarizes these constructs) (Invitrogen, 2007).

E. coli over-expression vectors



P. pastoris over-expression vectors

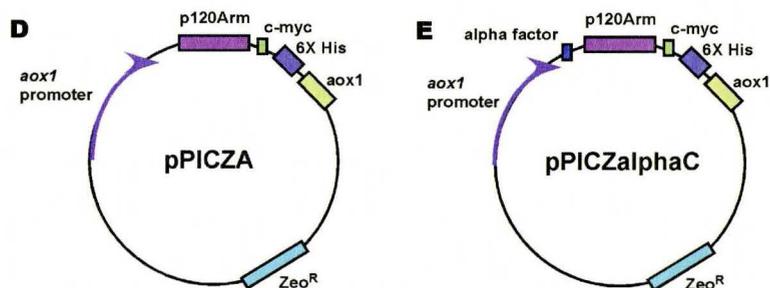


Figure 4: Protein over-expression vectors. Five vectors used to clone the genes encoding p120Arm, Hakai and Ecadc. *E. coli* over-expression vectors A, B and C possess either an N-terminal GST or StrepII affinity tag and carry the gene for carbenicillin resistance. pGEX4T1MM (B) was created in this thesis to possess a C-terminal hexa-histidine tag to facilitate dual-affinity purification. Vectors D and E are *P. pastoris* over-expression vectors. pPICZA (D) was used for intracellular expression regulated by the *aox1* promoter that drives expression of the gene encoding the heterologous protein and the downstream *aox1* gene. pPICZalphaC (E) was used for secreted expression via the alpha factor secretion signal N-terminal to the gene encoding the protein of interest. Hakai, p120Arm and Ecadc genes were cloned into both D and E to possess a C-terminal c-myc and hexa-histidine tag. Both vectors D and E carry a gene for Zeocin resistance.

4.2. Small-scale expression of target proteins to optimize yield and purity

4.2.1. E. coli over-expression:

Initially p12C1A, p120Arm, Hakai and Ecadc fusion proteins were over-expressed using nine conditions of protein overproduction (small-scale test expression) experiments in order to determine conditions for optimal expression of soluble protein. Three *E. coli* strains and a broad temperature range of protein production were attempted that was previously used to express eukaryotic proteins in bacteria (37 °C, 30 °C / 26 °C, or 15 °C) (Makagiansar *et al.*, 2002; Vedadi *et al.*, 2007; Zheng *et al.*, 2004). BL21 DE3 is a very popular bacterial over-expression strain and it is widely used for protein overproduction (Stratagene, 2006). BL21 Star DE3 has an additional advantage in that it carries a mutation in its RNase E gene that was engineered to stabilize the mRNAs of over-expressed genes (Invitrogen, 2002). Rosetta DE3 has a different advantage; this strain expresses six additional rare tRNAs (complementing codons: AUA, AGA, AGG, CCC, CUA, and GGA) (Novagen, 2006). TestX of the bacterial expression conditions for p120, p120Arm, Hakai and Ecadc commonly showed the highest level of expression in the host *E. coli* Rosetta DE3. An example of test expression in *E. coli* is shown in Figure 5 in which the optimal conditions for expression of the Strep-Ecadc variant (Y755E, Y756E) were determined to be in strain Rosetta DE3 at 37 °C for 3 h of induction, conditions that were selected for large-scale expression experiments. Perhaps this is the result of the expression of the additional tRNAs. In support of this, others have also made similar findings and used Rosetta DE3 for overproduction of human proteins such as G

protein coupled receptor (Bane *et al.*, 2007) and Propyl hydroxylase PHD3 (Fedulova *et al.*, 2007).

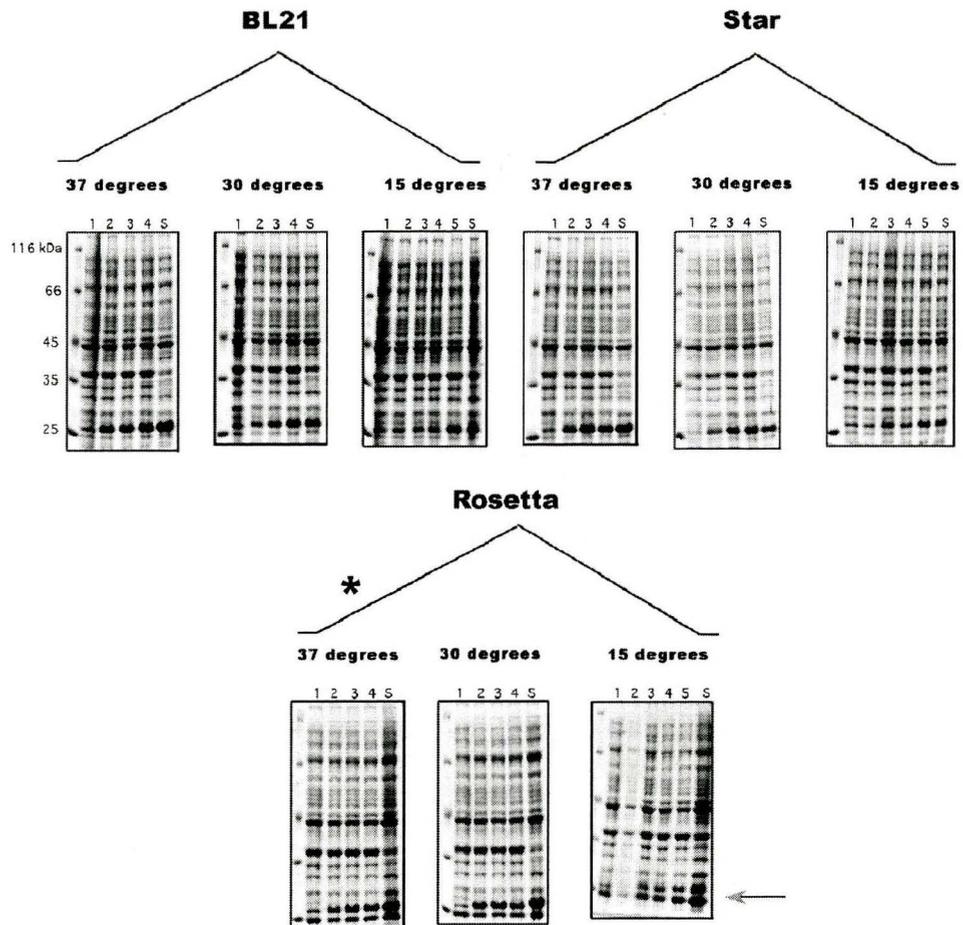


Figure 5: Small-scale protein test expression of Strep-Ecadc variant (Y755E, Y756E). Test expression was used to determine the conditions to obtain the highest amount of protein. Three *E. coli* strains **BL21** DE3, **Rosetta** DE3 pLysS, **BL21 Star** DE3, each cultivated at 37 °C, 30 °C, or 15 °C were used, thus creating nine conditions of expression from which the optimal conditions were selected. The arrow indicates the optimal expression of Strep-Ecadc variant (18 kDa) migrating at approximately 25 kDa in a soluble form. 1: Sample taken before induction, 2: Sample taken 1 h after induction with IPTG, 3: Sample taken 2 h after induction, 4: Sample taken 3 h after induction, 5: Sample taken after 16 h induction, S: Soluble fraction after cell lysis. Samples were analyzed on 10 % SDS-PAGE followed by Coomassie staining. The condition that produced the most soluble protein was in strain Rosetta at 37 °C (asterisk) and was chosen for large-scale overproduction. The molecular masses of the reference proteins are indicated on the left in kDa.

4.2.2. *P. pastoris* over-expression:

Expression in *P. pastoris* (a methylotrophic yeast) involves the cloning of the gene of interest downstream of the *aox1* gene required for recombination into the yeast genome. AOX1 is an alcohol oxidase, which oxidizes methanol to formaldehyde using molecular oxygen (Invitrogen, 2007). The promoter of *aox1* also drives expression of the downstream heterologous protein. Secretion and intracellular expression of the target proteins was attempted in both high and low expression yeast. GS115 Mut⁺ is a high expression strain in that it possesses both *aox1* and *aox2* (results in slower growth because AOX2 is not as effective at oxidizing methanol). These genes are required for *P. pastoris* to metabolize methanol as its sole carbon source (Koutz *et al.*, 1989). KM71H is a Mut^s strain in which the strain only possesses *aox2* rendering it unable to metabolize methanol at the same rate as GS115, thus, producing the recombinant protein in low amounts due to its slow growth. The initial obstacle was integration into the *P. pastoris* genome, which is necessary for subsequent expression of the heterologous protein. Integration occurs via a single crossover event between the loci of either two *aox1* regions on the vector and the genome. Multiple integrations can occur (1-10% of insertion events)(Invitrogen, 2007) and are most favourable for expression. Integration into the genome is required to retain Zeocin resistance, as there is no origin of replication on either vector (pPICZA and pPICαC).

Cloning the genes of interest into pPICZA and pPICαC preceded similarly to cloning for bacterial expression, however, transformation of these vectors into yeast did not readily yield stable integrations. Bacterial transformations require nanograms of DNA

while yeast transformations requires between 5-100 μg of DNA (Invitrogen, 2002; Invitrogen, 2007). GS115 and KM71H were made chemically competent for heat shock transformation as well as electrocompetent for electroporation and both methods were applied for transformation. To optimize integration, the vectors were restricted upstream of the *aox1* gene to assist in integration within the cell. The amount of DNA was varied (linear or circular DNA, amount of DNA, buffer exchange after digestion) as well as the amount of cells plated after transformation in order to find conditions favourable for integration (Invitrogen, 2007). Once colonies grew after transformation, these colonies were re-streaked onto fresh YPDS Zeocin agar and they were analyzed for the presence of the gene insert by colony PCR and their protein expression was tested as well. At this point, it was found that many clones contained the gene prior to test expression, however, after expression in liquid media, the gene was no longer detected in the cell pellets of a majority of the clones by PCR. To date the only clone that retained the gene of interest and produced heterologous protein was that expressing p120Arm inside the cells in the strain KM71H (Figure 6). An expression clone for Ecadc was identified but did not produce the protein in amounts superior to those in *E. coli* (data not shown). Use of this expression system was very time consuming and did not produce the desired protein level that was required in this work. It was therefore concluded that this system should no longer be attempted for the remaining Hakai as of June 2008.

P. pastoris was successfully applied for over-production of proteins for pharmaceutical applications, but the obstacle of finding the clone with multiple integrations was still present. Previous works expressing eukaryotic growth hormones

and human monocyte chemo-attractant protein-1 (MCP-1) presented *P. pastoris* to produce on average 100 mg/L of secreted protein that can further purified from the media (Beall *et al.*, 1998; Xu *et al.*, 2008). Therefore, to further optimize the expression in this work, aside from searching for a clone in the high expression strain GS115, varying other parameters could have increased the yield in KM71H. Initially, the OD at which expression was induced by methanol ranged between 0.5 and 3, however, KM71H could be induced at a higher OD since it is a slow growing strain. Secondly, the pH of the media could be altered to range between 5.5 and 8.0 as each protein has a specific pH at which it is stable in solution. Lastly, the level of methanol used in induction of over-expression could range between 0.5 (use in these experiments) to 3 % (Xu *et al.*, 2008). For KM71H p120Arm, the culture was induced at OD₆₀₀ 3; the media is unbuffered (MMH) and ranges between pH 5-7. As well, if more clones were able to be screened for mRNA production (northern blot), then perhaps effective expression clones may have been found for this work (Delroisse *et al.*, 2005). All these options may have been attempted if time permitted.

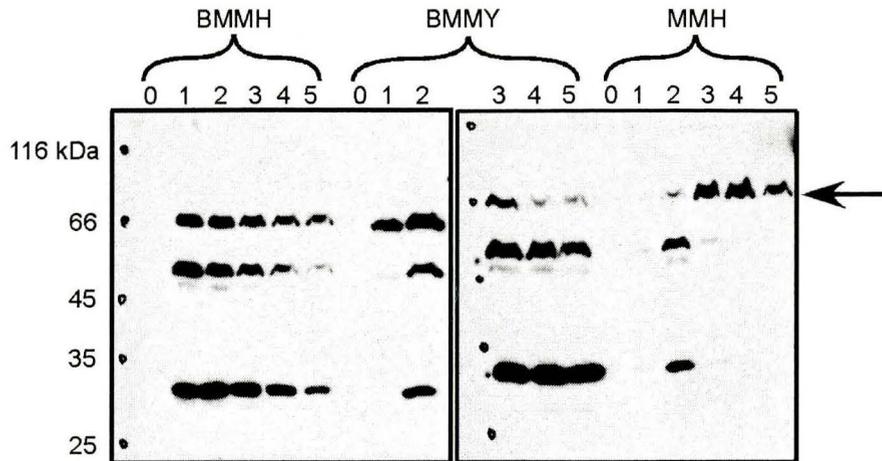


Figure 6: Test expression of *p120Arm* in *P. pastoris* KM71H. Three media were used: BMMH (buffered minimal media with histidine), BMMY (buffered rich media), MMH (minimal media with histidine). To induce gene expression 0.5 % methanol was added daily for 5 days. Sample time points 0-5 indicate times before induction with methanol (0) and subsequent days of induction (1-5). Cell pellets were lysed in sample buffer and subjected to electrophoresis on a 10 % Laemmli gel and followed by western blotting with p120 monoclonal antibody. Human p120Arm (C-terminal myc and 6X His tag) has a molecular mass of 60 kDa indicated by the arrow. Cultivation in MMH after 3 days of induction resulted in the production of p120Arm with minimal degradation products compared to the other media. Molecular masses of reference proteins are indicated on the left in kDa.

4.3. Large-scale expression of target proteins for protein-protein interactions experiments

4.3.1. GST-Hakai-His and GST-Hakai purification

Expression of GST-Hakai-His and GST-Hakai was attempted and appeared to give the highest yield in Rosetta DE3 at 15 °C after 16 h of IPTG induction. Purification required Ni-NTA affinity chromatography on a sepharose bead column followed by GST affinity chromatography. The full-length protein was detected by western blotting with GST monoclonal antibody, however, a protein of approximately 70-80 kDa was detected by Coomassie staining (indicated by the arrowhead in Figure 7). It is interesting to note

that the GST antibody did not detect the full-length Hakai fusion protein, but it detected fragments of approximately 25 kDa. Detection with a Hakai specific antibody as well as with a 6X His specific antibody (for the GST-Hakai-His) was attempted and both did not detect the protein at 80 kDa (data not shown). In attempts to find if the GST tag was hindering the recognition of Hakai by the antibody, the N-terminal tag was switched to Strep tag (Strep-Hakai), but we still did not detect anything but degradation products (data not shown).

