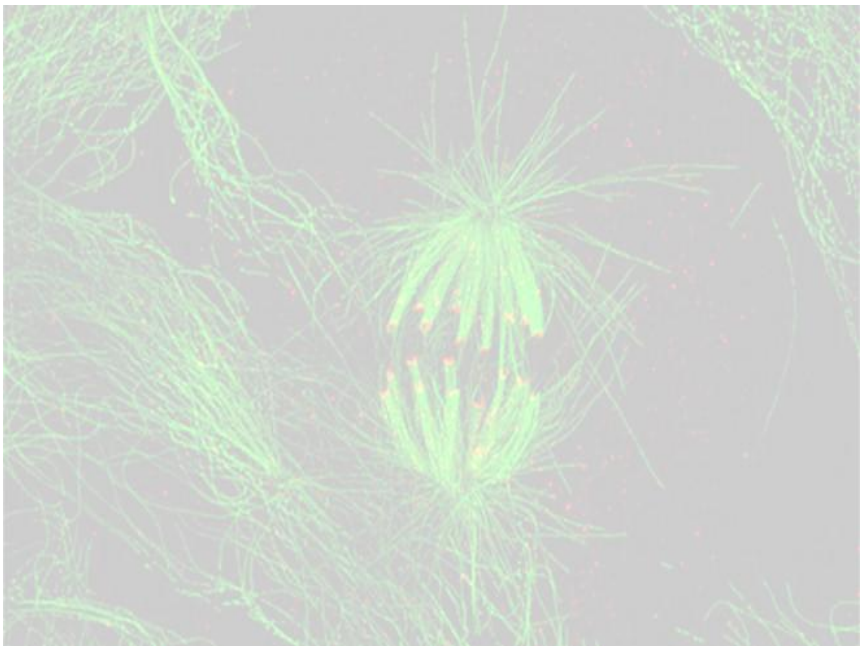

Biochemical characterization of interactions between kinetochore protein complexes

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O Presidente do Júri,

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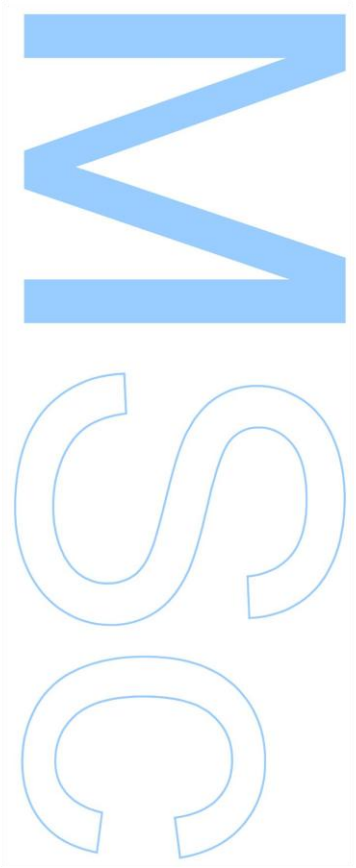


Abstract

During mitosis, the genetic material is segregated from a mother cell to a pair of daughter cells. All the events that happen during cell division have to occur in a synchronized fashion to allow correct chromosome segregation. This correct segregation is largely dependent on the formation of attachments between the spindle microtubules and kinetochores, during prometaphase (O'Connell and Khodjakov 2007). A crucial component of the kinetochore is the RZZ complex, which is composed of Rod – Rod-1 in *C.elegans* - (233,8 kD), Zwilch – Zwl-1 in *C.elegans* - (70,8 kD) and ZW10 – CZW-1 in *C.elegans* - (88,8 kD). None of these proteins have recognizable domains and the three subunits are interdependent for localization to kinetochores. RZZ promotes kinetochore recruitment of the minus end-directed microtubule motor complex Dynein and the Mad1-Mad2 complex, a component of the spindle assembly checkpoint (SAC)(Cheeseman, Niessen et al. 2004). Both Dynein and Mad1-Mad2 are recruited to kinetochores via the coiled-coil protein Spindly - Spdl-1 in *C.elegans* - (Griffis, Stuurman et al. 2007), which acts downstream of RZZ. Although we know the localization dependencies *in vivo*, the molecular basis of the interactions among RZZ subunits and between RZZ subunits and Spindly remain largely unknown. Given that incorrect segregation due to mutations in these genes can generate aneuploid cells promoting apoptosis or tumorigenesis(Wang, Cummins et al. 2004), understanding the underlying molecular mechanisms is of great interest.

With this project I wanted to better define the direct interactions among RZZ subunits, find *in vitro* evidence for a direct interaction between the RZZ complex and Spindly, and test whether Spindly is a dimmer. To do that, the techniques I used were Yeast two Hybrid analysis and in vitro pull down assays using purified recombinant proteins expressed in bacteria and insect cells.

From this work, three important discoveries were made: Zwl-1 interacts with the first 273 amino acids of Rod-1, CZW-1 interacts with Rod-1 between the amino acids 500 and 1203 and Spindly interacts with itself by the C-terminal.

In the future it will be important to be able to express and purify a soluble RZZ complex to test the interaction between this complex and Spindly, seen *in vivo* but not *in vitro.*

KEY WORDS: RZZ complex; Spindly; Rod; ZW10; Zwilch; SAC; Dynein; Kinetochores; Microtubules; Mitosis; Centromere; Cell Cycle; Yeast two Hybrid; Binding Assays with Purified Recombinant Porteins; Pull down assays; Bacteria expression; Baculovirus Expression

Resumo

Durante a mitose, o material genético é segregado da célula mãe para um par de células filhas. Todos os eventos que ocorrem durante a divisão celular têm de ocorrer de uma forma sincronizada para permitir a correta separação dos cromossomas. Esta separação é largamente dependente da formação de ligações entre os microtúbulos do fuso mitótico e os cinetocoros, durante a profase[1]. Um componente crucial do cinetocoro é o complexo RZZ, composto pelas proteínas Rod – Rod-1 in *C.elegans* - (233,8 kD), Zwilch - Zwl-1 in *C.elegans* - (70,8 kD) e ZW10 – CZW-1 in *C.elegans* - (88,8 kD). Nenhuma destas proteínas tem domínios padrão e as três subunidades são interdependentes para a localização no cinetocoro. O RZZ promove o recrutamento do complexo Dineína, uma proteína motora dos microtúbulos, e do complexo Mad1-Mad2, um componente do "spindly assembly checkpoint" (SAC)[2]. Ambos os complexos Dineína e Mad1-Mad2 são recrutados para os cinetocoros via uma proteína chamada Spindly - Spdl-1 in *C.elegans* - [3], que atua downstream do complexo RZZ. Apesar de se saber as proteínas responsáveis pelo recrutamento de cada proteína *in vivo,* as bases moleculares da interação entre as subunidades do complexo RZZ e entre as subunidades do complexo RZZ e a proteína Spindly, continuam por ser descobertas. Dado que a segregação incorreta dos cromossomas, devido a mutações nestes genes, podem gerar aneuploidias promovendo apoptose e células cancerígenas[4], perceber os mecanismos responsáveis por estas interações é de grande interesse.

Com este projeto o meu objetivo foi definir melhor as interações entre as subunidades do complexo RZZ, descobrir provas *in vitro* de uma interação direta entre o complexo RZZ e a proteína Spindly, e testar se a Spindly se comporta como um dímero na célula. Para fazer isso, as técnicas usadas foram Yeast two Hybrid e *in vitro* "pull downs" com proteínas recombinates purificadas expressas em bactérias ou em células de inseto.

Com este trabalho, três descobertas foram realizadas: Zwl-1 interage com os primeiros 273 amino ácidos da Rod-1, CZW-1 interage com a Rod-1 entre os aminoácidos 500 e 1203 e a Spindly interage com ela própria pelo C-terminal.

Futuramente, será importante ser capaz de expressar e purificar o complexo RZZ de forma a ser possível tertar a sua interação com a proteína Spindly.

Index

Figure Index

Table Index

Abbreviations

SAC – Spindle Assembly Checkpoint

RZZ complex – Rod-Zwilch-Zw10 complex

Rod – Rough Deal

ZW10 – Zeste-White 10

Mad – mitotic arrest deficient homologue

Bub – budding uninhibeted by benzimidazole

Cdkc – cyclin-dependent kinases complexes

KMN network – KNL1-Mis12 complex-Ndc80 complex network

KMT – Kinetochore Microtubules

CCAN – constitutive centromere associated network

GST – Glutathione S-tranferase

Ni-NTA resin – Ni- Nitrilotriacetic acid resin

CEN-P – Centromere Protein

bp – base pair

CATD – CENP –A targeting domain

GTP – Guanosine-5'-triphosphate

GDP - Guanosine-5'-driphosphate

CLIP – cytoplasmic linker protein

CLASP – CLIP associated protein

APC/C – Anaphase Promoting Complex/cyclossome

MCC –Mitotic Checkpoint Complex

MT – microtubulesCDC –Cell Division Cycle

C. elegans – *Caenorhabditis elegans*

RNAi –RNA interference

NRH – NAR-Rod homology

AcNPV - *Autographa californica* nuclear polyhedrosis virus

E. coli – *Escherichia coli*

SDS PAGE– Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis

K_{av} – partition coefficient

V_0 – Void Volume

V_T –Total Volume

V_e – Elution Volume

IPTG – Isopropyl β-D-1-thiogalactopyranoside

1. Introdution

1.1. Cell Cycle

The cell cycle is the series of events that take place in a cell leading to its division and duplication (replication) that produces two daughter cells. The cell cycle is divided into four major phases (Figure 1): G_1 phase, period when a cells grows in size, synthesizes mRNA and initiates DNA synthesis; S (synthesis) phase, were the chromosomes are replicated as well as the centrioles ; G_2 phase, after DNA Replication and before cell division; mitosis, also called the M phase, during which numerous events leading to cell division occur, which is divided into several stages (Figure 1). The G_1, S, and G_2 phases are collectively referred to as interphase, the period between one mitosis and the next. Most nonproliferating cells in vertebrates leave the cell cycle in G_1, entering the G_0 state.

Mitosis starts in the early prophase with the centrossomes, each with a daughter centriole, moving toward opposite poles of the cell. The chromosomes can be seen as long threads, and the nuclear membrane begins to disaggregate into small vesicles. During middle and late prophase, chromosome condensation is completed: each visible chromosome structure is composed of two chromatids held together at their centromeres. The microtubular spindle fibers begin to radiate from the regions just adjacent to the centrosomes, which are moving closer to their poles. Some spindle fibers reach from pole to pole. These fibers must attach properly at the kinetochores. During prometaphase chromosomes move toward the equator of the cell, where they become aligned in the equatorial plane. The sister chromatids have not yet separated. After proper positioning of all the chromosomes at the equatorial plate (metaphase), anaphase occurs and the two sister chromatids separate into independent chromosomes. Each chromatid contains a centromere that is linked by a spindle fiber to one pole, to which it moves. Simultaneously, the cell elongates, as does the spindle. Cytokinesis begins as the cleavage furrow starts to form. Telophase is characterized by the formation of the new nuclear membranes around the daughter nuclei. Cytokinesis is nearly complete, and the spindle disappears as the microtubules and other fibers depolymerize. Throughout mitosis the “daughter” centriole at each pole grows, so that by telophase each of the emerging daughter cells has two full-length centrioles. Upon

the completion of cytokinesis, each daughter cell enters the G1 phase of the cell cycle and proceeds again around the cycle.

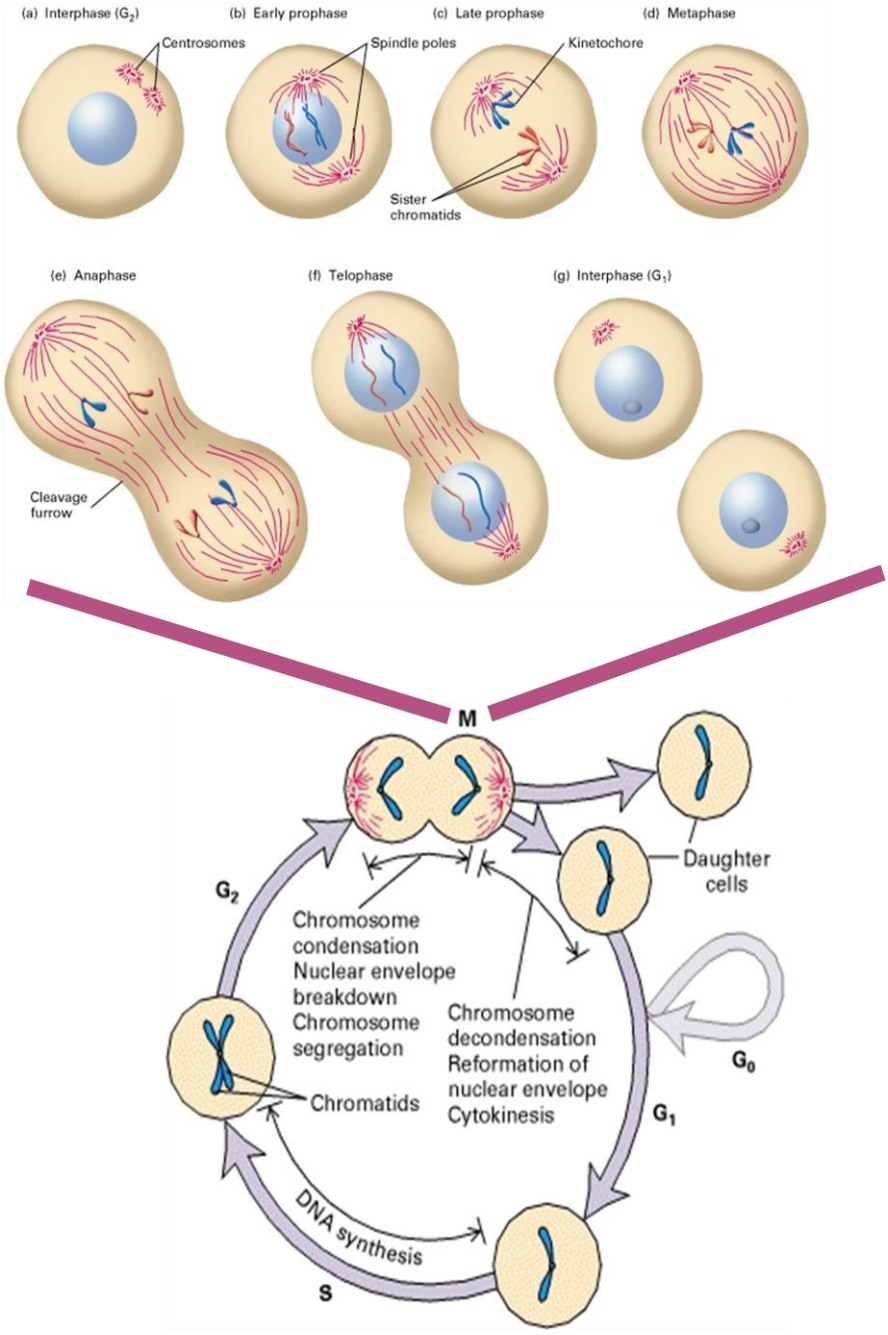


Figure 1 - Cell cycle and mitosis phases.