It was later determined in November 2007 that any attempts to over-produce Hakai in *E. coli* resulted in production of DnaK. DnaK (70 kDa) has been shown to interact with heat-shock chaperone proteins such as DnaJ and GrpE in *E. coli* involved in the assembly and disassembly of protein complexes (Hightower, 1980; Pelham, 1986). It was identified by Mass spectroscopy to be the protein detected by Coomassie staining at approximately 80 kDa (Figure 7). DnaK may have contributed to GST-Hakai degradation or perhaps deterred GST-Hakai production and folding within *E. coli*. Hakai overproduction was no longer attempted in bacteria; and due to time constraints and technical difficulties at obtaining integrated clones; we did not further pursue the yeast system.

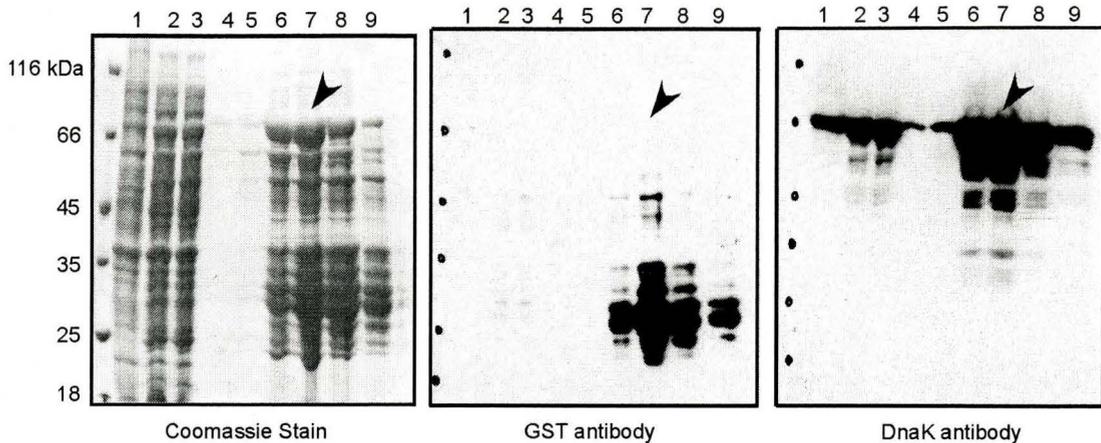


Figure 7: SDS-PAGE analysis of attempts to over-produce and purify GST-Hakai. The samples taken at different time points during the over-production experiment and purification were analyzed on a 10 % SDS-PAGE followed by Coomassie staining. GST-Hakai has a calculated molecular mass of 80 kDa. Lane 1: Before induction, 2: 16 h after induction, 3: all soluble fraction after lysis, 4-9: elutions from the GST affinity column. The arrowhead indicates the proposed location of full-length GST-Hakai protein (80 kDa). Molecular masses of the reference proteins are indicated on the left in kDa.

4.3.2. Purification of the Strep-E-cadherin cytoplasmic domain and variant Y755E, Y756E

Overproduction of Strep-Ecadc was most efficient in Rosetta DE3 at 37 °C and purification was performed using a Strep-Tactin superflow affinity column. Strep-Ecadc eluted from the Strep-Tactin column in fractions 3-4 (Figure 8 Coomassie stain lanes 6-7). A 400 mL culture yielded 2.2 mg of protein. DnaK was also detected in the elution fractions of this column (Figure 8) and further purification was attempted to remove a smaller degradation product with size exclusion chromatography. As Strep-Ecadc has a molecular mass of 18 kDa, the Superdex 75 column was used for this purpose. After size exclusion chromatography, Strep-Ecadc was further purified by anion exchange (pI of

Strep-Ecadc = 4.22) and these fractions were dialyzed in PBS with 50 % glycerol and 0.1 mM DTT for storage (Figure 9).

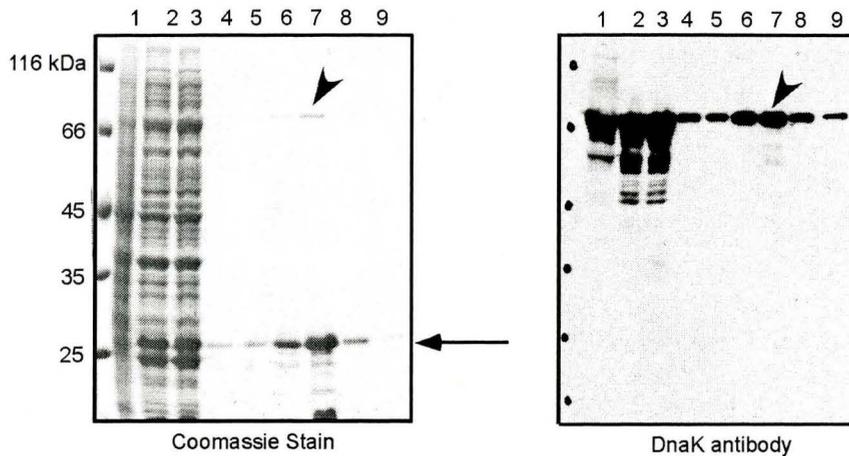


Figure 8: SDS-PAGE analysis of Strep-Ecadc large-scale growth and purification. The samples taken at different time points during the over-production experiment and purification were analyzed on a 10 % SDS-PAGE followed by Coomassie staining. Strep-Ecadc has a calculated molecular mass of 18 kDa, however it migrates on the SDS-PAGE approximately as a 25 kDa protein as indicated by the arrow. The arrowhead marks DnaK found in fractions of elution from the Strep-Tactin column both by Coomassie Staining and with a DnaK specific antibody. Lane 1: Before induction, 2: 3 h after induction, 3: all soluble fraction after lysis, 4-9: elutions from the Strep-Tactin affinity column. Molecular masses of the reference proteins are indicated on the left in kDa.



Figure 9: Size exclusion chromatography and Anion Exchange to purify Strep-Ecadc. Strep-Ecadc was initially purified by affinity chromatography, concentrated and subjected to size exclusion chromatography on the Superdex 75 column. Selected fractions were further purified and equilibrated in anion exchange buffer and then subjected to anion exchange using the HiTrap™ DEAE FF anion column. Elution fractions were analyzed on a 12 % SDS-PAGE followed by Coomassie staining. Strep-Ecadc has a calculated molecular mass of 18 kDa, however it migrates on the SDS-PAGE approximately as a 25 kDa protein as indicated by the arrow. Molecular masses of the reference proteins are indicated on the left in kDa.

The overproduction of Strep-Ecadc variant Y755E, Y756E (Strep-EV) was most efficient in strain Rosetta DE3 at 37 °C with 3 h IPTG induction and purification was performed as mentioned above. Strep-EV eluted from the Strep-Tactin column in fractions 3-4 (Figure 10 lanes 6-7) and a 400 mL culture yielded 1.15 mg of protein. This variant was generated to mimic phosphorylated tyrosines and enhance Hakai's ability to bind Ecadc *in vitro* (Fujita *et al.*, 2002). Further purification was performed as above with Strep-Ecadc (data not shown).

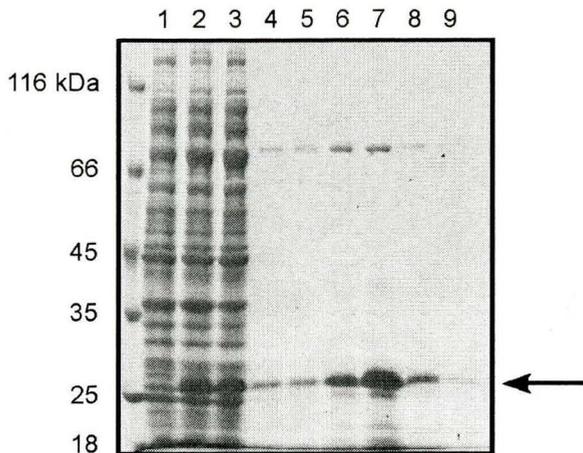


Figure 10: SDS-PAGE analysis of *Strep-EV Y755E, Y756E* large-scale growth and purification. The samples taken at different time points during the over-production experiment and purification were analyzed on a 10 % SDS-PAGE followed by Coomassie staining. *Strep-EV* has a calculated molecular mass of 18 kDa, however it migrates on the SDS-PAGE approximately as a 25 kDa protein as indicated by the arrow. Lane 1: Before induction, 2: 16 h after induction, 3: all soluble fraction after lysis, 4-9: elutions from the Strep-Tactin affinity column. Molecular masses of the reference proteins are indicated on the left in kDa.

4.3.3. *GST-p1201A* purification

Strep-p1201A was purified from strain Rosetta DE3 cultivated at 15 °C and isolated by Strep-Tactin affinity chromatography. The protein yield from 400 mL of culture was approximately 25 µg. Very little protein was recovered as seen by Coomassie staining (data not shown) and similar results were found with murine p120 with a GST tag. Therefore the GST tag based purification was not further pursued.

4.3.4. *GST-p120 Armadillo domain* purification

The highest protein yield for *GST-p120Arm* was obtained in strain Rosetta DE3 grown at 15 °C after 16 h of IPTG induction and it was isolated via GST affinity

chromatography in fractions 3-5 (Figure 11 lanes 7-9). The protein yield for a 400 mL culture was 2.1 mg. Further purification was attempted using Thrombin cleavage of the GST tag (as it degraded in solution easily, detected by western blotting) but we observed further degradation (data not shown). Cation exchange (pI of GST-p120Arm = 8.21) and anion exchange chromatography were attempted, but did not remove impurities or degradation products efficiently. Lastly, gel filtration was attempted and appeared to give the most stable protein, but some degradation was still observed (data not shown). Therefore, we tried to express the protein in an alternative expression system (*P. pastoris*) in order to obtain larger amounts and truly purified protein without degradation products.

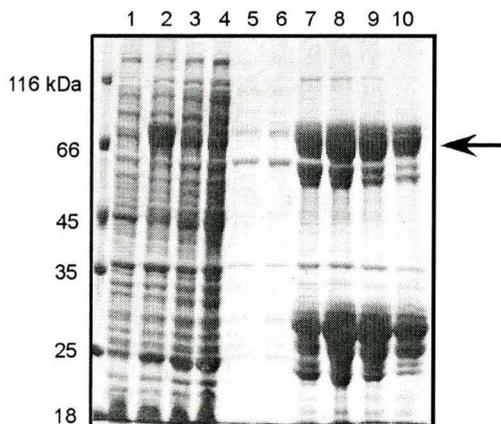


Figure 11: SDS-PAGE analysis of GST-p120Arm large-scale growth and purification. The samples taken at different time points during the over-production experiment and purification were analyzed on a 10 % SDS-PAGE followed by Coomassie staining. GST-p120Arm has a calculated molecular mass of 80 kDa as it migrates on an SDS-PAGE above the 66 kDa protein marker as indicated by the arrow. Lane 1: Before induction, 2: 16 h after induction, 3: all soluble fraction after lysis, 4: Flow through of GST column, 5-10: elutions from the GST affinity column. Molecular masses of the reference proteins are indicated on the left in kDa.

Large-scale expression of p120Arm in *P. pastoris* KM71H provided the highest yield with the least amount of degradation after 4 days in MMH (Figure 6). The protein was isolated by Ni-NTA affinity chromatography using increasing amounts of imidazole for elution (Figure 12). As this is a low expression strain, the starter culture had a volume of 2 L in MGYH media and was grown overnight to an optical density of approximately 4-6 and it was transferred into 400 mL of MMH media to increase the optical density before induction. This culture grew to an approximate optical density of 12-14 in 4 days. Figure 12 indicates that a majority of the protein did not bind the column as shown by the flow through and wash with 10 mM imidazole (Figure 12 lanes 3 and 4). Elutions 1-4 yielded approximately 5 mg of protein, however by examining the Coomassie stain relative to the western blot in Figure 12 it is evident that in elutions labeled A-D the amount of p120Arm is small. This protein requires further purification to remove the degradation products indicated by the protein signal in the Coomassie staining (Figure 12 (A)). The western blot in Figure 12 (B) shows two protein signals around the 66 kDa molecular marker. As these will be difficult to separate by size, cation exchange (pI of p120Arm-myc-6XHis = 8.70) was suggested to separate by charge. This procedure was not attempted, as there was very little recombinant protein seen during Coomassie staining.

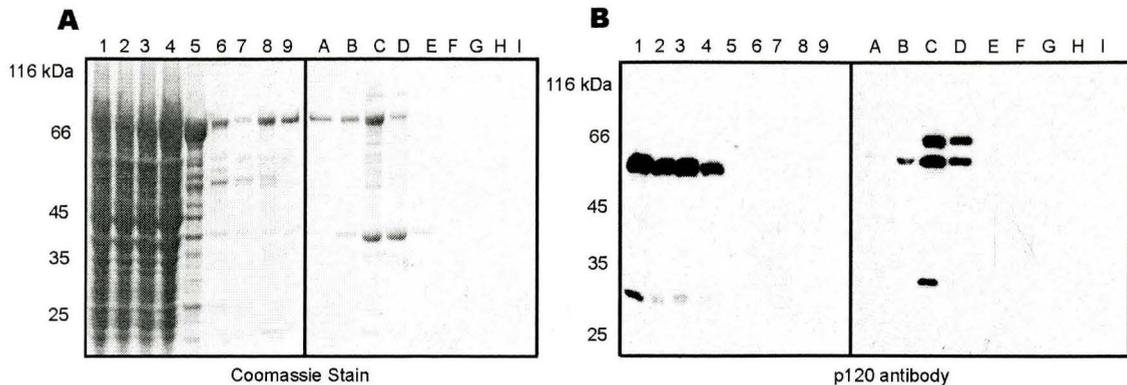


Figure 12: Over-production and purification of p120Arm in *P. pastoris* KM71H. Intracellular expression of p120Arm with C-terminal 6X His tag (60 kDa) purified by Ni-NTA column with increasing amounts of imidazole. (A) Coomassie Staining of purification Samples: 1- Cell lysate, 2- Soluble fraction of lysate, 3- Flow through of Ni-NTA column, 4-6- 10 mM imidazole washes, 7-9- 20 mM imidazole washes, A-D- 250 mM imidazole elutions, E- 300 mM imidazole elution, F- 350 mM imidazole wash, G- 400 mM imidazole wash, H- 450 mM imidazole elution, I- 500 mM imidazole elution. (B) Western using p120 monoclonal antibody, samples respective to A. Molecular marker is indicated on the left in kDa.

4.4. *In vitro* protein-protein interaction studies between E-cadherin and p120Arm

4.4.1. Co-precipitation

To examine protein-protein interactions between Strep-Ecadc and GST-p120Arm *in vitro*, we employed co-precipitation (pull-down) methodologies. The pull-down assay uses sepharose beads to sediment a tagged protein of interest together with its binding partners. The Strep-Tactin superflow beads bind Strep-Ecadc, via its N-terminal Strep tag. If GST-p120Arm had the ability to bind Strep-Ecadc *in vitro*, then both proteins would remain attached to the beads during washing steps and will co-elute. To test this hypothesis, the Strep-Tactin beads were incubated in pair-wise mixtures of GST-p120Arm and Strep-Ecadc. After washing the Strep-Tactin sepharose beads to remove

any weakly associated partners, the beads were suspended in 2X Laemmli sample buffer to co-elute the interacting proteins. As negative controls, only GST-p120Arm was incubated with the Strep-Tactin sepharose beads to ensure that it did not bind nonspecifically. The eluted protein samples were then analyzed by SDS-PAGE followed by western blotting with specific antibodies.

As indicated by Qi *et al.*, 2006 the stoichiometry between the proteins in a complex may not be 1:1 but perhaps 2:1 or 4:1. To address this possibility, the experiments incorporated different stoichiometries to study its effects on the protein-protein interactions with Strep-Tactin beads as an affinity matrix. The western blot analysis of the elutions from this pull-down experiment revealed an interaction when murine GST-p120Arm and Strep-Ecadc were incubated in a 4:1 stoichiometry (Melone, 2006).

This assay was improved using the human GST-p120Arm and Strep-Ecadc enriched from *E. coli*. We also attempted this experiment with the p120Arm purified from *P. pastoris*, but we did not detect an interaction. An interaction between the human fusion protein GST-p120Arm and Strep-Ecadc was observed when they were mixed in a 2:1 and 4:1 ratio (Figure 13). The signals observed in the negative controls GST-p120Arm indicate some non-specific binding to the Strep-Tactin beads, however, incubation with Strep-Ecadc coated beads co-precipitated much larger amounts of GST-p120Arm. This experiment establishes that GST-p120Arm and Strep-Ecadc do interact *in vitro* and this assay may be used in future to study peptide aptamers that disrupt this interaction.

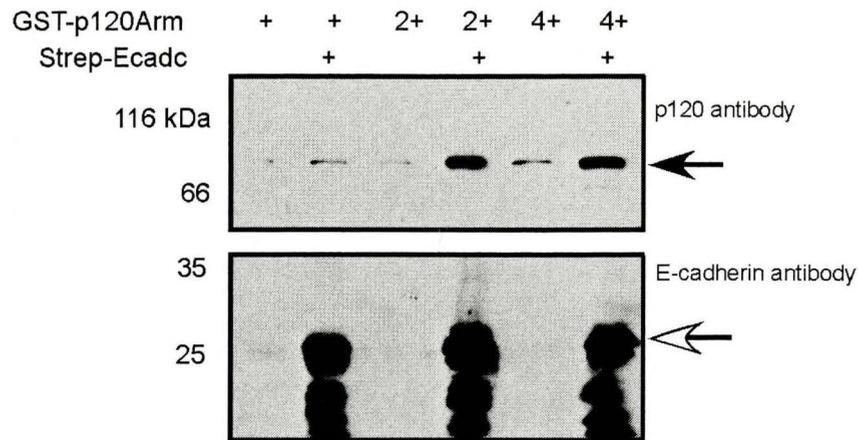


Figure 13: *Strep-Tactin pull-down assay with GST-p120Arm and Strep-Ecadc.* The GST-p120Arm and Strep-Ecadc were mixed in a 1:1, 2:1 and 4:1 ratio followed by pull-down in Streptavidin sepharose beads. SDS-PAGE and western blotting with specific antibody analyzed the elutions from the pull-down experiment. The black arrow indicates GST-p120Arm. The white arrow indicates Strep-Ecadc. As negative controls, GST-p120Arm alone was incubated with the matrix. The molecular masses of the reference proteins are indicated on the left in kDa.

4.4.2. Co-precipitation with methionine ³⁵S radiolabeled proteins

To enhance the ability to visualize the co-precipitated protein as well as decrease the amount of protein required for this experiment, the approach of *in vitro* transcription and translation (TnT) was adopted. *In vitro* TnT (Promega) incorporates radiolabeled ³⁵S methionine in addition to endogenous methionine and the resulting radiolabeled (TnT) protein is detected efficiently by autoradiography. The proteins generated as described in other parts of this thesis were produced in *E. coli*. In contrast, the *in vitro* TnT differs in that it is based on rabbit reticulocyte lysate (Promega, 2008) and the proteins generated may therefore be post-translationally modified which may favor interactions. Figure 14 A

depicts the results of co-precipitating $^{35}\text{SMet-GST-p120Arm}$ with Strep-Ecadc bound to Strep-Tactin resin. Increasing amounts of bait Strep-Ecadc were used to assess the effects on co-precipitation of $^{35}\text{SMet-GST-p120Arm}$. Figure 14 A indicates that 50 pmol of Strep-Ecadc was sufficient to co-precipitate a clearly detectable amount of $^{35}\text{SMet-GST-p120Arm}$ and the amount increased with increased amounts of Strep-Ecadc. A similar approach was taken with $^{35}\text{SMet-GST-Hakai}$ as shown in Figure 14 B, in which Strep-Ecadc and 50 or 100 pmol of Strep-Ecadc variant (Y755E, Y756E) (Strep-EcadcV) was used as bait. Figure 14 B indicates that neither Strep-Ecadc nor Strep-EcadcV co-precipitated $^{35}\text{SMet-GST-Hakai}$ as the protein signal was approximately equal to the negative control, showing that this assay was not suited to measure $^{35}\text{SMet-GST-Hakai}$ - Strep-Ecadc or Strep-EcadcV interactions. The development of this pull-down assay with radiolabeled protein was suggested as a more sensitive method to test peptide competition of binding, for example by peptides spanning repeats of p120Arm.

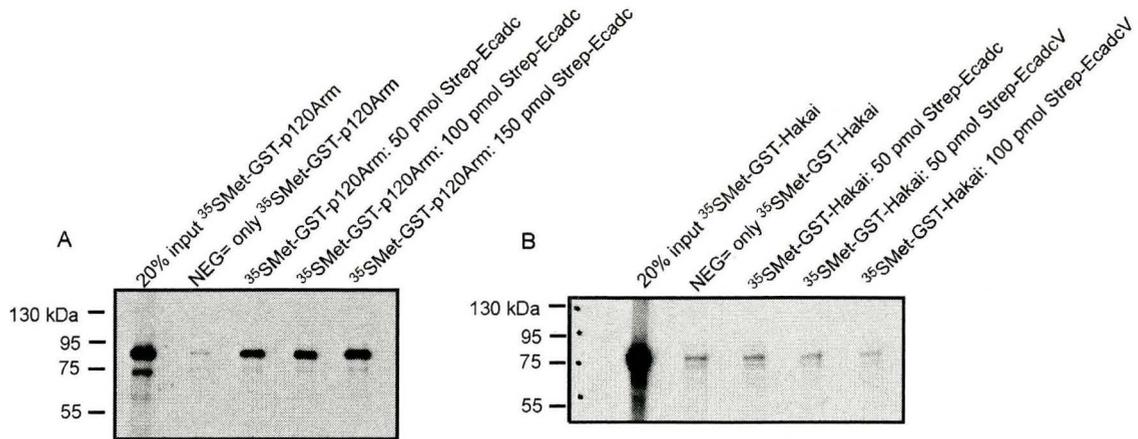


Figure 14: Co-precipitation of radiolabeled GST-p120Arm or GST-Hakai with Strep-Ecadc and Strep-EcadcV. *In vitro* TnT was used to produce ³⁵SMet-GST-p120Arm and ³⁵SMet-GST-Hakai. A pull-down assay was used to examine the protein-protein interactions and to optimize the amount of bait (Strep-Ecadc or Strep-EcadcV) required to pull down the potential binding partners using the Strep-Tactin affinity matrix. As a control to ensure the TnT reaction was successful, 20 % of the input protein was loaded. The negative controls were incubations of ³⁵SMet-GST tagged proteins alone with Strep-Tactin beads. Protein samples were resolved on 10 % SDS-PAGE, followed by gel drying and exposure to XAR film at -80°C , and the autoradiographs are presented. The molecular masses of the reference proteins are indicated on the left in kDa.

4.4.3. Analytical gel filtration

As another independent method for detecting the interaction between p120Arm and Ecadc, size exclusion chromatography (analytical gel filtration) was used to analyze complex formation. The principle of this method is that the Superdex 200 column is made of a matrix of beads that have small pockets within them. These pockets trap small proteins and retard their elution, while the larger protein complexes elute much faster. Using the Fast Protein Liquid Chromatography (FPLC) to detect protein interactions was previously shown to be extremely valuable in determining protein oligomerization (Jou *et al.*, 1995; Paschos *et al.*, 2006; Yuan *et al.*, 2005). By using gel filtration, the

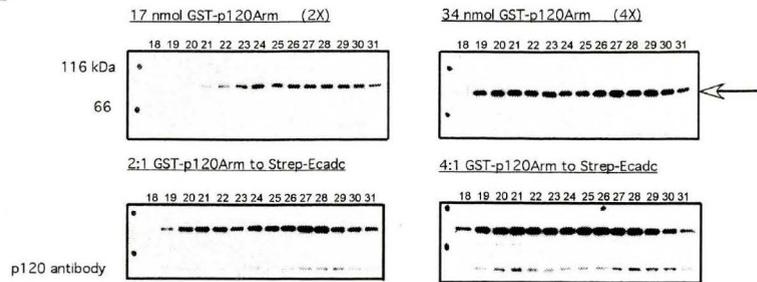
oligomerization state of the individual proteins alone and when combined with an interaction partner can be analyzed by western blotting. The elution profiles for human GST-p120Arm and Strep-Ecadc were first analyzed individually to determine the oligomeric state of the proteins (Figure 15 labeled in nmol of protein). Next, GST-p120Arm and Strep-Ecadc were mixed in a 1:1, 2:1, and 4:1 ratio and their ability to interact was determined by their elution from the Superdex 200 column. By examining the western blotting results (Figure 15 (A)), we showed that when 17 and 34 nmol of GST-p120Arm were loaded onto the column they eluted mostly in fractions 20-30 representing approximately 40-450 kDa. The large molecular mass complexes could result from protein aggregation or its interaction with the column resin. When we analyzed 8.5 nmol of GST-p120Arm the signals were too weak for detection by western blotting (data not shown). When a 2:1 ratio of GST-p120Arm and Strep-Ecadc was analyzed, GST-p120Arm was present in higher molecular mass fractions (19-28) corresponding to approximately 70-570 kDa as compared to the same amount of GST-p120Arm separated alone (fractions 24-29) approximately 50-180 kDa. This indicates a potential interaction with Strep-Ecadc since the complex elutes in earlier higher molecular mass fractions. A similar result was obtained when a 4:1 ratio of GST-p120Arm and Strep-Ecadc was separated, GST-p120Arm eluted in higher molecular mass fractions (approximately 50-720 kDa) as compared to the separation of 34 nmol GST-p120Arm alone (approximately 50-570 kDa).

The oligomerization of Strep-Ecadc was examined using 8.5 nmol of protein (Figure 15 (B)) and it eluted primarily in fraction 28 (approximately 70 kDa). When

GST-p120Arm was incubated with Strep-Ecadc in a 1:1 ratio, an evident retardation of Strep-Ecadc through the column and elution in higher molecular mass fractions began much earlier in fractions 24 (approximately 180 kDa). This result indicated a higher molecular weight complex formed between GST-p120Arm and Strep-Ecadc. Similarly, analysis of the separation of a 2:1 ratio of GST-p120Arm and Ecadc indicated a shift from fraction 28 (approximately 70 kDa) to fraction 19 (approximately 570 kDa) indicated in Figure 15 (B). When the proteins were mixed in a 4:1 ratio of GST-p120Arm and Strep-Ecadc, it was found that again Strep-Ecadc eluted in a higher molecular mass fraction similar to the 2:1 ratio. Similar results were previously observed in the case of the murine homologues (Melone, 2006).

Taken together, the results from the analytical gel filtration present a line of evidence substantiating the *in vitro* interaction between GST-p120Arm and Strep-Ecadc. However, this assay was not applied to study the disruption of protein-protein interactions with peptide aptamers, as we predicted that it might not be sensitive enough to measure small changes due to its own modest variability seen during independent repetitions.

A GST-p120Arm analytical gel filtration profiles



B Strep-Ecadc analytical gel filtration profiles

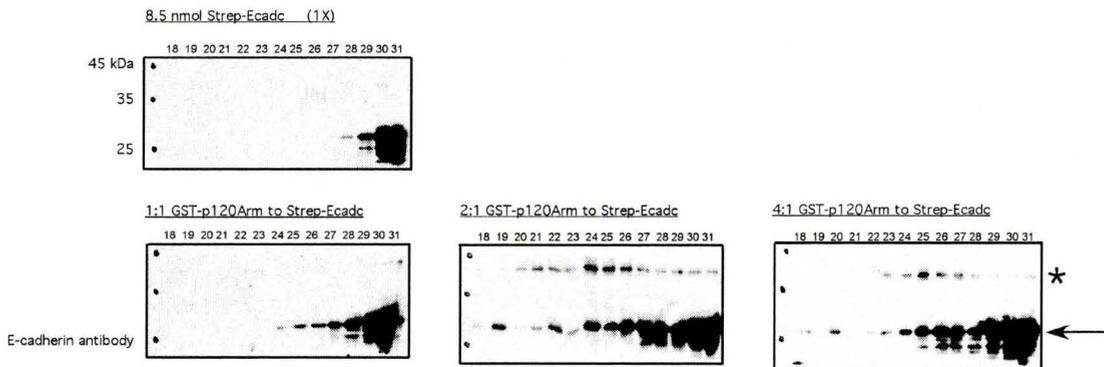


Figure 15: Analytical gel filtration analysis of GST-p120Arm and Strep-Ecadc interactions. The western blot analysis of the fractions collected from the Superdex 200 column. (A) The elution profile of GST-p120Arm was examined using 17 (2X) and 34 nmol (4X) of proteins to determine the protein's individual oligomerization. The white arrows indicate GST-p120Arm detected by specific antibody. The interaction between GST-p120Arm and Strep-Ecadc was examined using a ratio of 1:1, 2:1, and 4:1. These blots were also probed with p120 antibody. (B) The elution profile of Strep-Ecadc was examined using 8.5 nmol (1X) to determine the protein's oligomerization state and 17 and 34 nmol for complex formation experiments and detected with E-cadherin antibody. The black arrows indicate Strep-Ecadc. The star indicates potential dimers of Strep-Ecadc detected by the antibody. The fractions 18-31 out of 48 collected are indicated on top of the image. The molecular masses of the reference proteins are indicated on the left in kDa.