The complex macromolecular events of the eukaryotic cell cycle are regulated by a small number of heterodimeric protein kinases. The concentration of the regulatory subunits of these kinases, called cyclins, increase and decrease in phase with the cell cycle. Their catalytic subunits are called cyclin-dependent kinases (Cdks)

because they have no kinase activity unless they are associated with a cyclin. Each Cdk catalytic subunit can associate with different cyclins, and the associated cyclin determines which proteins are phosphorylated by the Cdk-cyclin complex. When cells are stimulated to replicate, G1 Cdk complexes are expressed first. These prepare the cell for the S phase by activating transcription factors that cause expression of enzymes required for DNA synthesis and the genes encoding S-phase Cdk complexes. The activity of S-phase Cdk complexes is initially held in check by a inhibitor, that is degraded in late G1, releasing the activity of the S-phase Cdk complexes, which stimulate entry into the S phase. S-phase Cdk complexes phosphorylate regulatory sites in the proteins that form DNA pre-replication complexes, that lead to DNA. Mitotic Cdk complexes are synthesized during the S phase and G2, but their activities are held in check until DNA synthesis is completed. Once activated, mitotic Cdk complexes induce chromosome condensation, breakdown of the nuclear envelope, assembly of the mitotic spindle apparatus, and alignment of condensed chromosomes at the metaphase plate.

To monitor and regulate the progress of the cell cycle, cells have to go through checkpoints that prevent progression of the cycle at specific points, allowing verification of necessary phase processes and repair of DNA damage. The cell cannot proceed to the next phase until checkpoint requirements have been met. One of those checkpoints is the spindle checkpoint, responsible for assuring the proper association of all chromosomes with spindle microtubules (Lodish H 2000).

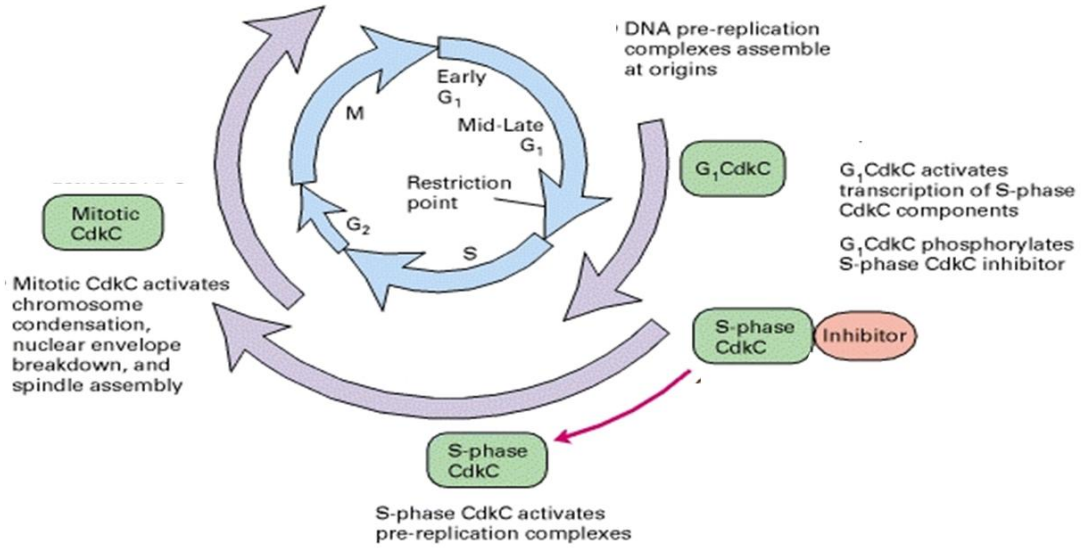


Figure 2 - Current model of regulation of the eukatyotic cell cycle by cyclin-dependent kinases complexes.

1.2. Kinetochores

Upon entry into mitosis, during prophase, replicated interphase chromosomes are compacted within the nucleus to facilitate their segregation within the dimensions of a cell (Figure 3). Concomitantly, chromosomes build a special structure to connect with spindle microtubules. In vertebrates, these connections occur at the site of the primary constriction of condensed chromosomes (the vertex of the familiar X shape). This site was initially called the centromere (from the Greek 'centro-', meaning 'central', and '-mere', meaning 'part') and later the kinetochore (from the Greek 'kineto-', meaning 'move', and '-chore', meaning 'means for distribution'). The centromere is now known as the region of chromosomal DNA that directs kinetochore assembly and the kinetochore as the proteinaceous structure that associates with this DNA.

The interactions between the kinetochore and spindle microtubules are central to the alignment and segregation of chromosomes on the spindle (Figure 3a,b). Following breakdown of the nuclear envelope, during prometaphase, kinetochores start to interact both laterally and in an end-on manner with spindle microtubules (Figure 3b). By metaphase, all chromosomes become bi-oriented, with sister kinetochores exclusively connected to microtubules that emanate from opposite spindle poles (Figure 3a). However, during the progression from prometaphase to metaphase, some chromosomes may be delayed in connecting to the spindle, whereas others may be inappropriately attached or have only one of their sister kinetochores connected (Figure 3b). To avoid loss of genomic information, the kinetochore monitors the attachment state and activates signalling pathways to prevent anaphase onset in the presence of incorrectly attached or unattached kinetochores (Figure 3b). Once bi-orientation occurs for all chromosomes in the cell, the machinery that separates sister chromatids is activated and the separated chromatids move to opposite spindle poles in anaphase occurs (Figure 3a). Then, during telophase, chromatids decondense, the nuclear envelope re-forms and a cortical actomyosin ring bisects the cell, between the separated chromatid masses, to generate two daughter cells with exact copies of the duplicated genome (Cheeseman and Desai 2008).

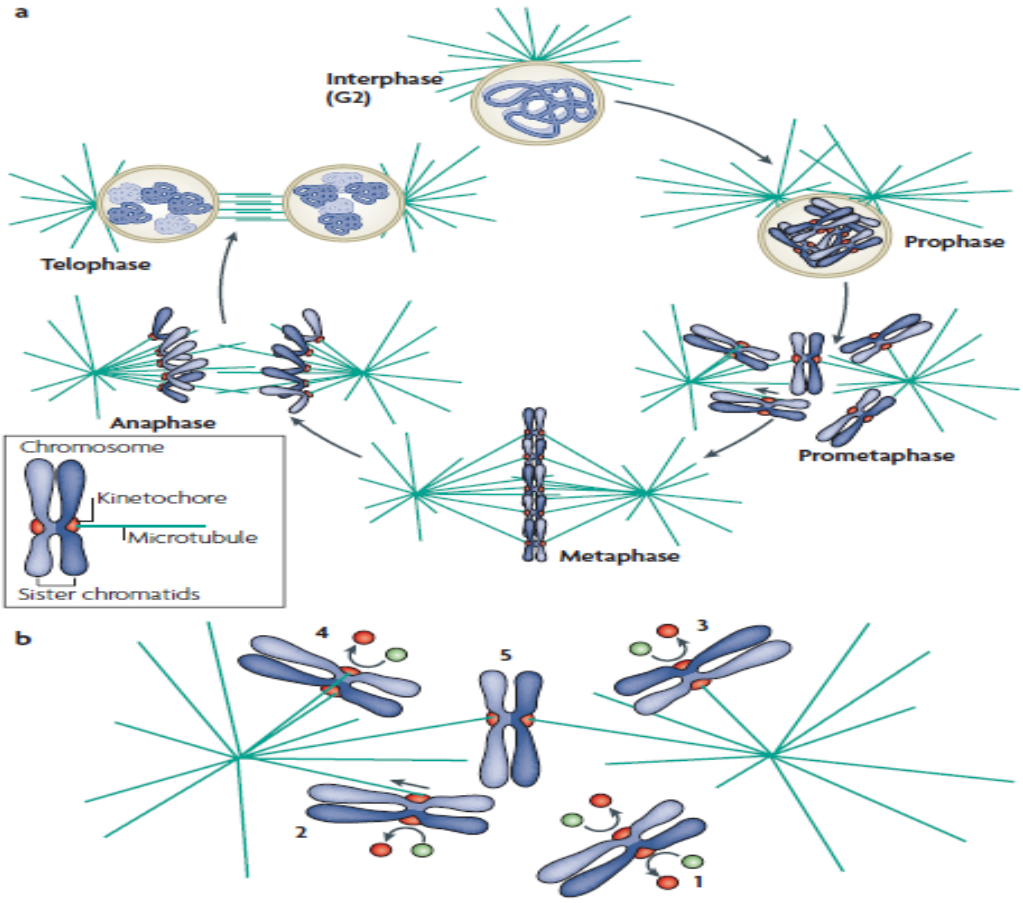


Figure 3 – Mitotic chromosome segregation: a – summary of chromosome-spindle interactions during the M phase of the cell cycle; b – detailed view of the prometaphase stage. Various intermediates (1-5) can be detected along the path from unattached (1) to bi-oriented (5) chromosomes. Lateral associations between kinetochores and spindle microtubules (2), which result in poleward chromosome movement, are frequently observed after nuclear envelope breakdown. Lateral attachments mature to end-on attachments, first with one kinetochore (3) and subsequently with both (5). Unattached kinetochores (as in 1, 2 and 3) catalyse the formation of an inhibitor (red circles) that prevents anaphase onset. Attachment errors, such as the one depicted in 4, are also common and are detected and eliminated to prevent chromosome loss (Cheeseman and Desai 2008).

1.2.1. Ultrastructure of kinetochore

Electron microscopy assays revealed that kinetochores have a trilaminar morphology: the inner kinetochore, which forms the interface with chromatin; the outer kinetochore, a 50–60-nm-thick region that forms the interaction surface for spindle microtubules; and the central kinetochore, the region between the inner and outer kinetochore (Figure 4). Electron microscopy analysis carried out in the presence of drugs that prevent microtubule polymerization shows a dense array of fibers, called the fibrous corona, that extend away from the outer kinetochore (Figure 4). The term inner

centromere refers to the chromatin that is located between the two sister kinetochores (Figure 4)(Brinkley and Stubblefield 1966).

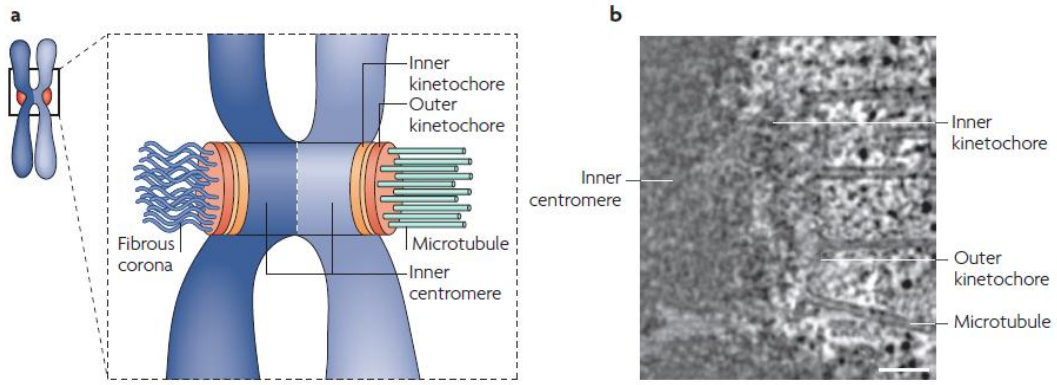


Figure 4 - Vertebrate kinetochore ultrastruture: a – schematic of a mitotic chromosome with paired sister chromatids, the chromatid on the right is attached to microtubules and the chromatid on the left is unattached; b – Electron micrograph of a human kinetochore (Scalebar 100 nm)(Cheeseman and Desai 2008).

1.2.2. Molecular composition of kinetochores

The first human kinetochore proteins were identified using human autoantibodies that recognized three major antigens, CENP-A, CENP-B and CENP-C (for 'centromere protein')(Earnshaw and Rothfield 1985). CENP-A is a variant of histone H3, one of the core subunits of nucleossomes (Palmer, O'Day et al. 1991). Around 80 kinetochore proteins have been identified in humans. Although there are some organism specific differences, the major themes in kinetochore composition and organization are conserved throughout eukaryotes.

1.2.2.1. Kinetochore Specification

If a chromosome fails to specify a site for kinetochore formation, it will be unable to attach to the spindle and will not be segregated during mitosis. Alternately, if multiple discrete sites of kinetochore assembly occur on a single chromatid, inappropriate attachments can connect that chromatid to both spindle poles, leading to its

fragmentation by spindle forces. Most organisms lack a precise DNA sequence that determines the site of kinetochore assembly. In humans, centromere regions are enriched in tandemly repeated arrays of a 171-base pair (bp) α-satellite DNA sequence. In this sequence, there is a 17-bp motif called the CENP-B box (Masumoto, Masukata et al. 1989), which can be bound by the inner centromere protein CENP-B. However, eliminating CENP-B from mice has no adverse effects on kinetochote funtions (Amor and Choo 2002). Other findings indicate that established centromeric loci can be stably maintained through mitotic and meiotic divisions in the absence of an interaction between CENP-B and CENP-B box or α-satellite DNA(Amor and Choo 2002). Therefore, in most eukaryotes, the site of kinetochore assembly is thought to be controlled primarily by epigenetic, rather than sequence based, mechanisms (Karpen and Allshire 1997).

The primary candidate for an epigenetic mark of kinetochore specification is the specialized chromatin that is present at centromeres. Centromeric chromatin consists of linearly interspersed regions of CENP-A nucleosomes and canonical histone H3 nucleosomes.(Blower, Sullivan et al. 2002) Therefore, CENP-A is a fundamental determinant of kinetochore identity.

A combination of mechanisms, including the targeted deposition of new CENP-A nucleosomes to regions of pre-existing CENP-A nucleosomes and the elimination of CENP-A nucleosomes from ectopic sites (Collins, Furuyama et al. 2004), ensure the maintenance of centromere identity (Figure 5). CENP-A itself is important for ensuring its targeting to centromeres. There is a striking structural difference in rigidity between CENP-A and histone H3 nucleosomes, which is mediated by a short CENP-A-targeting domain (CATD) in the histone fold (Black, Brock et al. 2007). This region confer an increased structural rigidity that is important for kinetochore function and for generating the unique chromatin environment at these regions.

Two extrinsic factors, Mis18 and KNL2 (also known as M18BP1), have been implicated specifically in CENP-A deposition and the maintenance of centromere identity (Figure 5) (Hayashi, Fujita et al. 2004; Fujita, Hayashi et al. 2007) (Fujita, Hayashi et al. 2007)' (Maddox, Hyndman et al. 2007). KNL2 contains a domain, which is commonly found in DNA-binding proteins and chromatin-remodelling complexes. CENP-A deposition at centromeres is inhibited by disrupting Mis18 function (Fujita,

Hayashi et al. 2007) and by disrupting KNL2 function(Maddox, Hyndman et al. 2007). The complex Mis18 and KNL2 localizes transiently to centromeres during a brief period of the cell cycle, starting in telophase and persisting through early G_1 phase(Fujita, Hayashi et al. 2007), which is consistent with the timing to when new CENP-A deposition occurs (Jansen, Black et al. 2007). So far, a direct association between these proteins and CENP-A has not been reported. One possibility is that the basal cellular machinery that is involved in chromatin assembly, such as the chaperone RbAp46/48, is adapted by Mis18 and KNL2 to direct CENP-A assembly (Figure 5)(Hayashi, Fujita et al. 2004).

Attached to CENP-A nucleosomes is present a network of proteins that are constitutively present at centromeres and that includes CENP-C and 13 interacting proteins (CENP-H, CENP-I, CENP-K–U). (Foltz, Jansen et al. 2006) This entire group of proteins is referred to as the constitutive centromere-associated network (CCAN).

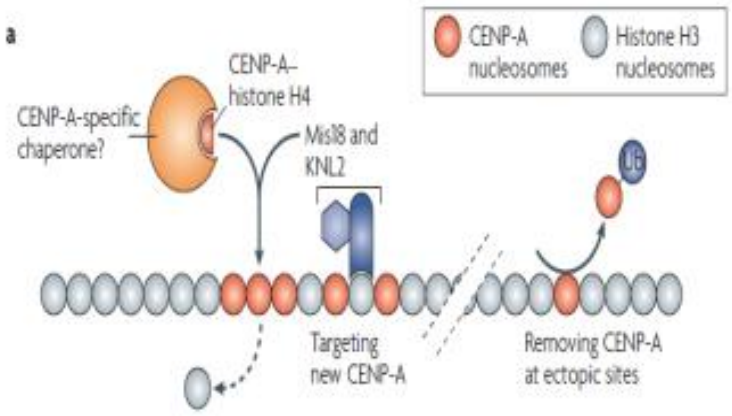


Figure 5 – Model showing the proteins and complexes that are implicated in kinetovhore specification (Cheeseman and Desai 2008).