4.5. Analysis of protein-protein interactions with the Bacterial-2-hybrid system

To test the interaction between p120, Hakai and Ecadc a bacterial adenylate cyclase 2-hybrid system (BACTH) was implemented. Once established, this system could be used to test a small molecule library for compounds that either enhance the interaction between p120 and Ecadc or inhibit the interaction between Hakai and Ecadc.

This system is based on the *Bordetella pertussis* adenylate cyclase (AC) composed of two complementary fragments. When the two fragments are joined based on fusion to interacting proteins this reconstitutes cAMP synthesis, which increases the expression of the reporter gene *lacZ* in an *E. coli cya* strain that is deficient in its endogenous AC. The interacting proteins are C-terminally fused to one of the two fragments of the AC (T18 and T25) and the resulting interaction between the proteins in questions will bring the T18 and T25 fragments in close proximity to convert ATP to cAMP. The subsequent *lacZ* expression may be measured with a β -galactosidase assay (Figure 16)(Karimova *et al.*, 1998; Miller, 1972).

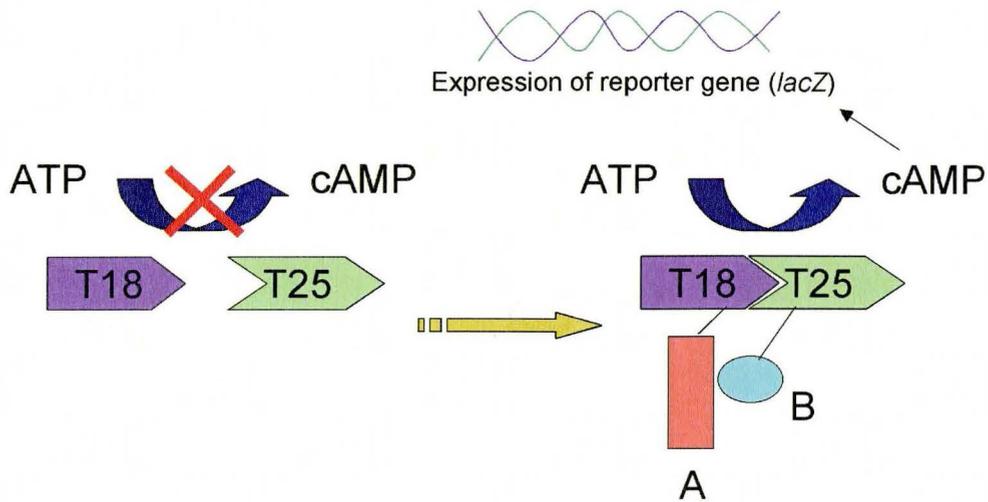


Figure 16: Principle of the Bacterial adenylate cyclase two-hybrid system (BACTH). The *B. pertussis* adenylate cyclase is separated into two catalytic fragments, but when brought into close proximity cAMP is synthesized and promotes the expression of a reporter gene (*lacZ*). Adapted from: (Karimova *et al.*, 2005; Karimova *et al.*, 1998)

The genes encoding p1201A, p120 Arm domain, Hakai, Ecadc and Ecadc variant (Y755E, Y756E) were cloned into both pUT18C and pKT25 as fusions to the T18 or T25 fragment could generate various protein conformations that may artificially enhance or inhibit the interactions between the proteins of interest (Table 6A lists all the constructs generated). The initial experiments were performed at 37, 30 and 26 °C to determine the optimal temperature for the interaction.

The interaction between Hakai and Ecadc, predicted by the literature (Fujita *et al.*, 2002), was not confirmed in this system, as the β -galactosidase activity resembled that of the negative control (T18, T25) (Figures 17 and 18). Similarly, mimicking phosphorylated tyrosines 755 and 756 on Ecadc by changes to glutamate did not increase Hakai's ability to bind Ecadc as detected by the BACTH system (Figure 17 and 18).

These results are depicted by the apparent lack of interactions measured on LB agar with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Figure 17) and by liquid ortho-Nitrophenyl- β -galactoside (ONPG) at 37, 30 and 26 °C (Figure 18).

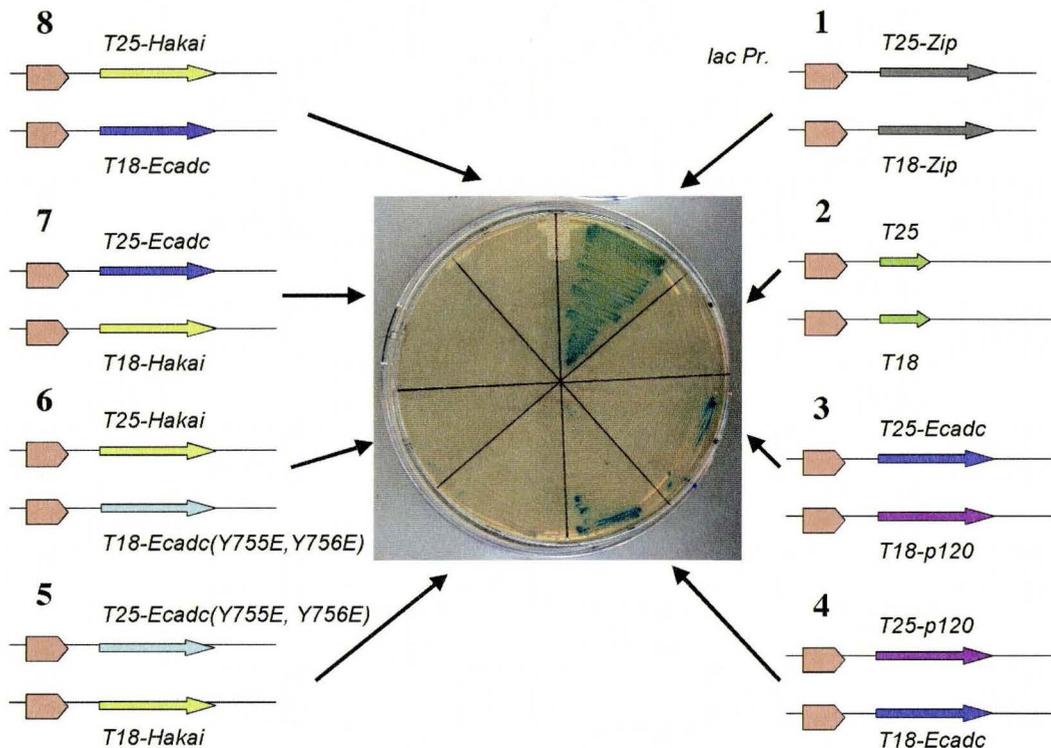


Figure 17: Bacterial-2-hybrid interactions detected on X-gal agar. This assay measures the ability of two fusion proteins interacting and thus promoting T18 and T25 to come into close proximity and subsequent X-gal breakdown. The positive control strain produced T18-ZIP, T25-Zip (1) and the negative control produced T18 and T25 fragments (2). The potential interacting proteins were p120 (p120A isoform), Ecadc (E-cadherin cytoplasmic domain), Hakai full length, Ecadc (Y755E, Y756E) double variant. There interaction was monitored by bacterial growth and colour production on X-gal LB agar 27 °C.

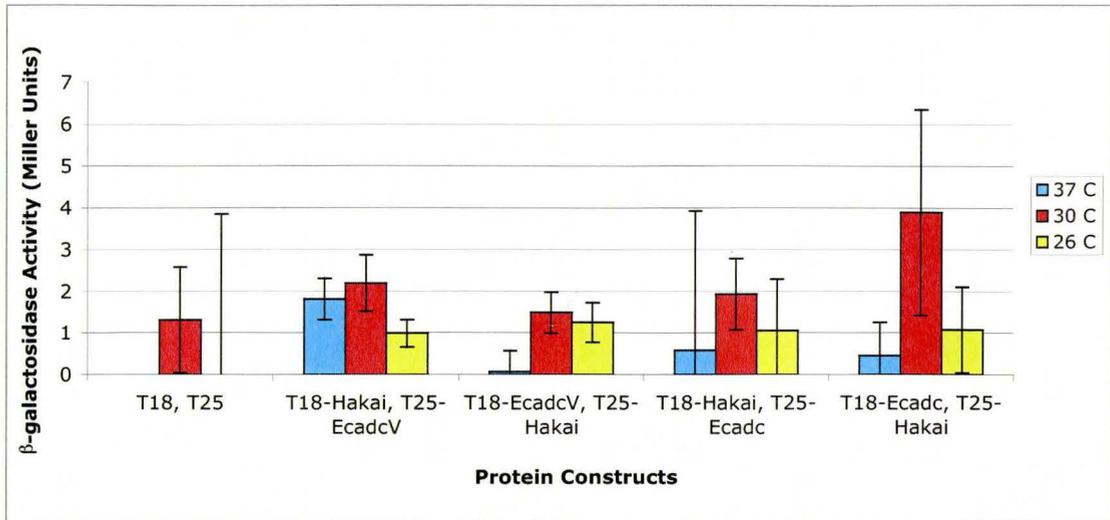


Figure 18: Bacterial-2-hybrid assay to measure interactions between Hakai and Ecadc and Ecadc Y755E, Y756E variant. To detect an interaction between Hakai and Ecadc (E-cadherin cytoplasmic domain) or EcadcV (variant Y755E, Y756E) constructs were created to express alternate T18 or T25 fusions and the protein expression was induced at 37, 20 and 26 °C to determine an optimal temperature for fusion protein expression and interaction. A β -galactosidase assay was performed to quantify the fusion protein interaction indicated in Miller units and the standard deviation from three experiments is shown.

The interaction between p1201A and Ecadc at 26 °C was detected in this assay, but it was relatively weak as compared to the positive controls in both the X-gal agar (Figure 17) and the liquid ONPG assay (Figure 19). The potential dimer formation of Ecadc (Shapiro *et al.*, 1995) was not detected using this method (Figure 19).

A reason for the lack of strong protein-protein interactions in the BACTH may be a lack of post-translational modifications in a bacterial system (phosphorylation sites in p1201A). Next, the p120Arm domain was expressed in the system and the interaction with Ecadc was tested. The interaction between p120Arm and Ecadc was stronger and found to be more elevated at 27 °C. The combination of T18-p120Arm and T25-Ecadc

expression vectors resulted in higher values (approximately 150 M.U.) than the opposite combination of T18-Ecadc and T25-Arm expressing vectors (approximately 80 M.U.) (Figure 19). In the positive control strain (T18-Zip, T25-Zip) the β -galactosidase activity reached over 2000 M.U at 37 °C (Figure 19). Similarly, assays conducted on X-gal agar demonstrated the interaction between T18-p120Arm and T25-Ecadc (construct 3) to be comparable to the positive control of T18-Zip, T25-Zip (construct 1) at 27 °C (Figure 22) substantiating the β -galactosidase activity seen with the liquid assay. Our next step was to test whether p120Arm repeats, and subsequent overlapping peptides of those repeats, could bind Ecadc effectively.

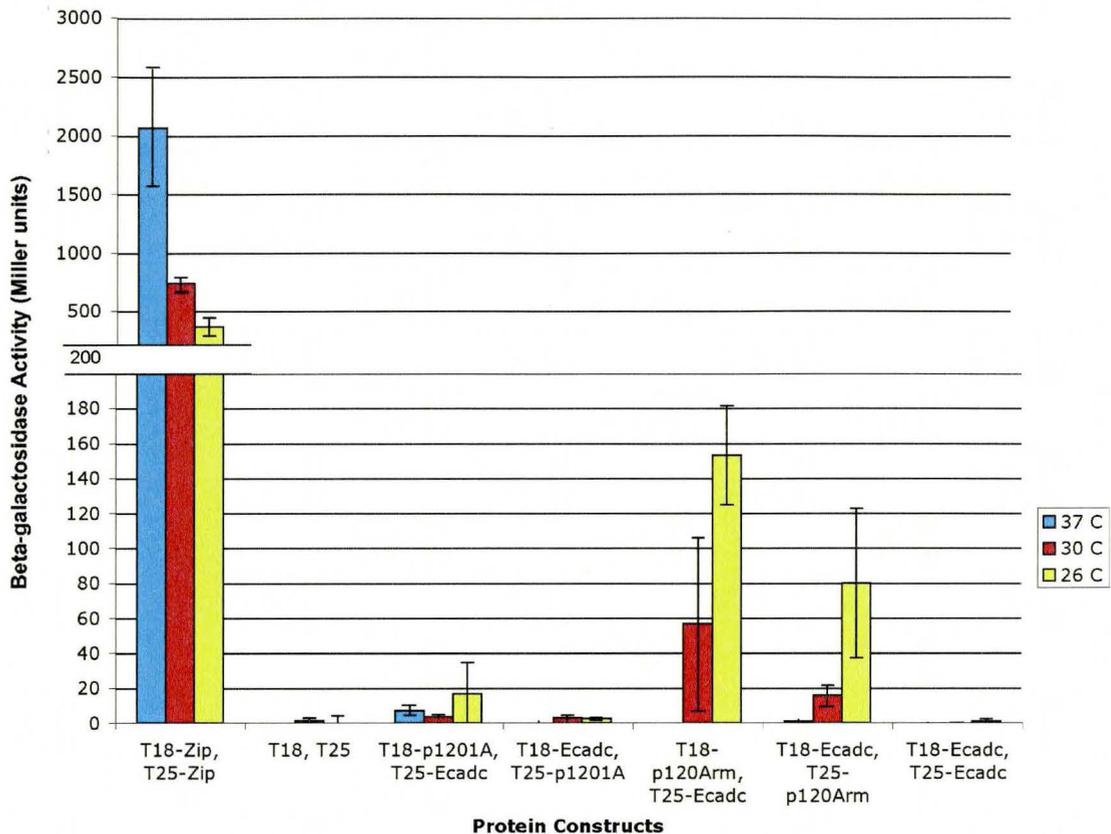


Figure 19: Bacterial-2-hybrid assay to measure the interactions between p120 1A isoform, p120 Arm domain and Ecadc. A β -galactosidase assay was performed after fusion proteins expression overnight at 37, 30 or 26 °C. The pair wise combination of fusion proteins to T18 and T25 fragments were p120Arm (p120 Arm domain), Ecadc (E-cadherin cytoplasmic domain) and p1201A (isoform 1A). The positive control of the assay was T18-Zip and T25-Zip and the standard deviation from three experiments is shown. The graph was cut at 200 Miller units to permit presentation of low β -galactosidase values.