1.2.2.2. Kinetochore assembly

The layered kinetochore ultrastructure observed by electron microscopy (Figure 4) is reflected by a step-wise assembly of kinetochore components from the chromatin to the outer kinetochore. A less linear hierarchy with complex feedback occurs at the outer kinetochore and fibrous corona, where assembly of components is transient and is influenced by the microtubule attachment state and local signalling pathways (Figure 6).

CENP-A chromatin forms the foundation for kinetochore assembly and the CCAN is constitutively associated with CENP-A chromatin throughout the cell cycle (Foltz, Jansen et al. 2006). Additional proteins are recruited and delocalized from kinetochores during specific stages of mitosis. Some of these other molecules that are involved in kinetochore assembly include the CENP-C and CENP-H/I/K subclasses of the CCAN, and KNL1 (also known as Spc105).

KNL1-family members are required for chromosome segregation and cell viability and for the localization of all known outer kinetochore proteins(Desai, Rybina et al. 2003). In vertebrates, CENP.C is upstream of most other kinetochore proteins, including the Mis12 complex(Liu, Rattner et al. 2006). CENP-H/I/K are not required for the mitotic localization of CENP-A and CENP-C, but contribute to the localization of outer kinetochore proteins, including components of the mitotic checkpoint (also known as the spindle-assembly checkpoint –SAC), the microtubule-binding Ndc80 complex and the coiled-coil-domain-containing protein CENP-F(Liu, Hittle et al. 2003). In humans, the Mis12 complex, KNL1 and the Ndc80 complex associate with Zwint(Cheeseman, Niessen et al. 2004), a coiled-coil protein that functions as a receptor for the transiently associated Rod–ZW10–Zwilch (RZZ) complex(Kops, Kim et al. 2005). The RZZ complex, in turn, recruits the minus-end-directed motor dynein to kinetochores(Karess 2005).

The amount of CCAN, Mis12 complex, KNL1 and Ndc80 complex at kinetochores does not change significantly along mitosis, fact that implies that the CCAN and the KNL1-Mis12 complex-Ndc80 complex (KMN) network that comprises the stable kinetochore structure observed by electron microscopy(Joglekar, Bouck et al. 2006).

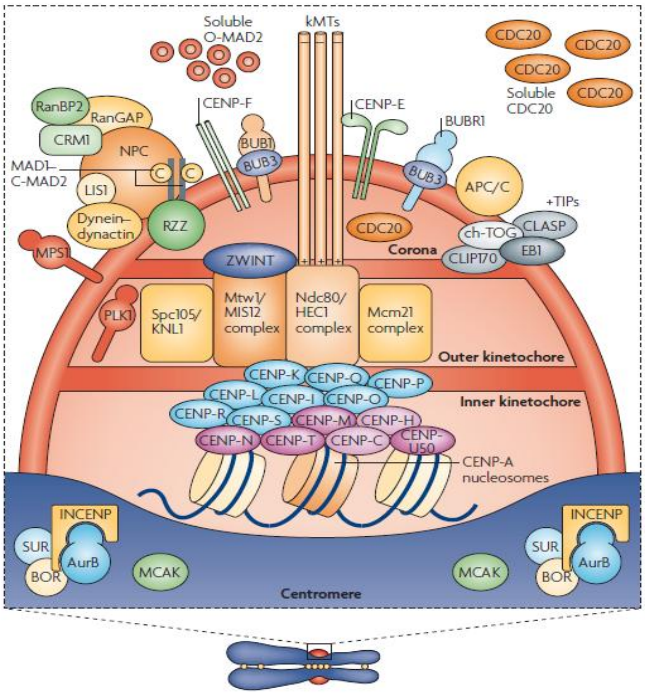


Figure 6 – The centromere kinetochore region composed by the inner kinetochore, outer kinetochore and the corona (Musacchio and Hardwick 2002).

1.2.2.3. Microtubule binding at kinetochores

Kinetochores function as an attachment site of spindle microtubules and to generate or transduce the forces that are required for chromosome segregation. There are multiple different microtubule-associated proteins function at kinetochores to generate a core attachment site affecting the polymerization dynamics of kinetochore-bound microtubules and driving translocation along spindle microtubules.

Microtubles are 25-nm diameter hollow polymers that are comprised of 12–15 protofilaments that are formed by head-to-tail association of αβ-tubulin dimers. The fixed orientation of the tubulin dimers makes the microtubule lattice polar — this polarity is central to the ability of motor proteins to move cargo to specific locations *in vivo*. Microtubules exhibit specialized non-equilibrium polymerization behaviour, termed dynamic instability, in which polymerizing and rapidly depolymerizing polymers coexist at steady state (Figure 7). Polymerization-triggered GTP hydrolysis on β-tubulin provides the energy source for this non-equilibrium behaviour; the resulting GDP is locked into the polymer lattice until depolymerization releases the subunit. The energy of GTP hydrolysis is stored as mechanical strain in the GDP–tubulin polymer lattice. A

polymerizing microtubule end is thought to persist because of a lag between subunit addition and nucleotide hydrolysis, which results in a stabilizing cap of GTP–tubulin (Figure7). Loss of this cap, either stochastically or by the action of external factors, triggers release of the stored mechanical strain and a switch to rapid depolymerization (Desai and Mitchison 1997).

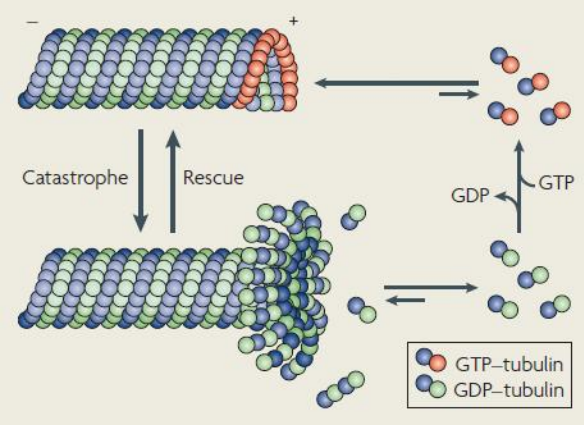


Figure 7 - Microtubule polymerization and depolimerization (Cheeseman and Desai 2008).

Kinetochore-microtubule attachments at the outer kinetochore must be robust enough to transduce forces to drive chromosome motility while also coupling the intrinsic dynamic instability of bound microtubule polymers to chromosome movement. The core kinetochore-microtubule attachment site is comprised by the KMN network and is likely to be formed by two apposed low-affinity microtubule-binding sites, one in the Ndc80 complex and a second in KNL1 (Figure 8a) (Cheeseman, Chappie et al. 2006).

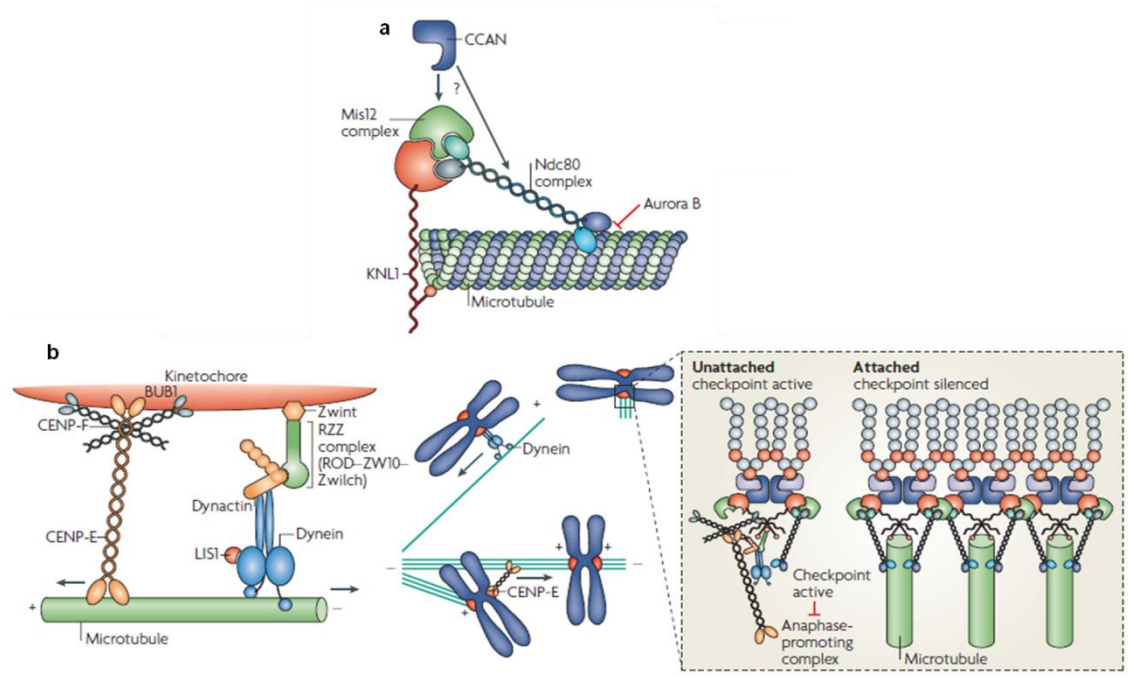


Figure 8 – Molecular mechanisms underlying specific microtubule-directed activities at the kinetochore: a – The core microtubule-attachment site is comprises the components of the KNL1–Mis12 complex–Ndc80 complex (KMN) network that are conserved throughout the eukaryotic kingdom. The association between the Mis12 complex and KNL1 generates a binding site for the Ndc80 complex. Both KNL1 and the Ndc80 complex directly bind to microtubules. In vertebrates and fungi, the constitutive centromere-associated network (CCAN) plays an important role in localizing the Ndc80 complex; b –Translocation along microtubules. The two motor proteins that are localized to kinetochores are CENP-E and dynein. Dynein translocates laterally associated kinetochores to the vicinity of spindle poles. CENP-E translocates along the kinetochore fibre of an already bi-oriented chromosome to move a mono-oriented chromosome towards the spindle equator. Dynein and the associated proteins LIS1 and the ROD–ZW10–Zwilch (RZZ) complex also contribute a poleward force at end-on attached kinetochores that contributes to the chromosome alignment and segregation. Different microtubule-binding proteins bing microtubules under different attachment states (Cheeseman and Desai 2008).

.

Functional analysis of the Ndc80 complex has demonstrated that it is essential for kinetochore–microtubule interactions(Desai, Rybina et al. 2003; McCleland, Gardner et al. 2003). This complex of four proteins forms structure with two globular heads at each end separated by a long coiled-coil region(Ciferri, De Luca et al. 2005) (Figure 8a). The globular regions of Ndc80 and Nuf2 localizes to the outer regions of the kinetochore(DeLuca, Dong et al. 2005) and binds directly to microtubules(Wei, Al-Bassam et al. 2007). The other end of the Ndc80 complex, composed of the globular regions of Spc24 and Spc25(Wei, Al-Bassam et al. 2007) is more closely apposed to the inner kinetochore. In *C. elegans*, SPC-24 and SPC-25 are required to associate with a kinetochore-bound receptor formed by the Mis12 complex and KNL-1(Cheeseman, Chappie et al. 2006). In vertebrates, both the Mis12 complex and the CCAN influence Ndc80 complex localization to kinetochores(Kline, Cheeseman et al.

2006). Ndc80, Mis12 and KNL-1 act synergistically to increase the microtubule-binding affinity(Cheeseman, Niessen et al. 2004). The two microtubule binding sites KNL-1 and Ndc80 are organized in a high density array, which provides a distributed series of intrinsically weak sites that make multiple contacts to a single microtubule. This distributed low-affinity interactions are very important because they would allow attachments to remain dynamic in response to growing or shrinking microtubules without resulting in the detachment of a kinetochore from the microtubules. Each kinetochore can bind several microtubules, contributing to the stable, yet dynamic, nature of the attachment.

Other proteins function in parallel to KNL1 and the Ndc80 complex to interact with microtubules. Two of those proteins are the motor proteins dynein and the kinesin CENP-E. Eliminating CENP-E reduces the number of microtubules bound to each kinetochore(McEwen, Chan et al. 2001). The RZZ complex targets dynein and its activator complex, dynactin, to kinetochores(Karess 2005). Perturbing dynein function globally by disrupting the dynactin complex or locally at the kinetochore using RZZ complex depletion does not prevent the formation of kinetochore–microtubule attachments, although perturbation does cause defects in the stability of attachments, chromosome alignment and segregation(Howell, McEwen et al. 2001; Yang, Tulu et al. 2007); (Yang, Tulu et al. 2007)

Movement of chromosomes towards the spindle poles is coupled to depolymerization of microtubules at kinetochores, whereas movement away from the poles is coupled to microtubule polymerization(Maiato, DeLuca et al. 2004). The microtubulebinding protein cytoplasmic linker protein (CLIP) - associating protein (CLASP) localizes to kinetochores and where it promotes the growth of kinetochore-bound microtubules(Maiato, Fairley et al. 2003; Cheeseman, MacLeod et al. 2005)' (Maiato, Fairley et al. 2003) (Figure 8). Kinetochores also associate laterally with and translocate along microtubules polymers (Figure 8b). Two microtubule-motor proteins implicated in this type of chromosome movement are dynein (minus-end directed) and CENP-E (plus-end directed)(Alexander and Rieder 1991). Dynein-powered poleward movement along the sides of microtubules is proposed to facilitate end-on interactions at the kinetochore outer plate by moving a chromosome into the proximity of the spindle poles, where the microtubule density is high(Figure 8b). Plus-end directed CENP-E motility along the kinetochore–microtubule fibres of already aligned

chromosomes facilitate movement of chromosomes that are trapped close to a spindle pole towards the spindle equator(Kapoor, Lampson et al. 2006) (Figure 8b).

1.3. Spindle-assembly Checkpoint (SAC)

To assure the accurate chromosome segregation there are multiple regulatory mechanisms, active in prometaphase, that include a set of checkpoint proteins as well as signaling proteins that monitor correct kinetochore-microtuble attachment preventing the precocious separation of sister chromatics and ensuring the fidelity of cell division. To do that, these proteins senses defects in kinetochore-spindle-microtuble attachments and prevent cell-cycle progression until all chromosomes are correctly connected to the spindle. This pathways constitutes the spindle-assembly checkpoint (SAC).

The definition of a checkpoint component is one that participates in the detection of kinetochore attachment status and the transmission of this information to the Anaphase Promoting Complex/Cyclossome (APC/C)(Musacchio and Hardwick 2002). This complex function as a multisubunit E3 ubiquitin ligase that triggers ubiquitination of a number of key cell cicle regulators targeting them for destruction by the 26S proteosome(Yu 2002). One of those proteins is Securin, that bind and inhibits the activity of separase, protein that will destroy Scc1, a cohesion subunit. Cohesin is the protein that link the two chromatids of the chromosome, and destroying this protein will allow sister chromatids separation(Yu 2002). APC/C also induces the proteolitic degradation of cyclin B, that is a cyclin responsible for activating CDK1, the primary kinase responsible for maintaining the mitotic state. The inactivation of cyclin B and CDK1 will trigger the onset of anaphase and the mitotic exit(Zhou, Yao et al. 2002).CDC20 is a co-factor of APC/C and is the target of SAC, specifically, that SAC negatively regulates the ability of CDC20 to activate the APC/C-mediated polyubiquitination of his targets(Hwang, Lau et al. 1998). So, by controlling CDC20, SAC prolongs prometaphase until all chromosomes have become bi-oriented between separated spindle poles on the metaphase plate. Chromosome bi-orientation will extinguish the checkpoint, allowing anaphase to occur.