4.6. Analysis of p120 Armadillo repeats - E-cadherin binding using bicistron expression

The BACTH system was next used to determine the binding ability of peptides when competing with their full-length protein counterpart. A bicistron vector was constructed in which the genes encoding full-length p120Arm along with that encoding a

p120-derived peptide (within the Armadillo repeats) was sub-cloned into the same expression vector separated by a Shine-Dalgarno sequence. This vector expressed an AC fragment (T18) fused to the full-length protein as well as a separate peptide aptamer (Figure 20 (A))(Karimova *et al.*, 2005). The BACTH system was used here to determine if individual or grouped Armadillo repeats competitively bind Ecadc in the presence of full-length p120Arm, thus diminishing the p120Arm- Ecadc interaction. These results were intended to indicate the Armadillo repeat(s) that determine the interaction between p120 and Ecadc.

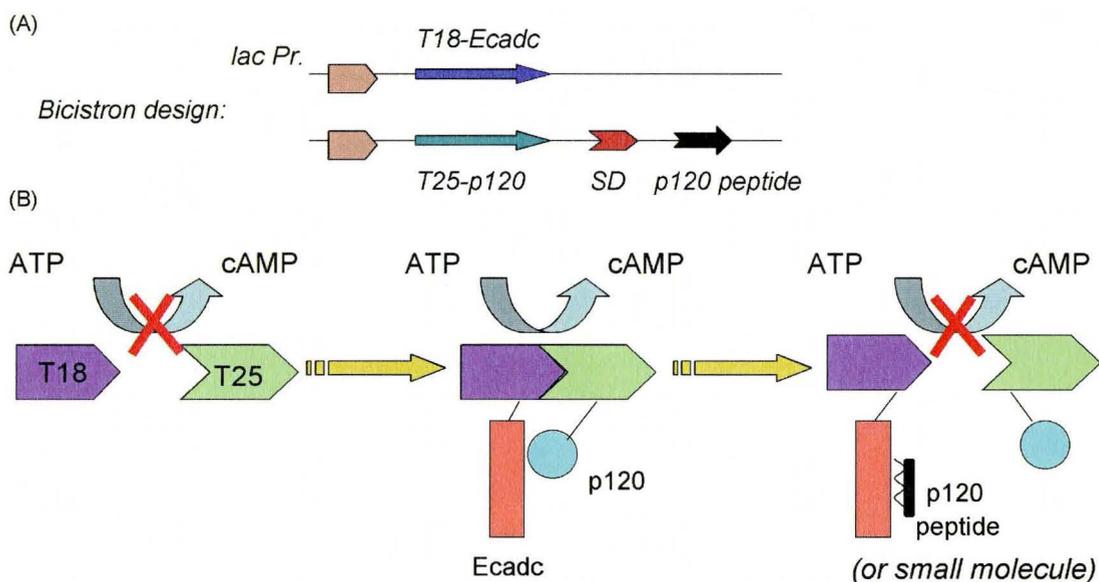


Figure 20: Bicistron expression experiments (A) The bicistron vector under the control of the *lac* promoter (*lac Pr.*) expresses the fusion protein (T25-p120) followed by a Shine-Dalgarno (SD) sequence and a separate peptide (p120 peptide). (B) If the peptide binds its target Ecadc, it will reduce the protein-protein interaction and the T18 and T25 fragments will not come into contact and cAMP will not be produced. Adapted from: (Karimova *et al.*, 2005; Karimova *et al.*, 1998).

4.6.1. Expression of linear p120-derived peptides

To determine which p120Arm repeats contribute to binding of E-cad, the first bicistron was created that fused the ten p120Arm repeats to the T18 fragment, followed by a Shine Dalgarno sequence and p120Arm repeats 3-5. This construct would test whether the p120Arm repeats 3-5 (aa 437-587) (previously shown to bind E-cad (Anastasiadis and Reynolds, 2000; Ireton *et al.*, 2002; Roczniak-Ferguson and Reynolds, 2003)) can displace the full-length p120 Arm repeats and decrease the measured β -galactosidase activity. However, the opposite effect occurred with the bicistron and we measured increased β -galactosidase activity in presence of the bicistron construct (an increase of approximately 40 Miller Units) at 27 °C (Figure 21) and similar results were obtained at other temperatures (Figure 23). A Student T-test confirmed that the increase in β -galactosidase activity by the bicistron compared to T18-p120Arm, T25-Ecadc was significant ($P=0.0067$) (Figure 21).

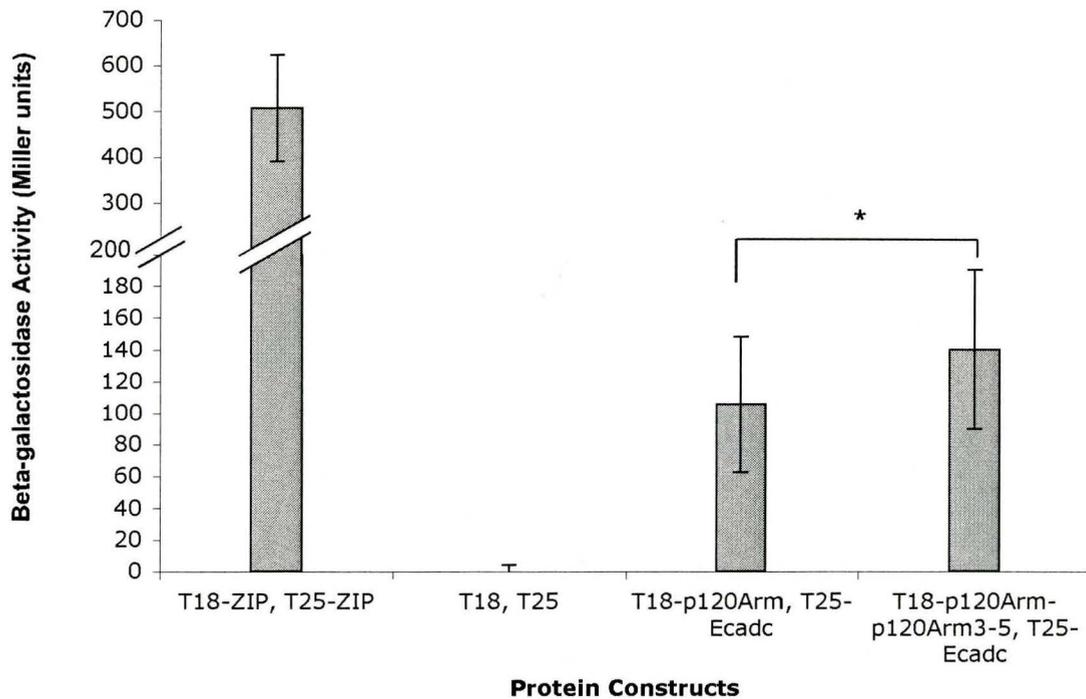


Figure 21: Bacterial-2-hybrid assay to measure interactions between E-cadherin cytoplasmic domain and p120 Armadillo domain (and only repeats 3-5) using bicistron expression. A β -galactosidase assay measured the interaction between the fusion proteins with or without the presence of the bicistron expression. The positive control for the assay was T18-ZIP, T25-Zip and the negative control was the two fragments expressed unfused to a protein. The potential interaction protein domains were p120Arm (full 10 Armadillo repeats of p120), Ecadc (E-cadherin cytoplasmic domain), p120Arm-A3-5 (full length 10 Armadillo repeats fused to T18 fragment, but also expressing untagged Arm repeats 3 to 5 of p120 = bicistron). Student T-test determines the increase in β -galactosidase activity in the presence of the bicistron was significant * $p < 0.0067$.

To test whether p120Arm 3-5 alone interacts with Ecadc, a new construct of p120Arm 3-5 fused to T18 (T18-p120Arm3-5) was created, but the data did not show an apparent interaction with T25-Ecadc (Figure 22 and 23) indicating that these repeats do not bind Ecadc in this assay. The next step we pursued was to design spanning peptides

of the p120 Armadillo repeats 2-6 (Figure 24) to cover more of the junctions between repeats and up to 4 repeats and to test their interaction with Ecadc.

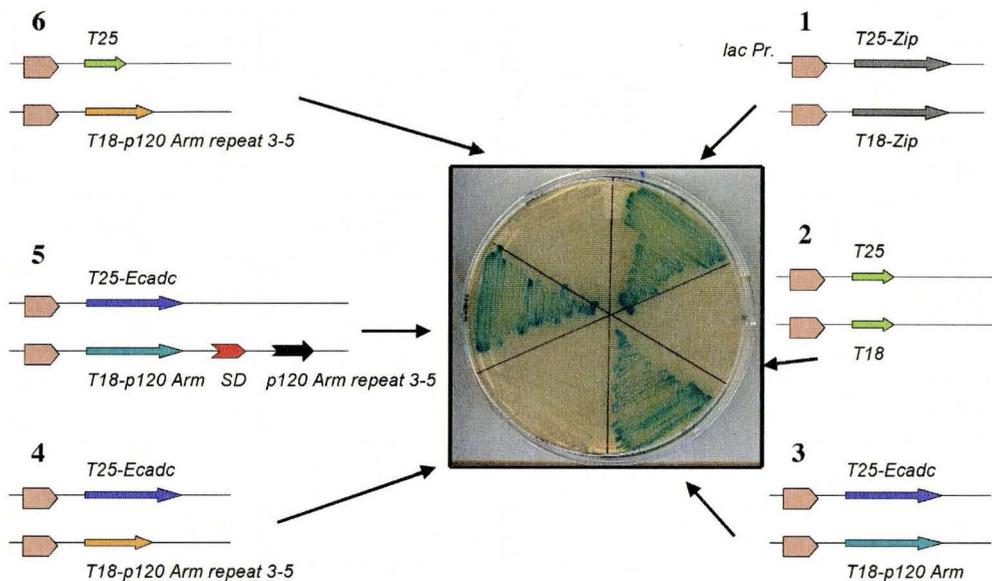


Figure 22: Bacterial-2-hybrid assay to measure the interaction between p120Arm repeats 3-5 and Ecadc through bicistron expression. Two fragments of adenylate cyclase (T18 and T25) were fused to potentially interacting protein domains and measured by β -galactosidase breakdown of X-gal. The positive control for the assay was T18-ZIP, T25-Zip (1) and the negative control was the two fragments expressed domains T18 and T25 (2). The protein domains fused to either T18 or T25 were p120Arm (full 10 Armadillo repeats of p120), Ecadc (E-cadherin cytoplasmic domain) and p120Arm repeat 3-5 (full length 10 Armadillo repeats fused to T18 fragment, but also expressing untagged Arm repeats 3 to 5 of p120 = bicistron) (3-5). Experiment (6) was a second negative control to verify that T18-p120Arm repeat 3-5 did not bind T25. The transformed strains were grown at 27 °C on LB agar with X-gal and blue colour change was observed indicating interactions.

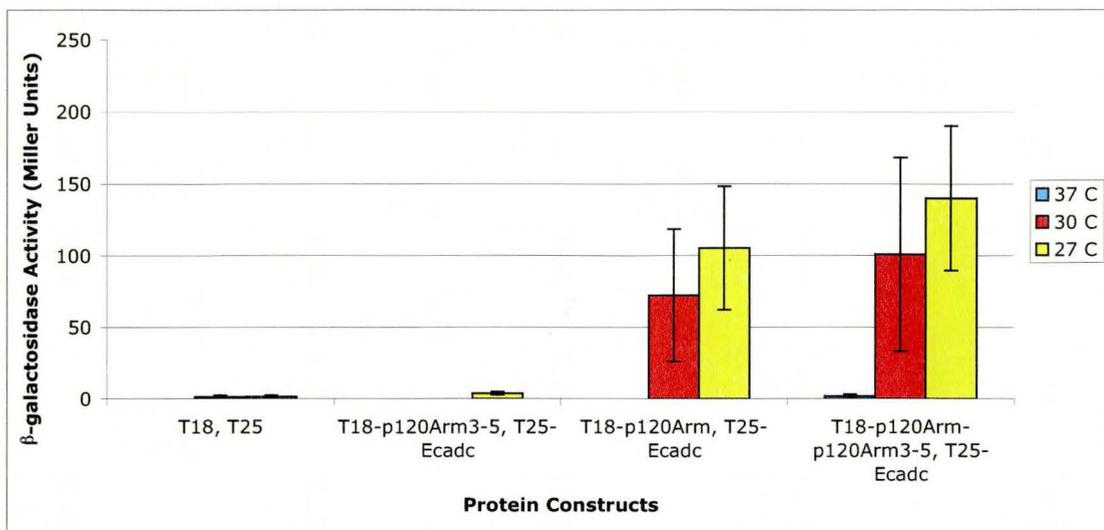


Figure 23: Bacterial-2-hybrid to measure the interaction between p120Arm repeats 3-5 and Ecadc. A β -galactosidase assay was performed to measure the interaction between T18 or T25 fused proteins. The proteins examined were p120Arm3-5 (Armadillo repeats 3-5 fused to T18), p120Arm (full 10 Armadillo repeats of p120), Ecadc (E-cadherin cytoplasmic domain), p120Arm-p120Arm3-5 (full length 10 Armadillo repeats fused to T18 fragment, but also expressing untagged p120Arm repeats 3 to 5 of p120 = bicistron). The protein constructs were expressed overnight at 37, 30 or 27 °C.

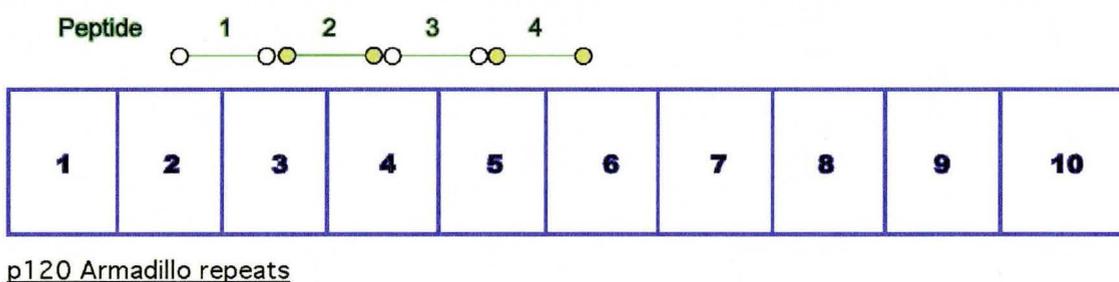


Figure 24: Spanning repeats of the p120 Armadillo domain tested. Scheme of the p120Arm peptides used to test the repeats 2-6 spanning the junctions between repeats. The exact amino acids in each peptide are outlined in Table 5.