CDC20 interacts directly, at least, with two different proteins: Mad2 and Mad3/BubR1. These two proteins, as well as BUB3, and CDC20 itself, are part of a complex called Mitotic Checkpoint Complex (MCC)(Fang, Yu et al. 1998). MCC is a

SAC effector, binds APC/C and abolish is ability of ubiquitin-ligase on securing and cyclin B(Fang, Yu et al. 1998).

The MCC is assembled at the unattached kinetochores(Scaerou, Starr et al. 2001) and his constituents cycle on and off kinetochores with high turnover rates (Howell, Hoffman et al. 2000). Kinetochore MAD2 consists of two roughly equal-sized pools: a more stably bound pool and a mobile, high-turnover pool (Shah, Botvinick et al. 2004). MAD2 binds to MAD1, a protein that is required for kinetochore localization of MAD2 and that is a stable kinetochore resident during prometaphase (Howell, Moree et al. 2004). BubR1 interacts and phosphorylates CENP-E (microtubule-plus end directed motor that contributes to chromosome alignment), by CENP-E activation (Mao, Abrieu et al. 2003). This activity is repressed when CENP-E binds to microtubules, which indicates that BubR1 kinase activity is high before kinetochore-microtubule formation and inactive following microtubule-kinetochore attachment. This two different complexes will then interact with CDC20, preventing anaphase to occur. The co-localization of checkpoint proteins and Cdc20 at the unattached kinetochore is essential for the assembly of the MCC and will create a diffuse APC/C inhibitory signal(Skoufias, Andreassen et al. 2001). The inactivation of the checkpoint is due to loss of kinetochore localization of Mad1 and Mad2 and by partial loss of BubR1/Bub3, CENP-E, and Cdc20 at the kinetochores, by free diffusion into the cytosol, or by motor assisted transport to spindle poles along MTs(Howell, Hoffman et al. 2000). So, when kinetochores interact with the MT, the complexes Mad2-Cdc20 and BubR1-Cdc20 dissociates and that lead to the activation off the APC-C/Cdc20, turning of the spindle checkpoint (Zhou, Yao et al. 2002). The activation of the APC/C by Cdc20 will promote the transition between metaphase and anaphase (Figure 9).

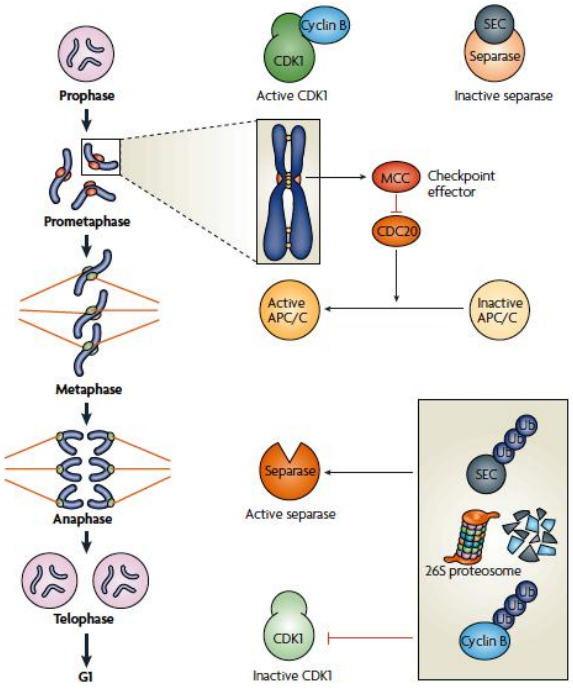


Figure 9 – Relationship of the SAC with the cell-cycle machinery (Musacchio and Hardwick 2002).

In addition to microtubule–kinetochore attachment, tension is important for SAC inactivation (Nicklas, Ward et al. 1995). Stretching of centromeric chromatin on bi-orientation increases kinetochore-to-kinetochore distance and kinetochore tension (Figure 10). Microtubule– kinetochore attachment is normally destabilized at low kinetochore tension and stabilized by high tension between bi-orientated sister kinetochores (Nicklas, Waters et al. 2001), being tension a fundamental criterion to discriminate against incorrect attachments. If both sister kinetochores attach to microtubules from the same pole (syntelic attachment; Figure 10b), not enough tension is generated and microtubule–kinetochore attachment is destabilized to correct the problem. Merotelic attachment occurs when one sister kinetochore becomes attached to microtubules from opposite poles. Bi-orientation of chromosomes with merotelic kinetochores produces sufficient occupancy and tension to turn off SAC activity. As a result, merotelic kinetochores, if left uncorrected, can produce lagging chromatids and potential chromosome mis-segregation in anaphase (Cimini and Degrassi 2005).

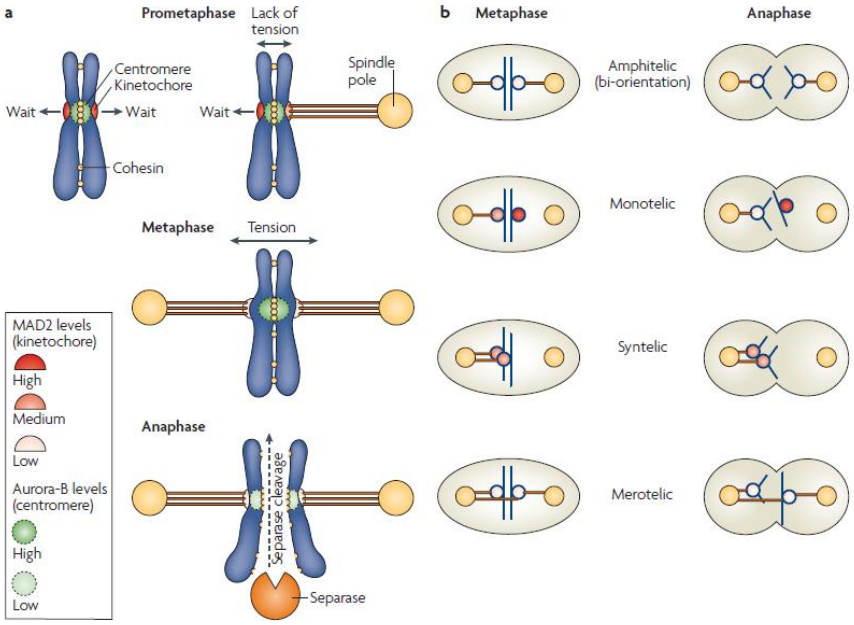


Figure 10 – Transition of Metaphase to Anaphase, the kinetochore attachment process: a – Unattached kinetochores generate a 'wait' signal and recruit the spindle assembly checkpoint (SAC) proteins. The levels of mitotic-arrest deficient homologue-2 (MAD2) are high at unattached kinetochores and moderately high at attached kinetochores in a monotelic pair. Under these conditions, the Aurora-B kinase concentrates at centromeres and is believed to be activated by the lack of tension between the sister chromatids. Bi-orientation depletes MAD2 (and budding uninhibited by benzimidazole (BUB)R1) from kinetochores and promotes the acquisition of tension in the centromere area, which is visualized as an increase in the inter-kinetochore distance between sister chromatids. When all chromosomes have achieved this situation, the SAC signal is extinguished and anaphase ensues thanks to the activation of separase, which removes sister-chromatid cohesion by proteolysing cohesion; b - Correct and incorrect attachments can occur during mitosis, and correction mechanisms exist to prevent incorrect chromosome inheritance (Musacchio and Hardwick 2002).

Mad1-Mad2 complex is recruited to the kinetochores by a very important complex called RZZ complex.

1.4. RZZ Complex

Besides proteins like, for example, Mad2, Mad1 and BubR1, there are other proteins that also carry out essential aspects of the spindle checkpoint. In Metazoan, 3 of those proteins are Rough Deal (Rod), Zeste-white (ZW10) and Zwilch. There proteins function as a unit and can be isolated in a stable complex called RZZ Complex(Scaerou, Starr et al. 2001). Rod and ZW10 genes originally identified in *Drosophila,* and are conserved

among multicellular eukaryotes(Karess and Glover 1989; Williams, Karr et al. 1992)'(Williams, Karr et al. 1992). Zwilch, the last protein of the complex, was identified by gel exclusion chromatography(Williams, Li et al. 2003). Null mutations of either gene cause similar chromosome segregation defects: lagging chromatids, nondisjunction and anaphase bridges, leading to aneuploidy (Scaerou, Starr et al. 2001). This data suggests a role for this gene product in spindle or kinetochore function. None of these proteins have obvious homologe among other proteins and no recognizable protein motif that might provide a hint as to their function. The combined mass of Rod, Zw10 and Zwilch (240, 85, 75 kDa, respectively) is about half the apparent mass of the complex. This suggests that the complex contains two copies of each protein, or perhaps that the complex, as isolated, is a stable dimer(Civril, Wehenkel et al. 2010). However for the moment, little else is known about the biochemistry and assembly of the RZZ complex.

Rod, Zw10 and Zwilch changes his localization during the course of mitosis. First, during interphase, they are cytoplasmic. In late prophase, during nuclear nuclear envelope breakdown, they enter the nucleus and begin to accumulate on kinetochores(Williams, Karr et al. 1992). Once the kinetochores microtubules (KMTs) have attached and the chromosomes are properly bi-oriented on the spindle, RZZ levels decline on kinetochores and its found distributed irregularly along the KMT fibers(Scaerou, Starr et al. 2001; Basto, Scaerou et al. 2004). This drastic redistribution of protein from kinetochores to KMTs is an example of kinetochore ‘shedding’, a dynein-dynactin-dependent process that removes outer domain components from the kinetochore and transport them along KMTs towards the poles(Howell, Hoffman et al. 2000; Howell, McEwen et al. 2001).

1.4.1. RZZ receptor

The kinetochore recruitment of many proteins is interdependent, and, the recruitment of the RZZ complex is dependent of the recruitment of a protein called Zwint-1. Zwint-1 is a coild coil protein, with 43 kD, and is recruited to the kinetochores in early prophase, before the detection of Zw10, and persist into mid-anaphase(Wang, Hu et al. 2004). The phenotype of Zwint-1 depleted cells is similar to that of zw10 and rod mutantas, suggesting that RZZ does no function when it cannot be recruited to the kinetochores(Wang, Hu et al. 2004). Zwint-1 is in association with the KMN

network(Kops, Kim et al. 2005). C.elegans homolog of Zwint, KBP-5, is not required for the binding of RZZ (Li, Armstrong et al. 2004)

1.4.2. The functions of RZZ

Is very important to understand well how the RZZ complex work because is known that this complex fulfills a lot of important functions in the cell: recruiting cytoplasmic dynein, Mad1-Mad2 complex and Spindly to kinetochores; maintain a functional spindle checkpoint; and participating in poleward movements of chromosomes during mitosis.

1.4.2.1. Recruitment of dynein-dynactin

The RZZ complex is directly required for the recruitment of cytoplasmic dynein and dynactin to the kinetochores(Starr, Williams et al. 1998). The interaction between this two complexes appears to be directly involving Zw10, since this protein is capable of interacting with the p50 subunit of the dynein-dynactin complex(Starr, Williams et al. 1998). Nevertheless, the whole RZZ is required for this interaction to occurs(Williams, Li et al. 2003).

Dynein levels are high on unattached kinetochores, and dynein is implicated in the rapid poleward movement of chromosomes after spindle capture(Alexander and Rieder 1991). After kinetochore capture of the MT, dynein as a role in the shedding and transport of outer domain proteins away from the kinetochores(Howell, McEwen et al. 2001). Dynein mediated shedding is part of the mechanism for shutting off the checkpoint by removing proteins like Mad2 from the properly attached kinetochores(Howell, McEwen et al. 2001), and RZZ complex, as discussed earlier. In summary, by recruiting dynein.dynactin complex to the kinetochores, the RZZ complex assures its own remove from the kinetochore, the remove of Mad2 and the inactivation of the checkpoint(Howell, McEwen et al. 2001).

1.4.2.2. Recruitment of Spindly

Spindly is a coild-coil protein that localizes in microtubule plus ends during interphase and to unattached kinetochores during mitosis. This protein is required for silencing the SAC and for recruiting dynein-dynactin complex to the kinetochores. Spindly is part of the corona region of the kinetochores and requires the RZZ complex to localize(Griffis, Stuurman et al. 2007). Resuming, the RZZ complex recruits Mad2, Spindly and dynactin to the kinetochores. Spindly and dynactin then work cooperatively to recruit dynein, wich then transports the whole complex towards the spindle pole and silences SAC signaling on the kinetochore(Griffis, Stuurman et al. 2007). In Caenorhabditis elegans, Spdl-1, and homologue of Spindly, depends on KNL-1, a highly conserved kinetochore protein, and Czw-1/Zw10 component of the RZZ complex, to localize. Spdl-1 is also required to induce the SAC-dependent kitotic delay and localizes the SAC protein Mad-1 to the kinetochore, function as a receptor of this protein to induce SAC functios(Yamamoto, Watanabe et al. 2008). So, Spdl-1 is recruited to the kinetochores by the RZZ complex, localizes downstream from this complex and is required for all the RZZ complex functions: spindle checkpoint activation; kinetochore recruitment of Mad2; and kinetochore recruitment of Dynein-Dynactin. Spdl-1 is nor involved in the assembly of the core kinetochore-MT.binding site constituted by the KMN network, but the RZZ complex is, ensuring a coordinated transition from transient lateral attachments made by dynein, which accelerate formation of end-coupled attachments of correct geometry, to stable load-bearing end-coupled attachments, important for chromosome segregation(Gassmann, Essex et al. 2008).

1.5. C. elegans

Caenorhabditis elegans is a transparent nematode of about 1mm in length. *C. elegans* embryo is a model organism used in study of mechanics of metazoan cell division, given that the syncytial gonad makes it possible to use RNA interference (RNAi) to generate oocytes whose cytoplasm is reproducibly (>95%) depleted of targeted essential gene products. There are also other unique important features regarding *C. elegans:* rapid and highly stereotypical mitotic divisions; the time between

the onset of DNA condensation and the completion of furrow ingression during cytokinesis is approximately 14 minutes; and the invariant nature of the first few divisions facilitates the development of methods to assess the consequences of molecular perturbations. These features make this system the model system used in our lab.

1.5.1. Kinetochores in *C. elegans*

Eukaryotes can be divided into two groups based on the architecture of their mitotic chromosomes: monocentric organism assemble kinetochores on a single localized chromosomal site, due to the presence of dedicated centromeric chromatin; holocentric organisms, like C. elegans, assemble diffuse kinetochores along entire poleward face of each sister chromatid (Figure 11).

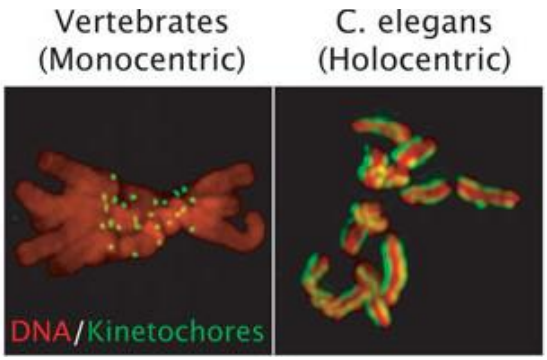


Figure 11 – Kinetochores localization in Vertebrates and in C. elegans.

In *C. elegans* the components of RZZ complex, Rod, ZW10 and Zwilch, are called Rod-1, CZW-1 and Zwl-1. Spindly is called Spdl-1.