The spanning peptides were generated to include the junctions between p120Arm domains 2-6 and each peptide encompassed one half of two Armadillo repeats (Table 5). The genes encoding the peptides were subcloned into the pUT18C-p120Arm bicistron and expressed at 30 °C and 27 °C. The error bars were much larger at 30 °C compared to 27 °C, however, a small decrease seen in the strains expressing the bicistrons with p120Arm 3-4 spanning peptide (known hereafter as Arm3-4sp) and Arm4-5sp was evident at 30 °C. The peptides in the bicistron were proposed to inhibit the interaction and decrease the M.U., which was not seen in Figure 25 at 27 °C, however at 30 °C, the M.U. for the peptide Arm4-5sp was 66.7 ± 59.5 M.U. compared to the control interaction of T18-p120Arm and T25-Ecadc at 74.4 ± 40.4 M.U. The expression of the next peptide that led to enzyme levels slightly above the control was Arm3-4sp at 85.2 ± 55.6 M.U. Whereas the very high standard deviation precluded clear conclusions from these experiments, we decided to further analyze whether the expression of peptides Arm3-4sp and Arm4-5sp in the constrained form would have more pronounced effects. The exact M.U. and standard deviations are presented in Appendix Table 1.

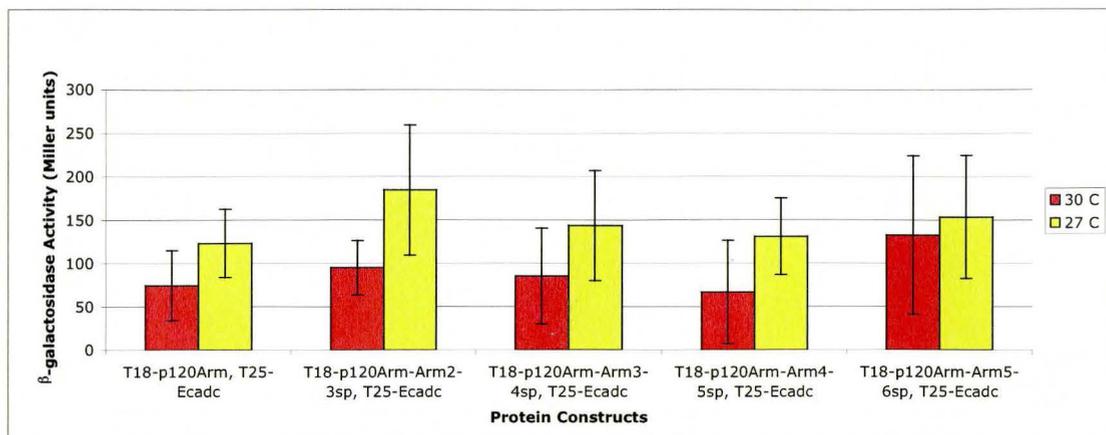


Figure 25: Bacterial-2-hybrid assay to measure interaction between p120Arm and Ecadc in the presence of linear p120Arm spanning repeat peptides. A β -galactosidase assay was used to measure the decrease in protein binding between the T18 and T25 fused proteins in the presence of linear peptides. The fusion proteins examined were p120Arm (full 10 Armadillo repeats of p120) and Ecadc (E-cadherin cytoplasmic domain). The linear peptides expressed as a bicistron were p120Arm-Arm2-3sp (full length 10 Armadillo repeats fused to T18 fragment, but also expressing untagged p120Arm spanning repeats of 2-3, 3-4, 4-5 or 5-6 of p120 = bicistron). The protein constructs were induced to express overnight at 30 or 27 °C.

4.6.2. Expression of constrained p120-derived peptides

To this end, two other bicistrons were generated to express *E. coli* thioredoxin A (Trx) protein as a scaffold protein to constrain the peptide. This approach was performed to express constrained versions of peptides Arm3-4sp and Arm4-5sp and their effects on the p120Arm-Ecadc interaction were tested next.

The constrained peptides appeared to increase the interaction between T18-p120Arm and T25-Ecadc. This may be the result of the expression of Trx as the negative control of T18-p120Arm-Trx which increased M.U. compared to strains without Trx in Figure 26. With that in mind, examining the effects of the constrained peptides, it was determined that the presence of Trx3-4sp (p120Arm spanning repeats 3-4 fused to Trx

active site) and Trx4-5sp produced lower M.U. at 27 °C than the linear peptides, but the opposite was seen at 30 °C. Nevertheless, both Trx3-4sp and Trx4-5sp did not restore the M.U. of the positive control T18-p120Arm and T25-Ecadc. Lastly, to examine if a constrained peptide without full-length p120Arm would bind to T25-Ecadc, the Trx4-5sp peptide was fused to T18 and examined using the ONPG liquid assay. Figure 26 indicates that T18-Trx4-5sp did not bind to T25-Ecadc in this assay implying that this region is not effective at binding Ecadc or that this assay may not be effective at measuring this interaction. The exact M.U. and standard deviations are presented in Appendix Table 2.

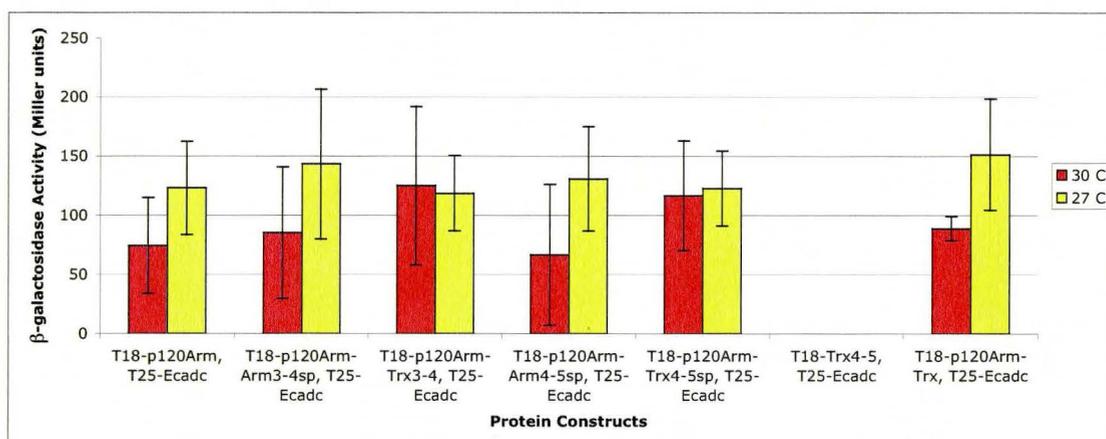


Figure 26: Bacterial-2-hybrid to measure the interaction between p120Arm and Ecadc in the presence of constrained p120Arm spanning repeat peptides. A β -galactosidase assay was performed to measure the dissociation of the interaction between T18-p120Arm and T25-Ecadc by constrained p120Arm spanning repeat peptides. The protein domains examined were p120Arm (full 10 Armadillo repeats of p120), Ecadc (E-cadherin cytoplasmic domain), p120Arm-Arm3-4sp (full length 10 Armadillo repeats fused to T18 fragment, but also expressing untagged p120Arm spanning repeats of 3-4 or 4-5 of p120 = bicistron). Trx is the Thioredoxin A scaffold creating a constrained peptide. The protein expression constructs were grown overnight at 30 and 27 °C.

4.7. Testing peptide binding ability in vitro

Based on the information gathered in the bacterial-2-hybrid bicistron experiments, the peptides that diminished the interaction between p120Arm and Ecadc would be candidates for further *in vitro* examination of binding to Ecadc. It was proposed that these peptides may bind Ecadc with higher affinity than the full length p120Arm, thus could be used to replace the full length p120Arm domain's function of binding Ecadc and potentially stabilizing it at the membrane *in vivo*.

To test this, the *in vitro* transcription/translation (TnT) system for mammalian protein was used as this assay incorporated radiolabeled methionine into proteins, which we hoped would increase the sensitivity of our co-precipitation assay. TnT required a SP6 or T7 promoter (Promega, 2008) for expression and the genes encoding p120Arm and Hakai were cloned into pT7GST that possessed a GST tag in the place of Strep otherwise identical to the original pT7-7 backbone.

The genes encoding linear peptides were also cloned into a pT7GST vector to enable purification via GST affinity chromatography from bacterial over-expression. Both the vectors pT7GST-p120Arm3-4 encoding (29 kDa peptide with tag) and pT7GST-p120Arm4-5sp (31 kDa peptide with tag) were constructed and the GST tagged peptides (referred to as 'peptides' noting that they are much larger than 20 aa) were purified from Rosetta DE3 after cultivation under induced conditions for 16 h at 15 °C (Coomassie Staining Figures 27 and 28). Both peptides were initially purified by GST affinity chromatography and then by anion exchange (pI for both approximately 5.7). After anion exchange chromatography and dialysis into storage buffer the yield of both

peptides was 3.1 mg from a 400 mL culture. The over-expressed peptides possessed the GST tag as confirmed by western blotting (Figures 27 and 28), but the p120 antibody did not recognize either GST-p120Arm3-4sp or GST-p120Arm4-5sp. These peptides were then used in experiments aimed at out-competing full length GST-p120Arm in a co-precipitation experiment with Strep-Ecadc. GST-p120Arm was produced using the *in vitro* TnT reaction to incorporate radiolabeled ^{35}S methionine ($^{35}\text{SMet}$).

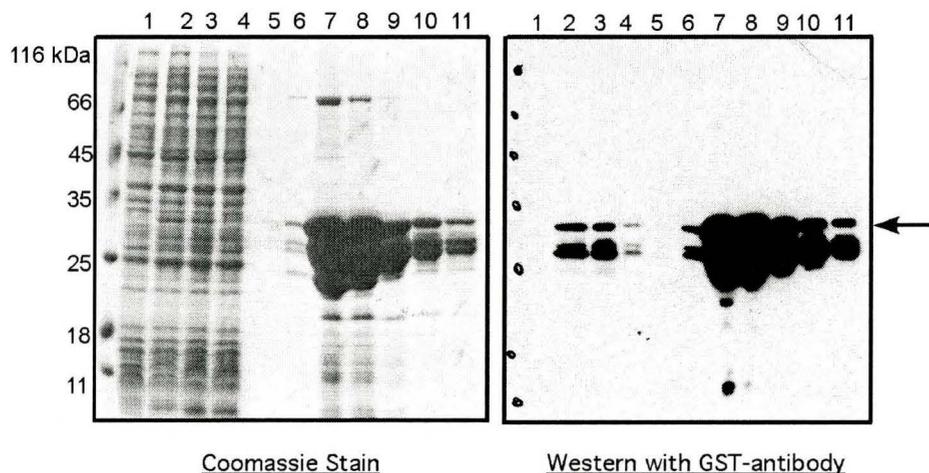


Figure 27: Purification of GST-p120Arm spanning repeats 3-4 linear peptide. The samples taken at different time points during the over-production experiment and purification were electrophoresed on a 12 % SDS-PAGE followed by Coomassie staining and western blotting with GST antibody. GST-p120Arm3-4 has a calculated molecular mass of 29 kDa indicated by the arrow. Lane 1: Before induction, 2: 16 h after induction, 3: all soluble fraction after lysis, 4: Flow through GST column, 5-11: elutions from the GST affinity column. Molecular masses of the reference proteins are indicated on the left in kDa.

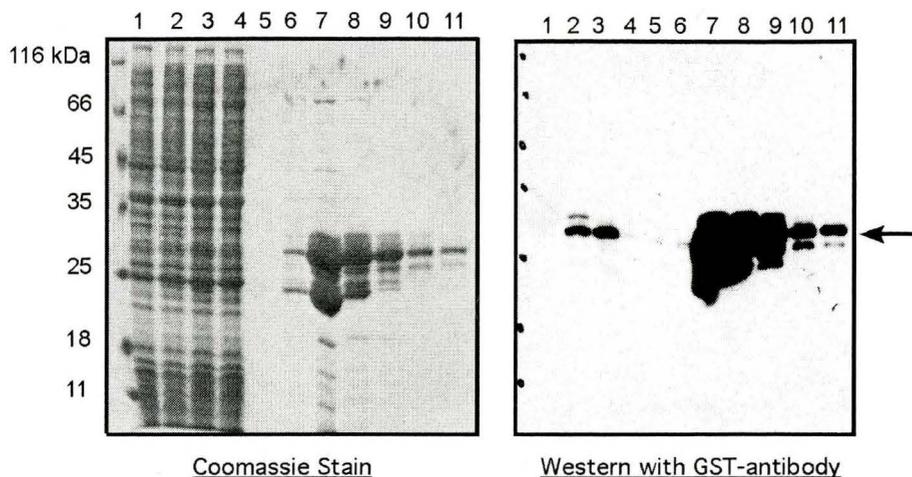


Figure 28: Purification of GST-p120Arm spanning repeats 4-5 linear peptide. The samples taken at different time points during the over-production experiment and purification were electrophoresed on a 12 % SDS-PAGE followed by Coomassie staining and western blotting with GST antibody. GST-p120Arm4-5 has a calculated molecular mass of 31 kDa indicated by the arrow. Lane 1: Before induction, 2: 16 h after induction, 3: all soluble fraction after lysis, 4: Flow through GST column, 5-11: elutions from the GST affinity column. Molecular masses of the reference proteins are indicated on the left in kDa.

To determine if the peptides could out compete the full length $^{35}\text{SMet-GST-p120Arm}$ in its interaction with Strep-Ecadc, a Strep-Tactin pull down assay was performed with increasing amounts of peptide. It was not possible to determine how much $^{35}\text{SMet-GST-p120Arm}$ protein was present in the TnT reaction, as the protein was not purified from the cell lysate. Thus, the amount of GST-p120Arm4-5sp was adjusted relative to the concentration of Strep-Ecadc as both were produced in *E. coli* and purified. The amount of peptide was chosen based on a previous study that reported that a 1:1 ratio was sufficient for GST-tagged peptides generated in *E. coli* to bind their target. However, in this study the GST tag was cleaved after purification, which may affect the conformation (Nouvion *et al.*, 2007). The peptide was applied in the range of 0.25-5X as

compared to Strep-Ecadc, but this did not result in any visible reduction of $^{35}\text{SMet-GST-p120Arm}$ binding to Strep-Ecadc as determined by autoradiography (Figure 29).



Figure 29: Co-precipitation assay to test competition with *GST-p120Arm4-5sp*. A pull-down assay was used to examine if increasing amounts of *GST-p120Arm4-5sp* would out compete the interaction between $^{35}\text{SMet-GST-p120Arm}$ (10 μL of TnT reaction) and Strep-Ecadc (50 pmol in reaction) as measured by co-precipitation on a Strep-Tactin sepharose affinity matrix. As a control to ensure that the TnT reaction was successful, 20% of the input $^{35}\text{SMet-GST-p120Arm}$ was loaded. The negative control comprised $^{35}\text{SMet-GST-120Arm}$ alone pulled down with Strep-Tactin beads. Increasing amounts of *GST-p120Arm4-5sp* co-incubated (0.25-5X as compared to Strep-Ecadc). Protein samples resolved on a 10 % SDS-PAGE, followed by gel drying and exposure to XAR film at $-80\text{ }^{\circ}\text{C}$, autoradiograph presented. The molecular masses of the reference proteins are indicated on the left in kDa.

We next increased the amount of the *GST-p120Arm3-4* peptide (10, 20 and 40 X the amount of Strep-Ecadc) and Figure 30 A shows the results. Neither 10, 20 nor 40 X excess of the peptide led to a notable reduction of $^{35}\text{SMet-GST-p120Arm}$ binding to Strep-Ecadc. Similarly, as shown in Figure 30 B increasing amounts of *GST-p120Arm4-5sp* had no visible effect.

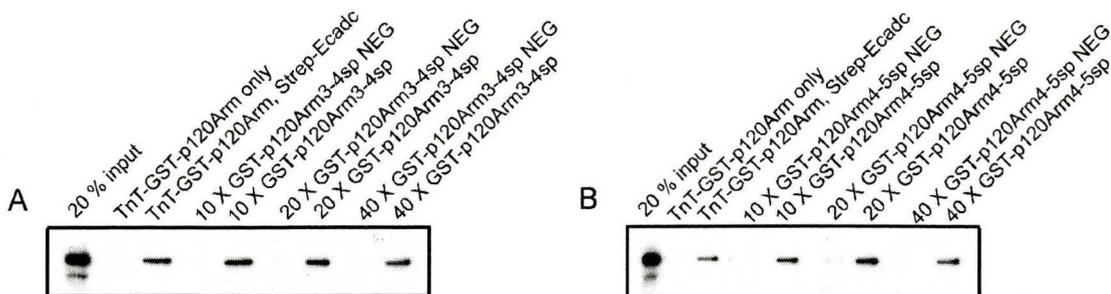


Figure 30: Co-precipitation assay to test competition with increasing amounts of GST-p120Arm3-4sp and GST-p120Arm4-5sp. A pull-down assay was used to examine if increasing amounts of GST-p120Arm3-4sp (A) or GST-p120Arm4-5sp (B) would out compete the interaction between $^{35}\text{SMet-GST-p120Arm}$ and Strep-Ecadc using Strep-Tactin sepharose affinity matrix. As a control to ensure that the TnT reaction was successful 20 % of the input $^{35}\text{SMet-GST-p120Arm}$ was loaded. The negative controls comprised $^{35}\text{SMet-GST-p120Arm}$ pulled down alone with Strep-Tactin beads. Also each increase in peptide was preceded by a negative control of $^{35}\text{SMet-GST-p120Arm}$ and peptide pull down without Strep-Ecadc (denoted NEG). Protein samples resolved on a 10 % SDS-PAGE, followed by gel drying and exposure to XAR film at $-80\text{ }^{\circ}\text{C}$, autoradiographs presented.

We also used densitometry to determine the decrease in $^{35}\text{SMet-GST-p120Arm}$ binding to Strep-Ecadc in the presence of peptides. This method revealed a small decrease of 3 % when 3 and 4 X excess of GST-p120Arm4-5 was added to the $^{35}\text{SMet-GST-p120Arm-Strep-Ecadc}$ pull down (Figure 29) and a decrease of 2 % when 40X GST-p120Arm3-4 was added (Figure 30 A). The densitometric analysis suggests that inclusion of the peptides modestly reduces the interaction between $^{35}\text{SMet-GST-p120Arm}$ and Strep-Ecadc, but more repetitions would be needed to substantiate these results.

5. DISCUSSION

5.1. Proposed Long-term approach

Originally, we planned to synthesize peptides that spanned the p120Arm repeats found to bind Ecadc to mimic full-length p120 and potentially restore *adherens junctions in vivo*. Peptides (20 amino acids in length) would either take a linear or constrained form. Linear peptides would be fused N-terminally to HIV Tat, a known cell-penetrating peptide (CPP) (Privé and Melnick, 2006), to mediate cellular entry and C-terminally to a Streptavidin affinity tag (Strep-tag) to facilitate purification and visualization during Western blot analysis. The constrained peptides would possess the same N-terminal CPP along with a C-terminal 6X His tag and would be displayed on the surface of a scaffold such as thioredoxin to stabilize the peptide conformation, increase binding affinity and provide proteolytic stability (Hoppe-Seyler *et al.*, 2004). If an interaction between Hakai and E-cadherin had been determined in the *in vitro* assays, peptides spanning the cytoplasmic domain of E-cadherin (Ecadc) would have been created to mimic the binding domain of Hakai. Ecadc (150 amino acids) was to be subdivided into 14 peptides of 20 amino acids, each overlapping the adjacent peptide by 10 amino acids. Theoretically, these could be used to sequester endogenous Hakai in the cell to allow E-cadherin to bind p120 effectively at the cell membrane.

In vitro studies of p120 peptides (overlapping design as described above with Ecadc) interacting with Hakai were to be conducted in order to identify peptide(s) with the highest binding affinity. Initially, the interactions studies such as co-precipitation,

chemical cross-linking and analytical gel filtration would be used to assess the binding ability of the peptides to the target protein. As well, binding affinity may have been quantified using ITC to distinguish which peptides would have the strongest affinity for their target. The successful candidate peptides from these studies would have then been further examined in the long-term approach *in vivo*.

5.1.1. Analyze the effects of peptides in vivo

The *in vitro* studies were to be validated using *in vivo* experiments to determine the effects of the peptides as well as of small molecules. These studies would have been conducted in collaboration with a student from Dr. Juliet Daniel's laboratory. The effect of inhibitory peptides on the morphology of the cell and the sub-cellular localization of E-cadherin would have been determined using immunofluorescence of E-cadherin and its complex proteins p120, α - and β -catenin. To complement this, immunoprecipitation experiments would validate *adherens junction* complex formation, as endogenous E-cadherin should successfully co-sediment its interaction partners (α - and β -catenin) as well as the p120 peptides. The co-precipitates from cellular extracts would be visualized through western blotting with protein-specific antibody and CPP-tag antibody, respectively. Similarly, these approaches would be used to visualize Ecadc peptides interacting with Hakai within the cell as well as co-sedimentation. Once we would have determined the most effective peptides and small molecules, their effect on inhibition of cell migration and invasion would have been studied primarily by Dr. Daniel's student who would verify, using Matrigel invasion assay and potentially nude mouse models, the effects of the peptides or small molecules on the inhibition of metastasis.

5.1.2. Rationale for long-term research goals

We anticipated pursuing a dual approach at identifying potential chemotherapeutics for solid tumours in the form of small molecule screens using the BACTH system as well as peptide aptamer drug therapy. We hoped that maintaining E-cadherin stability at the cell membrane would preserve *adherens junctions* and reduce tumour proliferation. The potential findings of this project therefore could have not only determined the importance of E-cadherin, p120 and Hakai for tissue integrity but also allowed academic scientists to use their knowledge to design effective and novel anti-metastatic therapies for cancer treatment (Prive and Melnick, 2006).

Whereas technical difficulties did not allow me to pursue most of the above mentioned approaches, we developed and applied novel assays for the analysis of the p120-Ecad interaction and those are discussed in the following.

5.2. Qualitative binding assays

5.2.1. Co-precipitation assay

Co-precipitation assays (pull-down) using affinity sepharose beads to trap the bait protein and the putative prey were attempted in two forms. Initially, proteins were generated in bacteria and we later used the TnT system to generate the prey protein *in vitro*. The GST tag is commonly used to perform *in vitro* pull-down assays with human proteins (Ferber *et al.*, 2008; Gloeckner *et al.*, 2000). Since it was previously determined that Strep-Ecadc bound nonspecifically to GST resin, StrepTactin resin was introduced as the matrix to bind the Strep tagged bait protein. Both a radioactive and a non-radioactive

co-precipitation assay were used to determine peptide binding and to try competition experiments.

The non-radioactive co-precipitation assay included GST-p120Arm and Strep-Ecadc produced by bacteria (Figure 13). The interaction between these two proteins was most evident when they were incubated in a 2:1 and 4:1 ratio and this was also previously observed with the murine protein counterparts (Melone, 2006). The radiolabeled GST-p120Arm (³⁵SMet-GST-p120Arm) also co-precipitated with 50 pmol of Strep-Ecadc as the bait (Figure 14). Proteins generated by TnT for these types of assays are commonly cytotoxic or difficult to produce in bacteria or eukaryotic systems (Spirin, 2004). TnT also has the advantage that small amounts of protein are required because of the sensitivity of radio-labeling and may be a quick strategy to assess whether two human proteins interact *in vitro* (Simone *et al.*, 2002). It was decided that this assay would be used to test the competition by p120Arm peptide aptamers.

5.2.2. Analytical gel filtration

Another technique attempted to study the protein-protein interaction between GST-p120Arm and Strep-Ecadc was analytical gel filtration that separates proteins based on size. This technique was previously shown in our lab to be successful at determining protein-protein interactions with the murine homolog (Melone, 2006). This analysis produced similar results with human proteins in that Strep-Ecadc eluted in higher molecular mass fractions when it was incubated with GST-p120Arm rather than when it was separated alone (Figure 15 B). This assay was originally to be used to test the binding ability of peptide aptamers with the expectation that peptides would decrease the

ability of GST-p120Arm and Strep-Ecadc to interact thus indicating competition by the peptide. However, this technique was not pursued further as the effects of peptides would have to be drastic to lead to different elution profiles and we already observed substantial variability in different experiments. If this technique were chosen to study the effects of peptide aptamers, further controls would have been included such as to test if Strep-Ecadc nonspecifically binds to the GST tag.

5.2.3. *Human proteins*

The lack of posttranslational modifications, which is due to use of a bacterial host strain for protein expression, may be a reason why stronger interactions have not been detected between p120 and Ecadc *in vitro*. p120A has been shown to possess tyrosine phosphorylation sites in the N-terminal region of the protein and may require post-translational modifications to interact with Ecadc (Daniel and Reynolds, 1997; Mariner *et al.*, 2001). It has been proposed that the kinases required to phosphorylate the 8 tyrosine residues N-terminal to the Armadillo domain are membrane bound linking these modifications to the binding of E-cadherin (Alema and Salvatore, 2006; Mariner *et al.*, 2001). Also, these proteins may be altered by mutagenesis to incorporate amino acids that mimic a phosphorylated tyrosine or serine shown to increase binding affinity between p120 and Ecadc as well as between Hakai and Ecadc (Fujita *et al.*, 2002; Roura *et al.*, 1999). An example of this was demonstrated with changes of serine residues to glutamate to mimic phosphorylated serine and enhance the binding affinity of E-cadherin to β -catenin (Gail *et al.*, 2005). Another method to over-come this limitation would be to implement a eukaryotic expression system, such as *P. pastoris*. However, the production

of the target proteins in this system has been very time consuming and showed minimal results with p120Arm, so that we decided not to pursue this approach.

5.3. Bacterial-2-hybrid assay

In general, the bacterial-2-hybrid (BACTH) has been shown to be useful for the analysis of interactions between mammalian proteins. Specifically, it was previously used to screen cDNA libraries for novel binding partners such as the cellular prion protein PrP^c that was found to bind TREK-1, a potassium channel protein. The authors continued their experiments to verify the interaction through co-immunoprecipitation and confocal microscopy (Azzalin *et al.*, 2006). Thus the BACTH assay was used as the initial screening tool.

The BACTH technique was initially established to determine the binding of p1201A to Ecadc as well as of Hakai to Ecadc. If successful, it would be further used to test the impact of peptide aptamers in an *in vivo* system. Initially, the analysis of the interactions between p1201A and Ecadc was attempted at various temperatures and in fusion to both N-terminal adenylate cyclase fragments (e.g. T18-p1201A, T25-Ecacd or T25-p1201A, T18-Ecacd). The results of the Hakai-Ecacd experiment did not produce any evidence of an interaction between the two proteins (Figure 17 and 18). Initially this was thought to be the result of a lack of post-translational modification in bacteria since Hakai has previously been shown to bind to phosphorylated tyrosines 755 and 756 on Ecadc (Fujita *et al.*, 2002). These residues were then changed from tyrosine to glutamic acid to mimic phosphorylated tyrosines creating Ecadc variant (Y755E, Y756E) (EV) (Zhang *et al.*, 1991) expecting to enhance the interaction between Hakai and Ecadc.

However, even with the glutamic acid in the place of tyrosine, the bacterial-2-hybrid did not indicate an interaction between Hakai and EV (Figure 17 and 18). It was then determined that Hakai is not stably expressed in *E. coli* and the presence of DnaK was up-regulated as a result indicating that Hakai over-production may strain the cells. Thus, in general, the assay was not efficient at demonstrating this protein-protein interaction between Hakai and Ecadc and this is likely due to the fact that Hakai cannot be expressed in *E. coli*.

Based on the knowledge that E-cadherin forms homodimers with adjacent E-cadherin proteins at the cell membrane (Shapiro *et al.*, 1995), it was thought that perhaps the cytoplasmic domain could contribute to dimerization and this possibility was tested in the bacterial-2-hybrid system. However, Ecadc dimerization was not detected using this assay (Figure 19). This result could simply imply that E-cadherin does not dimerize through its cytoplasmic domain at the cell membrane or that other proteins or molecules may be required for this to occur. It would be interesting to test in the future if the N-terminal region of E-cadherin, which is involved in binding E-cadherin molecules on adjacent cells (Shapiro *et al.*, 1995) dimerizes.

In contrast, the interaction between p1201A and Ecadc was detected with the bacterial-2-hybrid assay on plates with X-gal and with quantitative β -galactosidase assays after growth of the cells in liquid media. However, the β -galactosidase activity was lower than 50 M.U. and this indicated a weak interaction (Figure 17 and 19). It is worth noting that the interaction between p1201A and Ecadc could be enhanced by post-translational modifications internally performed by *E. coli*. Interestingly, mammalian signal cascades

can be analyzed in *E. coli* due to the lack of endogenous serine/threonine or tyrosine kinases that would constitutively phosphorylate proteins, as seen in yeast, making it difficult to specifically recognize which kinase is affecting the signaling cascade. *E. coli* signaling cascades are mostly regulated by histidine and aspartate, which makes it a good organism to study mammalian phosphoprotein interactions (Khokhlatchev *et al.*, 1997; Shaywitz *et al.*, 2002). To analyze the effect of a specific serine/threonine or tyrosine kinase, a third vector encoding a mammalian kinase could be introduced in addition to the BACTH system (bait and prey vectors). This approach could be pursued in future to analyze the p1201A interaction with Ecadc, as it was previously shown that p120 possesses an N-terminal phosphorylation domain that is a Src kinase substrate (Mariner *et al.*, 2001). This kinase could have been introduced to post-translationally modify p1201A and potentially increase the interaction between p1201A and Ecadc.