2. Goals

Rod and Zw10 were initially identified in genetic screens in *Drosophila melanogaster(Karess and Glover 1989)*. After demonstration that Zw10 and Rod worked together in a complex(Scaerou, Starr et al. 2001), immunoaffinity chromatography led to the discovery of Zwilch as an additional RZZ subunit(Gassmann, Essex et al. 2008). Both Zwilch and Zw10 bind to Rod(Civril, Wehenkel et al. 2010).

Rod is an unusual long protein (Figure 12). Rod is predicted to have a secondary β structure organized in putative WD40 β-propellers, in the N-terminus(Neer, Schmidt et al. 1994). This kind of structure is usually mediating protein-protein interactions(Neer, Schmidt et al. 1994). C-terminally to the β-propeller region, Rod is predicted to have a α-helical secondary structure, organized in stacked helical domains, called α-solenoids, arranged in a superhelical domain(Andrade, Petosa et al. 2001). Rod also has a region that is homolog of a protein implicated in vesicular transport, called NAG. The C-terminal region of the homologous segments correspond to the Sec39 domains, while the N-terminal portion constitutes a novel region of conservation between the two proteins that is called NAG-Rod homology (NRH) domain(Civril, Wehenkel et al. 2010).

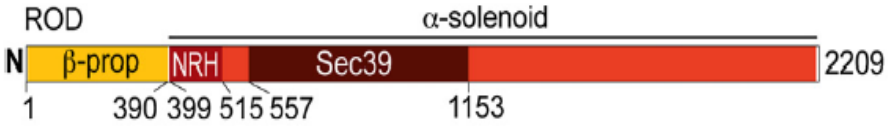


Figure 12 – Domain organization of ROD (Cheeseman, Chappie et al. 2006).

ZW10 is recruited to kinetochores by Zwint-1 and interacts with p50 dynamitin, a component of the cytoplasmic dynein activator dynactin(Starr, Williams et al. 1998).

Zwilch is composed of two domains and as a complex αβ structure, whose most prominent feature is a heavily arched 12 stranded β sheet forming an incomplete barrel (Figure 13). In the domain 2, Zwilch as extended conserved regions that might act as binding sites for other proteins. It is known that Zwilch and ZW10 do not interact. It is

also known that Zwilch interacts with the first 350 amino acids of Rod (β propeller region) since they bacterially coexpress and they copurify after Size Exclusion Chromatography (Civril, Wehenkel et al. 2010). So, Zwilch binds directly to Rod and do not need Zw10 as a binding factor. Zwilch as a highly conserved region (Figure 14).

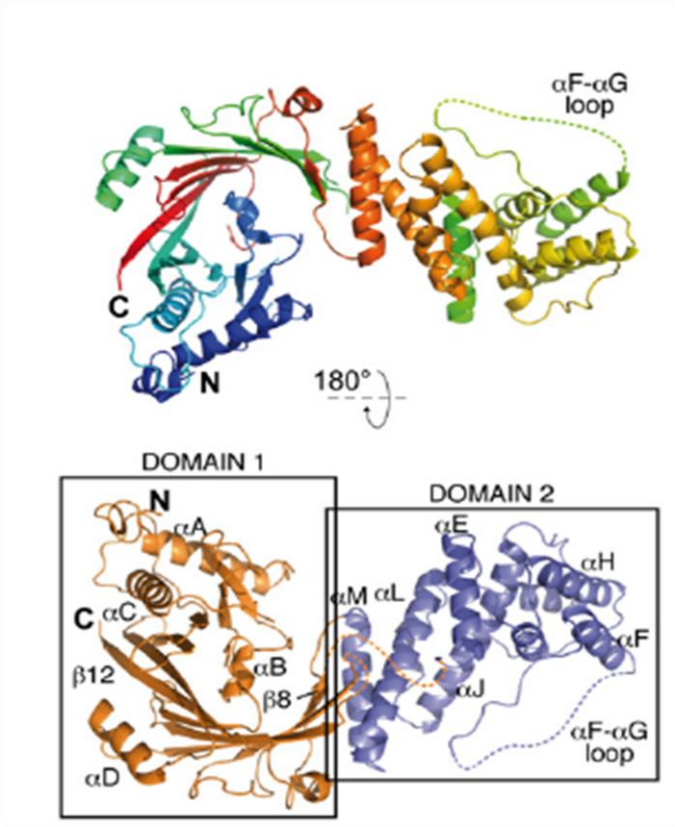


Figure 13 – Cartoon model of a crystal structure of Zwilch and of the two structural domains (Cheeseman, Chappie et al. 2006).

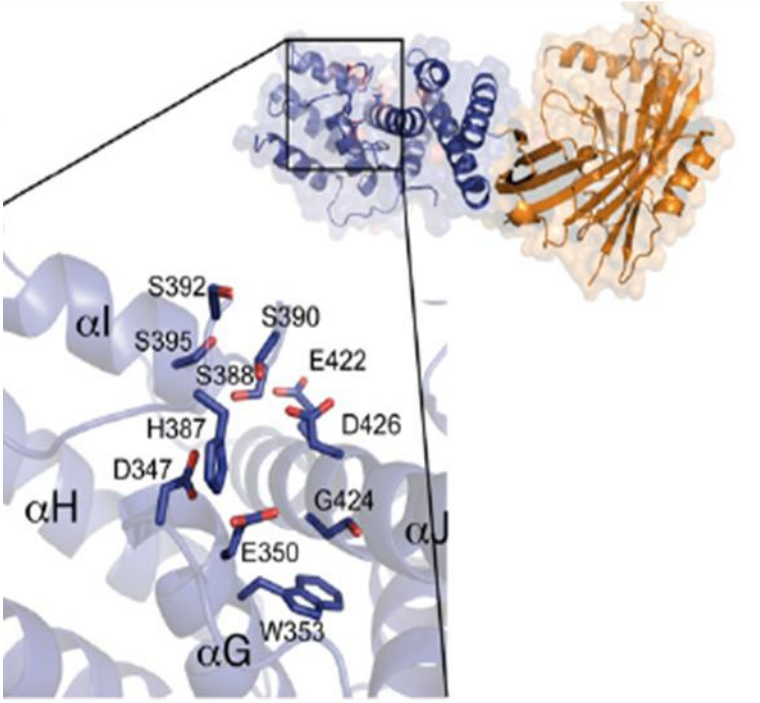


Figure 14 - Cartoon model of Zwilch evolutionary conserved residues **(Cheeseman, Chappie et al. 2006).**

Little is known about the interaction between RZZ complex and Spindly protein, and which of the proteins of the complex interacts directly with Spindly. After testing a series of hypothesis, my lab discovered that mutating two amino acids of the *c. elegans* Zwilch in the high conserved region, abrogates the *in vivo* localization of Spindly (data not published), without perturbing RZZ localization and function. The two aminoacids mutated were glutamic acid 433 and 437 to an alanine.

After this discovery, the next step in the investigation is to express and purify Spindly and RZZ complex, or a part of this complex, that will allow us to test the *in vitro* interactions between these two components to prove that in fact this interactions exist.

Knowing this, the goals of my project were: define the interaction between the RZZ complex and Spindly; find if spindly, like the RZZ, is behaving as a dimer in the cell and find out what part of spindly is responsible for this dimerization; it is known that Rod interacts with Zw10 and Zwilch, but it is not known what fragments of Rod is responsible for this interaction, so the other goal of my work is to find out what fragments of rodare interacting with Zw10 and Zwilch. To try to achieve these goals we will divide the work in two major approaches: Yeast-two-Hybrid assays and binding assays with purified recombinant proteins.

3. Material & Methods

3.1. Baculovirus Protein Expression

Recombinant baculovirus have been used as vectors to express heterologous genes in cultured insect cells. These genes are placed under transcriptional control of the polyhedron promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). In most cases, the recombinant proteins expressed are processed, modified, and targeted to their appropriate cellular localations, where they are functionally similar to their authentic counterparts. Other advantages of this method are: high level of heterologous gene expression, compared to other eukaryotic expression systems; the recombinant proteins are usually soluble and easily recovered from the insect cells; the cells can be co-infected with different virus in order to obtain expression of hetero-oligomeric protein complexes; and baculoviruses have a restricted host rage, limited to specific invertebrate species.

The main concept of this technique is a site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid bMON14272) propagated in *E. coli*. Recombinant bacmids are constructed by transposing a mini-Tn7 element form a donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 functions are provided *in trans* by a helper plasmid (pMON7124). Insertions of the mini-Tn7 into the mini-attTn7 attachment site on the bacmid disrupts expression of the lacZα peptide, so colonies containing the recombinant bacmid are white, in a background of blue colonies (Figure 15).

The bacmid (bMON14272) contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the *lac*Zα peptide from a pUC-based cloning vector. Inserted into the N-terminus of the lacZα gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame of the lacZα peptide. The bacmid propagates in Escherichia coli DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue (Lac^+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG. The helper plasmid (pMON7124) confers resistence to tetracycline and encodes the transposase. The mini-Tn7 in the donor plasmids

contains an expression cassette consisting of a Gm^r gene, a baculovirus-specific promoter, a multiple cloning site and an SV40 poly(A). The bacmid also contains a YFP reporter gene that allows to follow the protein expression by fluorescence.

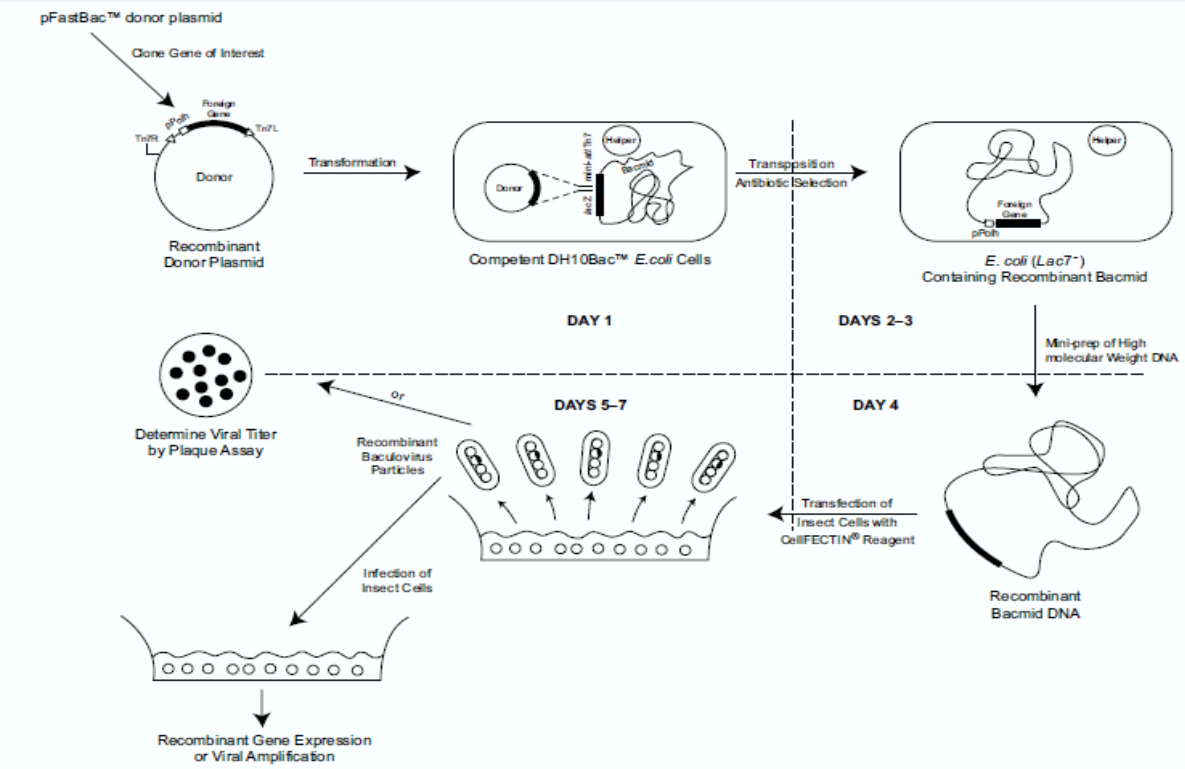


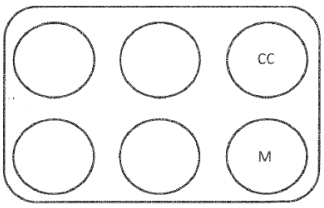
Figure 15 – Generation of recombinant baculoviruses and gene expression with the Bac-toBac® Expression System.

3.1.1. Protocol

C. elegans open reading frames were amplified from N2 cDNA and cloned into the pFastBacHta. The plasmid was transformed into DH5α cells. 100 ng of the purified plasmid was transformed in competent DH10EMBacY cells. Add 1 mL of LB and leave o.n. at 37ºC. Cells were diluted using LB to 10^{-1}, 10^{-2} and 10^{-3} and placed 100 µL of each dilution on LB/Agar plates containing 50 µg/ml kanamycin, 7 µg/ml

Figure 16 – Plate with blue and white colonies.

gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. Cells were incubated for 48h at 37ºC. 8 white colonies and 1 blue colony were picked in a fresh plate to verify the phenotype (Figure 16). Take 2 white colonies and set up two 2 mL liquid cultures containing antibiotics (kanamycin, gentamicin and tetracycline) for isolation of recombinant bacmid DNA and grow o.n. at 37ºC. Pellet cells, remove supernatant and resuspend each pellet in 0.3 ml of Solution I [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A], by gently inverting the tube up and down. Add 0.3 ml of Solution II (0.2 N NaOH, 1% SDS) and gently mix (inverting the tube up and down 6 times). The samples were incubated on ice for 5 min and slowly add 0.3 ml of 3 M potassium acetate (pH 5.5). Invert the tube up and down 6 times. Place the samples on ice for 5 to 10 min. The samples were centrifuged for 10 min at 14,000 × g. Transfer supernatant to a new tube and centrifuge for more 3 min at 14,000 × g. Transfer supernatant to a new tube, add 0.7 ml of absolute isopropanol drop by drop and invert tube until the solution is well mixed. The samples were centrifuge for 10 min at 14,000 × g. Discard supernatant and add 250 µl of 70% ethanol drop by drop with the tube horizontally. The samples were centrifuged for 5 min at 14,000 × g. Discard supernatant and add 50 µl of 70% ethanol. DNA can be stored or used right away. Before using the DNA, centrifuge for 5 min at 14,000 × g. From now on the all the steps are made in the sterile hood. Remove all the ethanol. Let dry out for 10 minutes. Pipette 30 µL of sterile water and hit the tube 10 times. In an eppendorf tube prepare 200 µL of media HyClone (FM4Insect – Thermo medium) plus 20 µL of X-tremeGEN. Add 100 µL of the mixture plus 200 µL of medium to each DNA tube and mix gently. In a 6 well plate (Figure 17) seed 0.5 x 10^6 Sf 21 cells in each well (leave one well for the “only medium” control) in a total of 3 mL volume. Leave a well to “Cell only control”. Prepare two wells for each bacmid. In each well add 150 µL of the mixture (media+ xtremeadasd+bacmid) and put the plate in the dark box. After 60 hours remove the supernatant from the 6 well plate and store the 3+3 mL from each clone together in the same tube stored at 4ºC - Virus V_0. At the same time, do a protein expression test experiment by gently adding 3 mL of new media to the 4 wells and store the box in the dark. Wait 2 days and remove all the medium of the plate. Resuspend the cells attached to the bottom of the well with 500 µL of PBS and test protein expression on



CC - cell control (uninfected cells)
M - media only (3 mL)

Figure 17 – **6 well plate.**

the Fluorimeter at 530 nm (values higher than 10^6). This is not a quantitative bat a qualitative test, just to know is the protein if being expressed. If the protein is expressed, infect 25 mL of 0.5 x 10^6 cells with V_0. Count the number of the cells every day until they stop growing, if they are still growing dilute the cells to 0.5 x 10^6 cells per flask. The day after the cells stop growing is called day after proliferation arrest. 24 hours after this day, centrifuge culture gently for 3 minutes at 800 x g in a 50 mL falcon tube. Take supernatant in a new 50 mL falcon tube – Virus V_1. Use this cells to test if the protein expressed is the protein of interest and if is soluble, by running an SDS-Page Gel: take samples of the cells if and without virus (1 x 10^6 cells per sample); the cells were centrifuged for 3 minutes at 800 x g; pellet the cells in 1 mL of PBS; lysate cells in sonicator with microtip 3 x 10 s intervals (1 min between intervals). If the protein expresses and is soluble, use virus V_1 to infect cells for protein expression in big scale.