To further investigate the binding between p1201A and Ecadc, analysis of the interaction of T18-p120Arm (T18-Arm) and T25-Ecacd was attempted. p120Arm interacted more strongly as compared to p1201A with Ecadc as seen in Figures 19 and 22. A concern evident when examining the data was that the standard deviation was large despite the fact that all experiments were conducted at least 3 times. Each culture was grown in 3 separate tubes and each tube was tested in 3 separate wells of the 96-well plate. The average of all these experiments still had a high standard deviation and we have no explanation for this finding.

5.4. Bacterial bicistron analysis to test the effects of p120Arm spanning repeats peptides in vivo

5.4.1. Examining the p120Arm repeats 3-5

In continuation of the results on the interaction between p120Arm and Ecadc using the bacterial-2-hybrid system, we wanted to assess which p120 Armadillo repeats were required to bind Ecadc. In the context of this work, p120Arm repeats 3-5 (aa 437-587)(Anastasiadis and Reynolds, 2000) were chosen based on the literature demonstrating that the deletion of these repeats has the consequence that p120 is not able to bind to the JMD of Ecadc and the *adherens junctions* disassemble (Anastasiadis and Reynolds, 2000; Ireton *et al.*, 2002; Rocznik-Ferguson and Reynolds, 2003). These three repeats were cloned downstream of the Shine Dalgarno (SD) sequence on the pUT18C-p120Arm vector and the bicistrons were expressed *in vivo* along with pKT25-Ecadc. In case of bicistron fusions, the pUT18C vector expresses the T18 fused protein and a peptide from a downstream SD sequence. The bicistron approach was pursued to assess the interaction between T18-p120Arm and T25-Ecadc could be diminished by the expression and competition of p120Arm3-5. However, the opposite effect was observed (Figure 21 and 22). To test the interactions further, p120Arm3-5 was fused to the T18 fragment to determine if it could directly bind to T25-Ecadc, but this was not the case as there was no apparent interaction between T18-Arm3-5 and T25-Ecadc. A potential explanation could be that the p120Arm3-5 did not competitively bind but perhaps it increased the interaction between T18-p120Arm and T25-Ecadc by binding cooperatively to the two fusion proteins.

5.4.2. p120Arm spanning repeats

5.4.2.1. Linear peptides

The fact that p120Arm3-5 modestly increased the bacterial-2-hybrid interaction between T18-p120Arm and T25-Ecadc led to further investigation. It was purposed that smaller peptides should be cloned in the bicistron as p120Arm3-5 (18 kDa) may be too large or take an undesirable conformation. Perhaps the effects of peptide binding would be detected if smaller domains of p120Arm were analyzed. It was suggested that the junctions between p120 Armadillo repeats should be examined, as they may be significant for binding Ecadc. Therefore peptides spanning the junctions between p120Arm repeats 2-6 were designed to encompass a repeat junction and one half of two repeats of p120Arm repeats 3-5 (Figure 24). We anticipated that the peptides may decrease the binding between T18-p120Arm and T25-Ecadc, but this was only observed to some extent with peptide p120Arm 4-5sp and p120Arm 3-4sp (Figure 25). It is apparent that the linear peptides did not strongly inhibit the T18-p120Arm - T25-Ecadc interaction. This may be due to the random conformation they may take when produced in the bacterial cell. We therefore tested constrained peptides next.

5.4.2.2. Constrained peptides

To maintain a fixed conformation peptides are fused to scaffolds, usually a stable protein. This does not guarantee the most favourable conformation, but it is proposed to decrease the random structures of linear peptides (Berndt *et al.*, 2008). *E. coli* thioredoxin A (Trx) protein is one such scaffold used for the expression of the constrained peptides.

Trx regulates protein folding within the bacteria cell specifically by reducing disulfide bonds. Independently of its redox potential, Trx also functions to solubilize proteins by avoiding inclusion body formation and it protects against thermal degradation (Berndt *et al.*, 2008). In the context of this work, Trx was used as an inert scaffold protein to display peptides in a constrained form. It was previously shown that Trx fused peptides were able to bind their target in a yeast-2-hybrid assay and that they possess enhanced binding ability to their target *in vitro* as determined by their dissociation constants (Colas *et al.*, 1996).

With this potential in mind, p120Arm 3-4sp and p120Arm 4-5sp were cloned into the bicistron vector with T18-p120Arm and expressed as fusion to the active site of Trx. The effect of the two constrained peptides were tested on the T18-p120Arm - T25-Ecadc interaction, but a reduction was not observed (Figure 26). Instead, it was found that the constrained peptides modestly increased the interaction between T18-p120Arm and T25-Ecadc. We also tested whether Trxp120Arm 4-5sp fused C-terminally to the T18 fragment bound T25-Ecadc (Figure 26), but this was not the case. As a control, the bicistron of T18-p120Arm-SD-Trx was tested to assess whether the Trx alone would have an effect and it appeared to increase this interaction as seen in Figure 26. To improve this control a random peptide should be expressed as fusion to Trx because the scaffold could have cellular effects that are not relevant to this assay. Nevertheless, it was interesting to note the increase in binding seen by Trx, perhaps this result is due to its function as a chaperone protein that assists protein folding and stability (Berndt *et al.*, 2008). Regardless, the linear and constrained peptides tested in this system were not

efficient in decreasing the binding between T18-p120Arm and T25-Ecadc. Other peptides could have been tested individually to bind T25-Ecadc but time and evidence to pursue this approach further were not available. If time had permitted, an alternative, a yeast-2-hybrid assay may have been attempted since it was previously used to test protein-peptide interactions (Yang *et al.*, 1995). A yeast-2-hybrid may have had the advantage of producing eukaryotic post-translational modifications, a factor that lacked from the bacterial-2-hybrid system (Hoppe-Seyler *et al.*, 2001).

5.5. Cloning and purification of GST-p120Arm spanning repeats peptides

In order to test the impact of p120Arm peptides *in vitro*, the fragments encoding peptides p120Arm3-4sp and p120Arm4-5sp were cloned into bacterial expression vectors behind a T7 promoter. This was done to ensure that further experiments aimed at testing the binding of the peptides to Ecadc could be done by radiolabeling the peptide in the TnT system. The pΓ7GST vector was used and these proteins were over-expressed and purified from *E. coli* in order to out-compete the binding of radiolabeled ³⁵SMet-GST-p120Arm to Strep-Ecadc in a co-precipitation assay. The over-expressed GST-tagged peptides were on average 29-31 kDa in size because of the relatively large GST tag (25 kDa). This approach was previously pursued to examine the interactions of CDC6, a protein involved in DNA replication and cell cycle progression. Peptides (27-35aa) encoded by chicken genomic DNA that bind CDC6 were discovered using the BACTH system. The interaction with these peptides was then further examined by an *in vitro* co-precipitation in which the peptide was fused by an N-terminal GST and purified from *E. coli* BL21. The peptide was GST affinity purified from *E. coli* and incubated with lysate

from HeLa cells transfected with a plasmid expressing CDC6 to GST co-precipitate them together *in vitro*. It was eventually shown that one peptide had the ability to inhibit CDC6 and may be used as a cell-cycle inhibitor (Zhu *et al.*, 2000). We had hoped to obtain similar results in the context of this work, but the analysis of the binding ability of the p120Arm peptides could not be completed. Initially, the GST affinity chromatography purification of the p120Arm-derived peptides showed some degradation as indicated by the GST western blot (Figures 27 and 28). Perhaps, the GST tag is cleaved from the peptide by cellular enzymes. A similar result was also noted in the paper studying CDC6 mentioned above, however, we thought that degraded peptides could produce unspecific results and they were therefore removed. To remove these degradation products the elutions were further purified by anion exchange chromatography. These peptides were designed to be linear, but it was originally proposed to later study constrained peptides but we could not complete this work due to time constraints. We realize that the large GST tag may be reason for concerns, perhaps a smaller tag should have been used such as a 6X His. However, at the time, a vector with a T7 promoter that would permit hexahistidyl tag fusions was not available. Another alternative could have been to synthetically produce peptides and this would eliminate the need of an affinity tag (Miranda and Alewood, 1999).

5.6. In vitro Strep-pull down assay

As a result of difficulties in protein purification and the identification of the most effective p120Arm domain peptide, much of our initially planned work was not conducted. Nonetheless, the information gained above was used to attempt *in vitro*

experiments with p120Arm spanning repeats 3-4 and 4-5. These co-precipitation experiments revealed a small decrease in the interaction between ³⁵SMet-GST-p120Arm and Strep-Ecadc with 40X excess of peptide GST-p120Arm3-4sp and 3-4X excess of peptide GST-p120Arm4-5sp depicted in autoradiographs of Figures 29 and 30. However, the small decrease (ranging 2-3 % measure by densitometry) requires further validation. Additional optimizations were not attempted due to time constraints.

The lack of a more drastic inhibition by the peptides could have several reasons. The ability of the GST-p120Arm-derived peptides to bind Strep-Ecadc directly *in vitro* was not tested. These peptides were always examined in the presence of ³⁵SMet-p120Arm, and therefore, we do not know if the GST-p120Arm-derived peptides have any affinity for the Strep-Ecadc. This was not attempted due to time restraints. Secondly, previous work showed that bacterial made peptides usually have the fusion tag cleaved before they are used as inhibitors and we did have time to pursue this approach (Nouvion *et al.*, 2007). Thirdly, the effective concentration may not have been found yet, as the amount of ³⁵SMet-p120Arm protein cannot be quantified in the reticulocyte lysate. Lastly, perhaps the full p120Arm domain takes a conformation that permits binding to Strep-Ecadc and the torsion or strain required to fold into a favourable binding conformation is not accomplished with only two halves of an Arm domain. Also, constrained peptides could be used to increase the rigidity of conformation that could be required for effective protein-peptide binding.

5.7. Additional future experimental techniques

Chemical cross-linking

A method to potentially analyze the disruption of protein-protein interactions by peptide aptamers could be chemical cross-linking (Paschos *et al.*, 2006). This technique was employed to detect murine GST-p120Arm and Strep-Ecadc interactions in previous work of our lab (Melone, 2006). In brief, two chemical cross-linking agents may be used. Firstly, DSS which is an irreversible cross-linking agent that cross-links lysine residues at a distance of 11 Å (Montesano *et al.*, 1982). Alternatively, a second cross-linking agent is formaldehyde, which is a reversible cross-linker that cross-links at lysine and arginine residues as close as 2 Å apart (Jackson, 1999). First, the proteins would be subjected to cross-linking individually to detect oligomerization of the purified protein. These cross-links would then be compared to the pattern resulting after cross-linking of pair-wise mixture of Strep-Ecadc and GST-p120Arm. If a unique cross-linking product was formed that corresponds to the sum of the molecular masses of GST-p120Arm and Strep-Ecadc then this could be taken as evidence for an interaction. Chemical cross-linking could be attempted when the human proteins are purified further from their degradation products that can also cross-link. This would produce many cross-linking products that are not representative of the full-length protein.

Isothermal Calorimetry

Measuring affinity constants between proteins is a powerful tool in determining the strength of interactions and the impact of small molecules or peptide aptamers. A common quantitative technique to determine binding constants is through Isothermal

calorimetry (ITC). ITC measures the direct thermodynamic parameters of a binding reaction (ΔG , ΔH and ΔS), and also measures the dissociation constant (K_d) (Velazquez-Campoy *et al.*, 2004). It was previously found that *in vitro* phosphorylation of p120 with pp60^{c-src} increased the binding ability of p120 to the cytoplasmic domain of E-cadherin as compared to the non-phosphorylated p120 by a factor of 4 (as indicated by the association constants determined by densitometry of the affinity tag co-precipitation assay) (Roura *et al.*, 1999). ITC has advantages in determining binding constants and it considers the solvent effects and any conformational changes that may occur upon binding (Ababou and Ladbury, 2006). This technique has also previously been shown to be an excellent tool for examining protein interactions among proteins of the Wnt signaling pathway (Liu *et al.*, 2006). This technique may be employed to quantitatively measure changes in protein-protein interactions with the presence of peptide aptamers. Before attempting this, the current proteins will need to be purified further to remove any degradation products as it would be difficult to verify if the peptides or the degraded protein products are producing the changes in thermodynamics.

6. CONCLUSION

The hypothesis that peptides spanning p120Arm could bind Ecadc and effectively replace the need of the full-length p120Arm *in vitro* could not be substantiated in the context of this thesis. It was found that bacterial expression of Hakai is not possible, but expression of p120Arm and Ecadc was feasible. Peptide aptamers of p120Arm did not bind to Ecadc, as measure by competition of the interaction with p120Arm, but further investigation should be conducted to effectively use peptides to bind E-cadherin in the absence of p120Arm. The overall hypothesis was not validated, however, this thesis did confirm the interaction between p120 and E-cadherin via the Armadillo domain using co-precipitation, analytical gel filtration and the bacterial-2-hybrid system. These assays were not previously used to demonstrate this interaction and could be applied for more thorough analysis in future.

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8. APPENDIX

Data for Figure 25: Linear peptides to inhibit T18-p120Arm and T25-Ecadc interaction				
Construct	30 °C		27 °C	
	Average	Std dev	Average	Std dev
T18-p120Arm, T25-Ecadc	74.4	40.4	123.0	39.4
T18-p120Arm-Arm2-3sp, T25-Ecadc	95.0	31.1	184.4	74.8
T18-p120Arm-Arm3-4sp, T25-Ecadc	85.2	55.6	143.4	63.4
T18-p120Arm-Arm4-5sp, T25-Ecadc	66.7	59.5	130.9	44.1
T18-p120Arm-Arm5-6sp, T25-Ecadc	132.6	91.5	153.6	70.7

Appendix Table 1: Bacteria-2-hybrid averages and standard deviations as depicted in Figure 25.

Data for Figure 26: Constrained peptides to inhibit T18-p120Arm and T25-Ecadc interaction				
Construct	30 °C		27 °C	
	Average	Std dev	Average	Std dev
T18-p120Arm, T25-Ecadc	74.4	40.4	123.0	39.4
T18-p120Arm-Arm3-4sp, T25-Ecadc	85.2	55.6	143.4	63.4
T18-p120Arm-Trx3-4, T25-Ecadc	125.1	67.0	118.6	31.8
T18-p120Arm-Arm4-5sp, T25-Ecadc	66.7	59.5	130.9	44.1
T18-p120Arm-Trx4-5sp, T25-Ecadc	116.7	46.3	122.9	31.5
T18-Trx4-5, T25-Ecadc	-5.8	1.8	-5.8	1.2
T18-p120Arm-Trx, T25-Ecadc	89.0	10.1	151.6	47.0

Appendix Table 2: Bacteria-2-hybrid averages and standard deviations as depicted in Figure 26.