3.2. Yeast two Hybrid

The Yeast two Hybrid assay is used to test protein-protein interactions. This assay is done in *Saccharomyces cerevisiae* (yeast) and relies in the ability of two proteins to activate the transcription of a reporter gene by reconstitution of a transcription factor called GAL4. Every transcription factor has two domains: one for DNA binding and one for activation. In this assay, we will use two different plasmid vectors: bait vector that expresses a gene as a fusion to the GAL4 DNA-binding domain (DNA-BD), and another vector called prey that expresses a gene as a fusion to the GAL4 activation domain(Fields and Song 1989). When bait and prey proteins interact, the DNA-BD and AD are brought into proximity, thus activating transcription the reporter gene. This reporter gene will confer selectivity in a certain medium. The vectors used are pGBKT7 and pGADT7. The pGBKT7 is a bait vector, that expresses a protein fused to GAL4 DNA-BD, conferring selection in a minus TRP medium, and is resistant to kanamycin. The pGADT7 is a prey vector, that expresses a protein fused to GAL4 DNA-AD, conferring selection in a minus LEU medium, and is resistant to ampicilin. The two different yeast strains used are Y187, transformed with pGADT7, and AH109, transformed with pGBKT7. This two strains are mating partners, and will grow in a medium without LEU and TRP when the mating ocurs. The strain AH109 contain the reported genes HIS3 and ADE2 genes, that are part of the histidine and adenine biosynthetic pathway, respectively. If the GAL4 promotor is activated,

meaning, the bait and the prey proteins interact, the yeast will be able to grow in the absence of histidine and adenine. In this assay are used two different nutritional stringencies for the growth of the yeast: Low stringency, tryptophan minus/leucine minus/histidine minus, will be used to test for weak interactions between the bait and the prey proteins and, high stringency, tryptophan minus/leucine minus/histidine minus/ adenine minus will be used to test for strong interactions between the bait and prey proteins. This method is sensible to interaction with dissociation constants (Kd) above ~70 µM.

3.2.1. Protocol:

Inoculate two 100 mL YPDA flacks (Yeast Extract, Peptone Dextrose, and adenine) with a 2-3mm colony of AH109 and Y187 (grown in YPD Plates - Yeast Extract, Peptone and agar). Grow to OD600 0.5 at 30 ºC. Spin down cells, ressuspend cells in 10 mL TEL (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0,1 M LiAc), spin again and add 1 mL TEL to the pellet. Add 50 µg of Salmon Sperm DNA to 100 µL of competent cells. Add 1 µg of DNA plasmid to the suspension. Incubate 30 minutes 200 rpm at room temperature. Add 0.7 mL of PLATE solution (40% PEG 3350, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0,1 M LiAc) and resuspend. The samples were incubated for 1 hour 200 rpm at room temperature. Heat schok samples at 42ºC for 15 min. Chill cells on ice and spin down for 5 min at 1000 x g. Resuspend cells in 200 µL of ddH_2O. Plate AH106 strains in minus tryptophan plates (Difco Yeast Nitrogen Base without Amino Acids, Agar and Clontech DO supplement without tryptophan and glucose) and Y187 strains in minus leucine plates (Difco Yeast Nitrogen Base without Amino Acids, Agar and Clontech DO supplement without leucine and glucose). Let the cells grow for 3 days at 30 ºC. Pick one single colony of bough strains and mate them in 1mL YPDA. Let the cells mate over night room temperature at 200 rpm. Take 50 µL of each mating and inoculate in double selection plates (Difco Yeast Nitrogen Base without Amino Acids, Agar, Clontech DO supplement without leucine, tryptophan and glucose). Let the cells grow for 3 days and re-streak them to new double selection plates. Let the cells grow for 3 days, pick a single colony and let it grow overnight in double selection medium (Difco Yeast Nitrogen Base without Amino Acids, Clontech DO supplement without leucine, tryptophan and glucose). Equal the OD of each mating and put a 10 µl drop in a triple selection plate (Difco Yeast Nitrogen Base without Amino Acids, Agar, Clontech DO supplement without leucine, tryptophan and histidine and glucose). Wait 5

days to see strong interactions and 10 days to see weak interactions. The positive control is pGBKT7 and pGADT7 encoded fusion between the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively (Li and Fields 1993). The negative controls are between constructs and empty vectors.

3.3. GST Gene Fusion System Protein Purification

The Glutathione S-transferase (GST) Gene Fusion System is an integrated system for the expression, purification and detection of fusion proteins produced in E. coli. The concept is expression of genes or gene fragments as fusions with Schistosoma japonicum GST (Smith and Johnson 1988). For that we use a series of vector called pGEX vectors that are designed for inducible, highlevel intracellular expression of fusion proteins. Protein expression from a pGEX plasmid is under the control of the tac promoter, which is induced using the lactose analog isopropyl b-D-thiogalactoside (IPTG). GST occurs naturally as a 26 kDa protein that can be expressed in E. coli with full enzymatic activity. Fusion proteins that possess the complete amino acid sequence of GST also demonstrate GST enzymatic activity, meaning that they can undergo dimerization (Parker, Lo Bello et al. 1990). Fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose Beads. Using Glutathione Sepharose 4B (Figure 3), fusion proteins can be purified to >90% in a single chromatographic step. Fusion proteins are recovered from the matrix under mild elution conditions (10 mM glutathione), which preserve antigenicity and functionality of the proteins. Induced cultures are allowed to express GST fusion proteins for several hours, after which time cells are harvested and then lysed by mild sonication. The bacterial lysate is cleared of cellular debris by centrifugation and the cleared lysate is ready to be applied directly to Glutathione Sepharose 4B. After the fusion proteins are bound to the matrix, it is washed with buffer to remove non-specifically bound proteins. Bound GST fusion proteins can then be eluted from the beads with reduced glutathione or cleaved. Cleavage of the desired

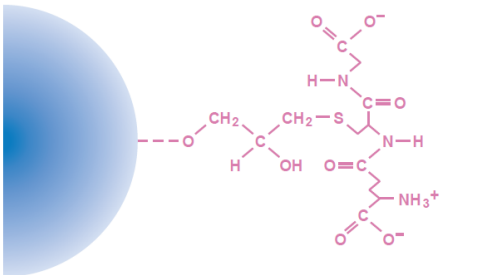


Figure 18 – Schematic of Glutathione attached to Sepharose 4B.

protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site. pGEX6P1 (Figure 1 of supplement data) is a bacterial vector for expressing GST fusion proteins with a PreScission Protease site.

3.3.1. Protocol:

Inoculate a BL21 starter culture and grow overnight at 37ºC in LB + Antibiotic. Dilute this culture 2/100 in LB + Antibiotic flasks and let it grow to OD600 ~0.6 at 30ºC. Add IPTG to 0.1 mM final and continue to grow cells at 30°C for 3 h or, alternatively, grow cells overnight at 18°C. Pellet cells at 4000 x g for 30 min. Remove supernatant (freeze pellets at -80°C if needed). Ressuspend the pellets in Lysis Buffer (1X PBS, 10 mM EGTA, 10 mM EDTA, 0.1% Tween, 250 mM NaCl, 1 mM PMSF and 2 mM Benzamidine-HCl). Add lysozyme to 1 mg/ml and leave for 15 min on ice. Sonicate the lysate using output settings between 6 and 9, 50% duty cycle, in 4 x 30 s intervals (1 min between intervals). Add DTT to 5 mM final. The lysate was centrifuged at 8 000 x g for 20 min. Repeat this step. Transfer cleared lysate into a tube containing the equilibrated glutathione agarose beads. Rotate at 4ºC for 1 hour. Wash beads with 4 x 50 ml Wash Buffer (1X PBS, 250 mM NaCl, 0.1% Tween 20, 1 mM DTT and 2 mM Benzamidine-HCl). Remove Wash Buffer from the beads completely using a Poly-Prep Chromatography Column. Add 6 mL of Elution Buffer (50 mM Tris pH 8, 75 mM KCl and 10 mM reduced glutathione) to the column and incubate for 15 min at 4ºC. Collect the Elution Fraction. Desalt the protein using the desalting columns Econo-Pac® 10DG Columns. The desalting buffer (25 mM Hepes pH 7.4, 150 mM NaCl) is the buffer used to apply the sample in the gel filtration column. Concentrate the sample using Amicon Ultra-15 Centrifugal Filter Units, to the volume of 500 µL (desired volumn of sample to apply in the gel filtration column).

3.4. 6xHis Fusion Protein Purification

The 6xHis system is a tool for affinity purification of recombinant proteins. The concept is to express proteins recombinant proteins tagged with a 6xHistidine tail. The amino acid histidine as a high affinity to immobilized nickel ions, so, the 6x Histidine-tag fusion protein purification system is based on the selectivity and high affinity of Ni-NTA

(nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive Histidine residues [1,2]. This interaction allows purification of tagged proteins from <1% to >95% homogeneity in just one step. The association between the tag and the NI-NTA resin allows contaminants to be washed away easily under stringen conditions, while the bound protein can be eluted with by competition with imidazole.

3.4.1. Protocol:

Inoculate a BL21 starter culture and grow overnight at 37ºC in LB + Antibiotic. Dilute this culture 2/100 in LB + Antibiotic flasks and let it grow to OD600 ~0.6 at 30ºC. Add IPTG to 0.1 mM final and continue to grow cells at 30°C for 3 h or, alternatively, grow cells overnight at 18°C. Pellet cells at 4000 x g for 30 min. Remove supernatant (freeze pellets at -80°C if needed). Ressuspend the pellets in Lysis Buffer (50 mM Na-Phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.1% Tween, 1 mM PMSF and 2 mM Benzamidine-HCl). Add lysozyme to 1 mg/ml and leave for 15 min on ice. Sonicate the lysate using output settings between 6 and 9, 50% duty cycle, in 4 x 30 s intervals (1 min between intervals). Add DTT to 5 mM final. Centrifuge lysate in 8 000 x g for 20 min. Repeat this step. Transfer cleared lysate into a tube containing the equilibrated glutathione agarose beads. Rotate at 4ºC for 1 hour. Wash beads with 4 x 50 ml Wash Buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole). Remove Wash Buffer from the beads completely using a Poly-Prep Chromatography Column. Add 6 mL of Elution Buffer (20 mM Tris pH 8.0, 100 mM NaCl and 250 mM imidazole) to the column and incubate for 15 min at 4ºC. Collect the Elution Fraction. Desalt the protein using the desalting columns Econo-Pac® 10DG Columns. The desalting buffer (25 mM Hepes pH 7.4, 150 mM NaCl) is the buffer used to apply the sample in the gel filtration column. Concentrate the sample using Amicon Ultra-15 Centrifugal Filter Units, to the volume of 500 µL (desired volumn of sample to apply in the gel filtration column).

3.5. Gel filtration

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties. Gel filtration or size exclusion chromatography separates the molecules according to their differences in size. This technique can be applied to separate components of a sample in two major groups

according to their size range, in order to remove molecular contaminants, to desalt or exchange buffer, and to isolate with a high resolution one or more components of a sample, so separate monomers from aggregates and to determine molecular weight.

For the separation to occurs, molecules pass through a gel filtration medium packed in a column. This molecules don't directly bind to the medium or the column. The medium is a porous matrix form of spherical particles, very stable and chemically inert. The matrix is filled with buffer. The liquid inside the pores is referred to stationary phase and this liquid is in equilibrium with the liquid outside the particles, or the mobile phase. The samples are eluted isocratically, with no need to use different buffers during the separation.

The elution profile or chromatogram if shown in Figure 19. Large molecules are eluted in or just after the void volume (V_0), as they pass to the column at the same speed as the flow of the buffer. The void volume is equivalent to approximately 30 % of the total column volume. Small molecules such as salts that have full access to the pores move down the column, but do not separate from each other. These molecules usually elute just before one total column volume (V_t) of buffer passed through the column. The proteins are detected by monitoring their UV absorvance (usually at A280nm) and salts are detected by monitoring the conductivity of the buffer.

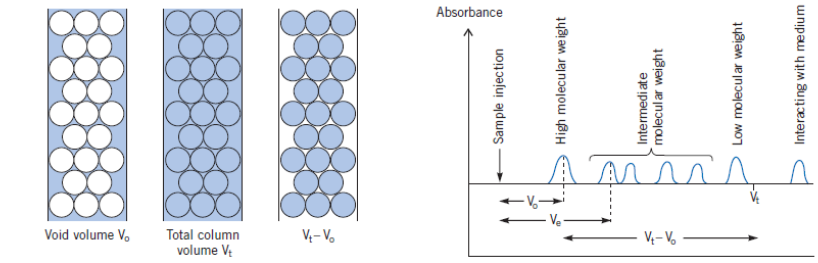
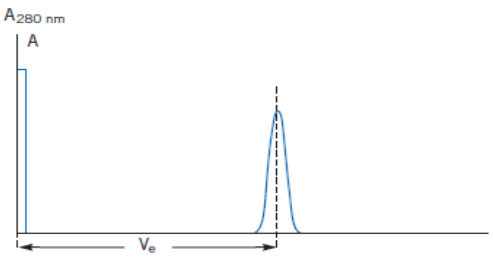


Fig. 2. Common terms in gel filtration.

Figure 19 – Common terms in gel filtration and an example of a chromatogram.



A. Sample size negligible compared with volume of packed bed.

Figure 20 – Elution Volume (Ve).

Molecules with partial access to the pores of the matrix elute from the column in order of decreasing size, and this are the molecules that we can separate. The behavior of each component can be expressed in terms of its elution volume (V_e), determined by direct measurement from the chromatogram. The volume of sample load in the column in this experiment (500 µL) is very low compared to the elution volume, so the elution volume is measured as exemplified in the Figure 20. The behavior of a specie in the column can be described by is partition coefficient (K_{av}). This coefficient represents the fraction of the stationary phase that is available for diffusion of a given molecular specie:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

The column used in all the experiment of this work was Sepharose 6, so the behavior of the sample can be defined by V_e values. K_{av} is related to the size and shape of a molecule. In this work we use this technique as a last step to purify proteins.

3.5.1. Protocol:

Before connecting the column, pump wash the machine (ÄKTA Purifier 10) with filtered distilled water. Insert the column (Superose 6, 10/300 GL) and wash the column with 2 volumes of filtered distilled water (2 x column volumes), and after equilibrate the column with filtered running buffer (25 mM Hepes pH 7.4, 150 mM NaCl) (1 x column volume). Use the same running rate that will be used in the run of the sample (0,2 ml/min). Wash the loading tube with the running buffer (4 x tube volumes). Load the sample in the tube. Start the running program. Major topics of the running program: inject sample in the beginning of the run; rate of 0,2 ml/min; max pressure 1,5 MPa; start collecting 0,5 ml fractions after 7 ml (expected void volume). After purification, the desired fractions were collected and the concentration of protein was measured using the AppplyChem kit.

3.6. Plasmids

All constructs were generated by PCR using a corresponding full length *C. elegans* cDNA clone as template, using Phusion® High-Fidelity DNA Polymerase (NEB). Except Rod-1(aa 1-418) with Zwl-1 6xHis tagged (Table 1), in pACYCDuet-1, that was given to our lab. All the plasmid where prepped using Zyppy™ Plasmid Miniprep Kit (Zymo Research).

pACYDuet-1 (Figure 2 of the supplement data) is a vector designed for the coexpression of two target genes. The vector contains two multiple cloning sites (MCS), each of which is preceded by a T7 promoter/lac operator and ribosome binding site. So, the different proteins are transcript to 2 different mRNAs. One of the cloning sites is positioned downstream and in frame with a polyhistidine tag, where they inserted Zwilch, and in the other they inserted Rod.

pRSETA (Figure 3 of the supplement data) is a vector designed for high-level expression of a target protein, in *E.coli*, due to the presence of the T7 promoter. DNA inserts are positioned downstream and in frame with a polyhistidine tag. Spindly fragments inserted in this vector are named in Table 1.

pST39 is a vector constructed to facilitate the studies of multicomponent protein complexes, in *E. coli*. It is composed by 4 cassetes, allowing the expression of until 4 polypeptides, in one mRNA. This vector tries to solve the fact that some genes might have internal restriction sites for the subcloning sites of the vector by provide alternate restriction sites with compatible ends (Figure 21). Rod-1 (aa 1 – 418) was fused with GST of pGEX6P1 by overlap PCR, and cloned into cassette 2 of pST39 with EcoRI and HindIII. Zwl-1 wild type and Zwl-1 mutant was cloned into pST39 by cutting the vector with XbaI and BamHI, but the insert with NheI and BamHI.

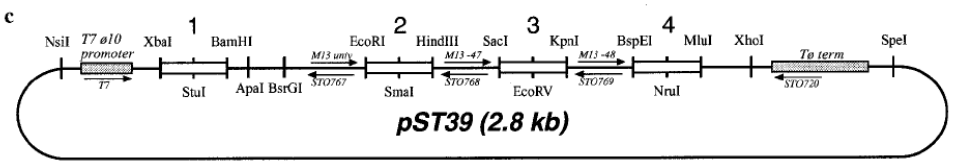


Figure 21 – pST39 polycistronic expression system.

pFastBacHta (Figure 4 of supplement data) is designed for use as part of the Bac-to-Bac® Baculovirus Expression System for the expression and purification of histidine-tagged recombinant proteins in Sf9 cells.

Construct	Vector	Affinity tag	Expression system	Restriction Enzimes
spdl-1 full length	pFastBac1	6xHis	baculovirus	EcoRI | SalI
spdl-1 full length	pGEX6P-1	GST	bacteria	EcoRI | SalI
rod-1(aa 1-418)::GST + zwl-1(Wild Type)	pST39	GST	bacteria	-
spdl-1(aa1-180)	pRSET A	6xHis	bacteria	Pst I | EcoRI
spdl-1(aa181-361)	pRSET A	6xHis	bacteria	Pst I | EcoRI
spdl-1(aa362-479)	pRSET A	6xHis	bacteria	Pst I | EcoRI
spdl1(aa401-479)	pRSET A	6xHis	bacteria	Pst I | EcoRI
spdl-1(aa1-360)	pRSET A	6xHis	bacteria	Pst I | EcoRI
spdl-1(aa362-479)	pGEX6P-1	GST	bacteria	BamHI | EcoRI
spdl-1(aa1-360)	pGEX6P-1	GST	bacteria	BamHI | EcoRI
rod-1(aa 1-418) + zwl-1::6xHis(Wild Type)	pACYCDuet-1	6xHis	bacteria	Rod-1 - EcoRI | HindII
rod-1(aa 1-418) + zwl-1::6xHis(E433A/E437A)	pACYCDuet-1	6xHis	bacteria	Zwl-1 – NheI | BamHI

Table 1 – Constructs used for Recombinant Protein Expression.

3.7. In Vitro Pull Down Assays

The pull-down assay is an in vitro method used to determine a physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein-protein interaction predicted by other research techniques and as an initial screening assay for identifying previously unknown protein-protein interactions. In a pull-down assay, a bait protein is tagged and captured on an immobilized affinity ligand specific for the tag, thereby generating a "secondary affinity support"' for purifying other proteins that interact with the bait protein. The secondary affinity support of immobilized bait is then incubated with a protein source that contains

putative "prey" proteins, such as a cell lysate or a purified recombinant protein.

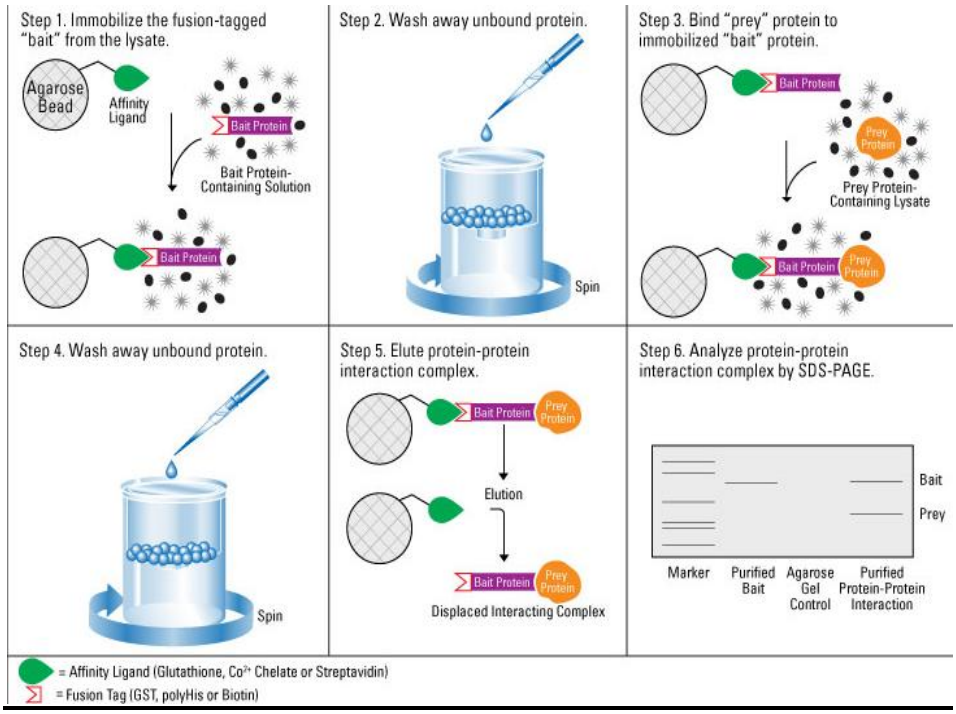


Figure 22 – General schematic of a pull down assay.

The bait protein used in this assays is a recombinant fusion-tagged proteins, tagged with GST. The prey protein as no tag. The identification of bait-prey interactions requires that the complex is removed from the affinity support and analyzed by standard protein detection methods. The metod used was sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a harsh treatment that will denature all protein in the sample.

3.7.1. Protocol:

Wash 20 µL bead volume of glutathione agarose beads with 3 x 1 mL Pull Down Buffer (50 mM Hepes pH 7.4, 50 mM NaCl, 0.1 % Triton X-100 and 1 mM DTT). Make a 50 % slurry of the beads with Pull Down Buffer. Put 20 µL of the 50% bead slurry in a 1.5 mL eppendorf tube, then add the right amount of protein bewaring the relative molecular weights of the recombinant proteins and the equimolarity of the components. The total amount of volume per pull down reaction is 100 µL. Rotate the tubes at 4 °C for 1 h. Wash with 3 x 500 µL Pull Down Buffer (50 mM Hepes pH 7.4, 50 mM NaCl, 0.1 % Triton X-100, 1 mM DTT and15 mM glutathione). After the last wash, remove all buffer with a gel loading tip. Elute bound proteins with 25 µL Elution Buffer

by rotating at room temperature for 15 min. Remove eluted fraction with a gel loading tip. Add 6 µL of 4 x SDS-PAGE sample buffer to the elution fraction and heat to 70 °C for 3 min. Run a gel (load 10 µL per lane). For the input, run the individual purified protein fractions in separate lanes (33% of what you put into the pull down reaction).

3.8. Western Blot

After running an SDS-Page gel, the gel was transferred to a membrane for 2 hours at 4 ºC with transferring buffer (25 mM Tris; 190 mM glycine; 20 % methanol; 0,1 % of SDS) at 220 mA, with agitation. Stain the membrane with Ponceau S (0,1 %, w/v, Ponceau S; 5 %, v/v, acetic acid) for a few minutes. Wash with water and, with a pen, mark the molecular markers. Wash with transferring buffer to remove the Ponceau S. Wash the membrane with TBS (50 mM Tris; 150 NaCl), 0,1 % triston X-100. Block the membrane with Blocking Buffer (TBS (50 mM Tris; 150 NaCl); 0,1 % triston X-100; 5% milk). Do an overnight incubation at 4 ºC with the primary antibody at the chosen concentration in blocking buffer. Wash 2 times quickly with TBS (50 mM Tris; 150 NaCl), 0,1 % triston X-100. Wash 15 minutes with blocking buffer. Wash 3 times for 10 minutes with TBS (50 mM Tris; 150 NaCl), 0,1 % triston X-100. Apply the secondary antibody in blocking buffer at the chosen concentration for 1 hour at room temperature. Wash 2 times quickly with TBS (50 mM Tris; 150 NaCl), 0,1 % triston X-100. Wash 15 minutes with blocking buffer. Wash 3 times for 10 minutes with TBS (50 mM Tris; 150 NaCl), 0,1 % triston X-100. Reveal the membrane with the Pierce ECL Plus Western Blotting Substrate.

4. Results and Discussion

4.1. Yeast Two Hybrid Analysis

Previous studies in multiples organisms have demonstrated that Zwilch and ZW10 interact with Rod to form RZZ Complex (Scaerou, Starr et al. 2001). However it remains not completely clear what are the regions of Rod that interact with these proteins. To address this question, I build several constructs with different fragments of Rod, and tested the interaction between these fragments and Zwilch and ZW10 (Table 1 of Supplement Data).

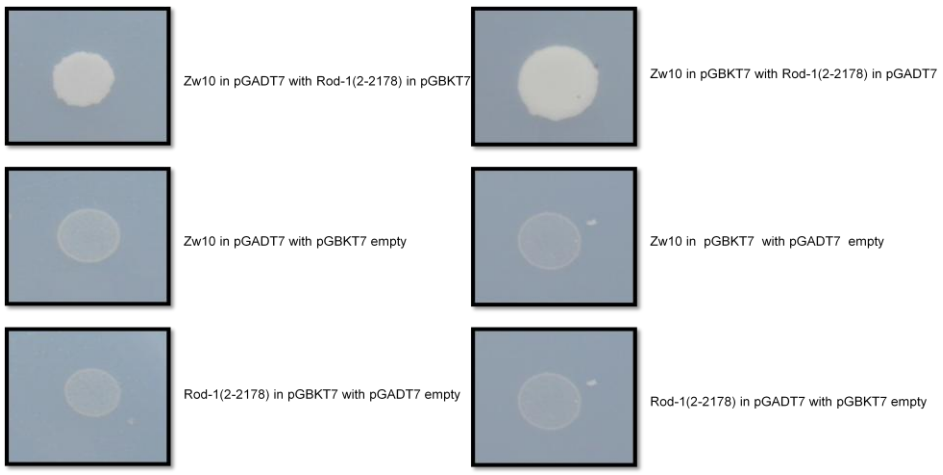


Figure 23 - Result of the interaction between Zw10 and Rod-1 a.a. 2-2178 (full length).

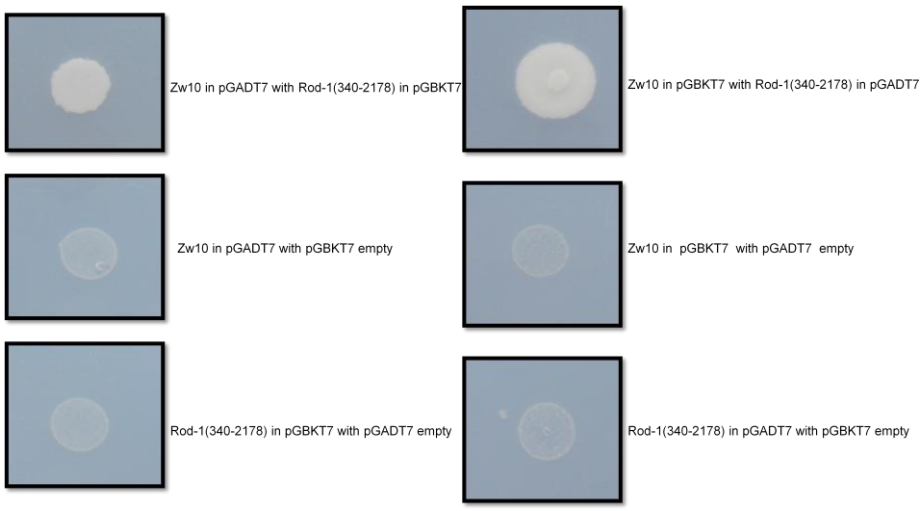


Figure 24 - Result of the interaction between Zw10 and Rod-1 a.a. 340-2178.

Figure 25 - Result of the interaction between Zw10 and Rod-1 a.a. 2-1710.

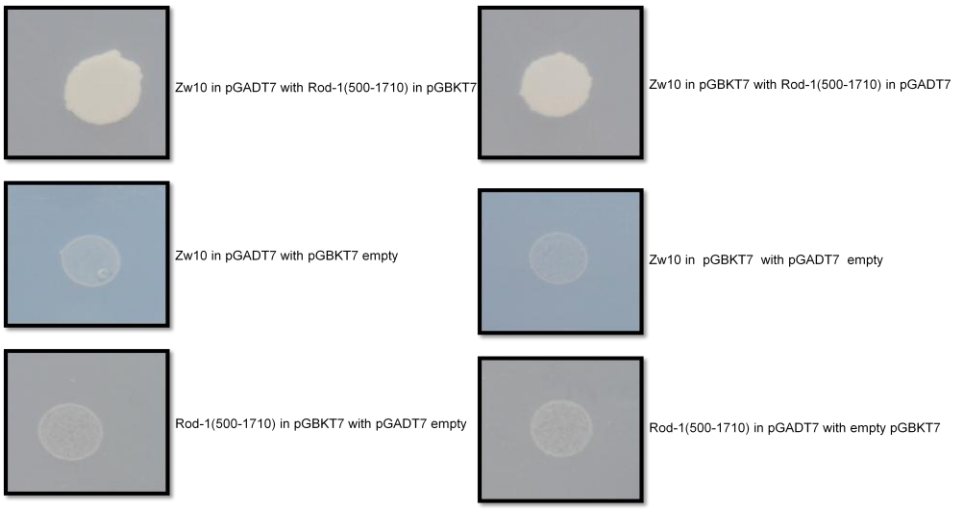


Figure 26 - Result of the interaction between Zw10 and Rod-1 a.a. 500 to 1710.

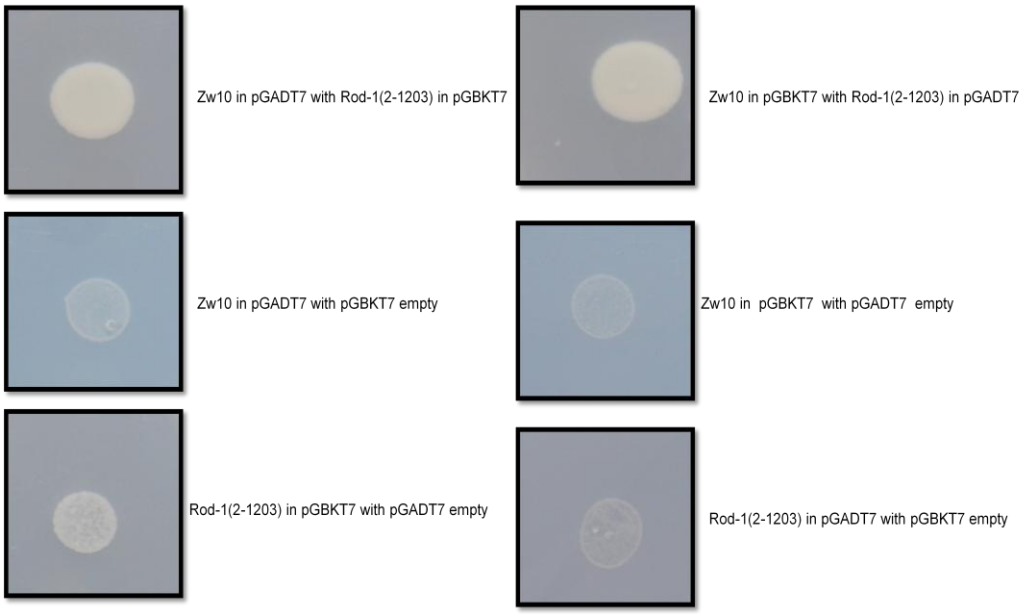


Figure 27 - Result of the interaction between Zw10 and Rod-1 a.a. 2 to 1203.

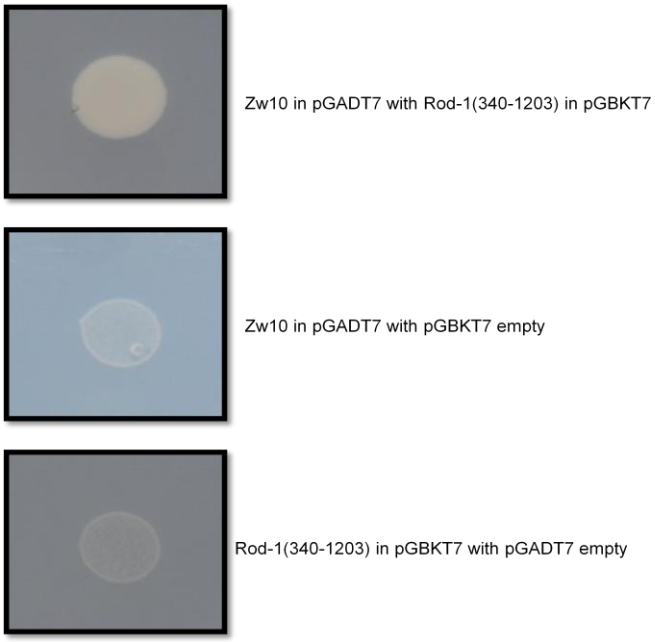


Figure 28 - Result of the interaction between Zw10 and Rod-1 a.a. 340 to 1203.

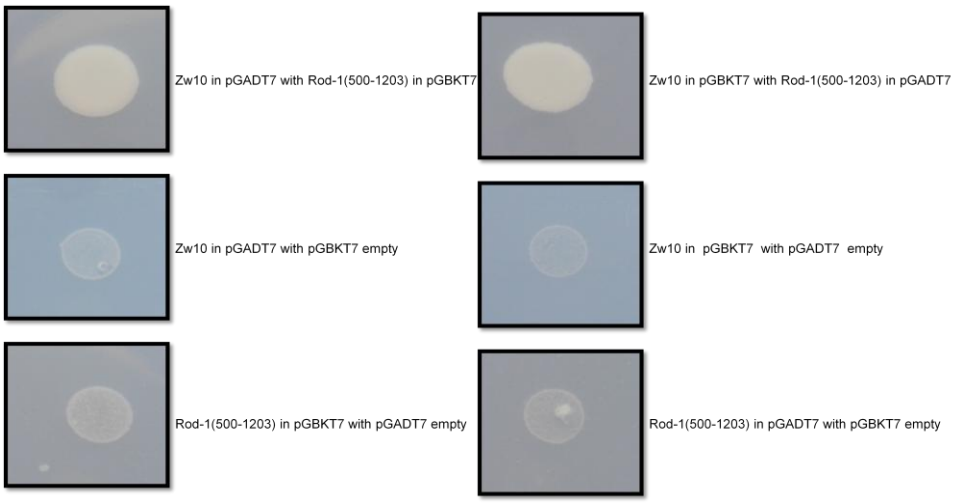


Figure 29 - Result of the interaction between Zw10 and Rod-1 a.a. 500 to 1203.

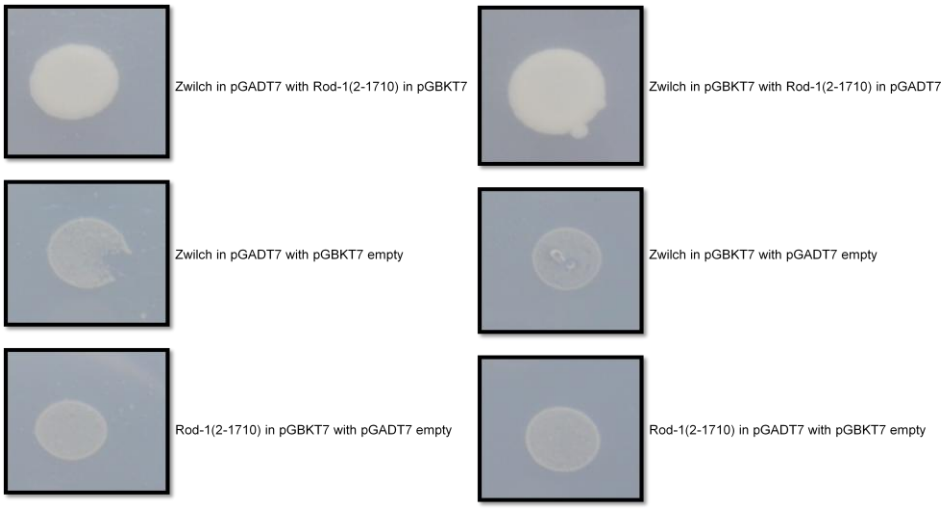


Figure 30 - Result of the interaction between Zwilch and Rod-1 a.a. 2 to 1710.

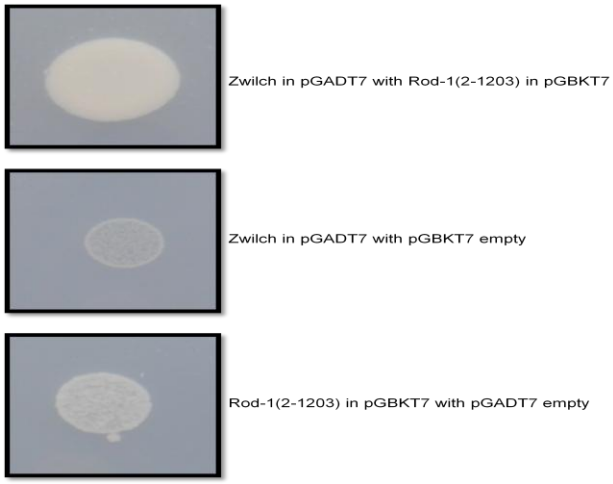


Figure 31 - Result of the interaction between Zwilch and Rod-1 a.a. 2 to 1203.

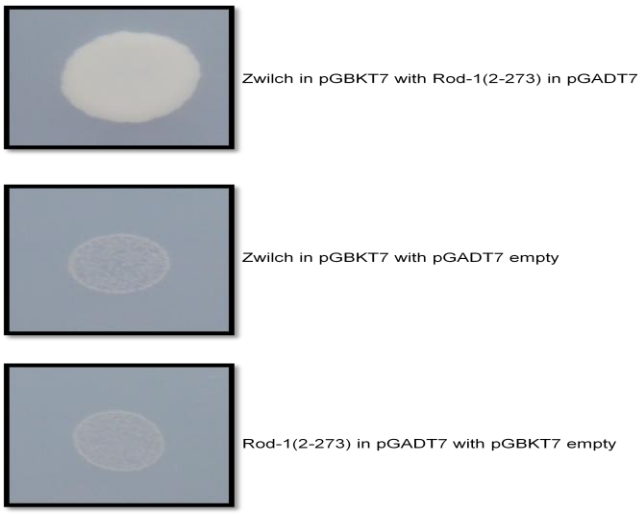


Figure 32 - Result of the interaction between Zwilch and Rod-1 a.a. 2 to 273.

The results (Figure 23 to 32) indicate that Zwilch interacts with the first 273 amino acids of Rod, that correspond to the β-propeller domain of the protein. This kind of domain is known to mediate protein-protein interactions (Neer, Schmidt et al. 1994) data that is consistent with our findings. These same experiments also suggest that ZW10 interacts with Rod between the amino acids 500 and 1203, that correspond to the Sec39 domain of the protein. Rod has a long C-terminal tail of around 1000 amino acids possibly organized as α-solenoids. The purpose of this domain in the function of the protein/complex is still unclear. Figure 33 is a scheme of the fragments of Rod (Table 1 of the supplement data) created and the ones that interact with Zwilch and ZW10.

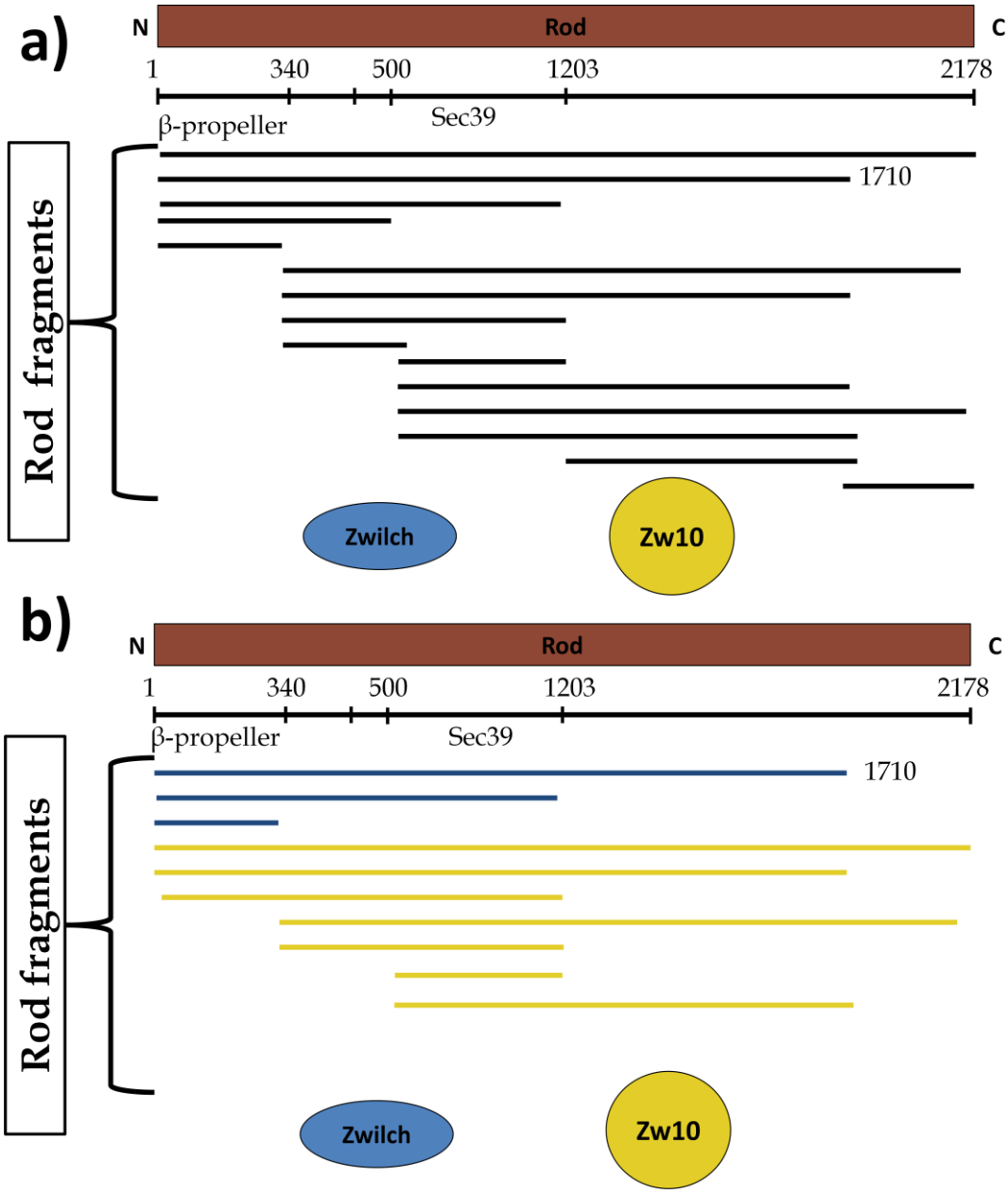


Figure 33 – Scheme that represent the Rod-1 fragments that interact with Zwilch and with Zw10 : a) all Rod fragments created; b) Rod fragments that interacted with Zwilch (in blue) and with Zw10 (in yellow).

After noticing that the N-terminal β-propeller domain of Rod interacts with Zwilch, I built a construct of Zwilch with two point mutations, that abolish Spindly recruitment to kinetochores in *C. elegans* embryos (data unpublished), to see if this fragment is still interacting with Rod. From Figure 34 is possible to conclude that, in fact, Zwilch is still interacting with Rod, meaning that the point mutation is interfering with localization of Spindly but not with the organization of Rod (Figure 34).

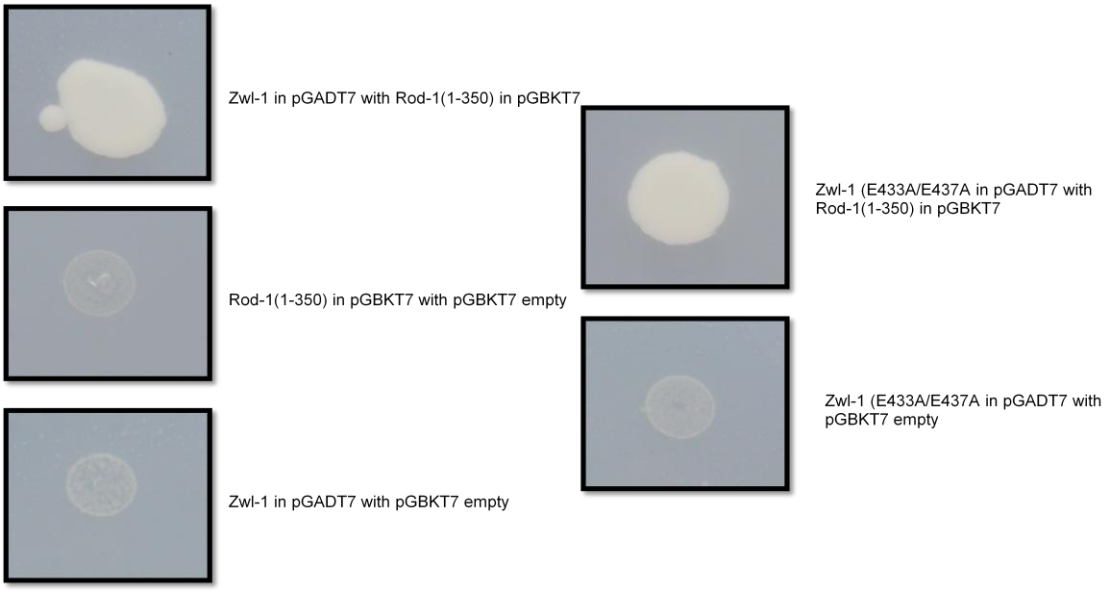


Figure 34 – Result of the interaction between Zwilch/Zwilch mutant and Rod-1 a.a. 1 to 350.

Spindly interacts with the RZZ complex (Griffis, Stuurman et al. 2007). However, it still not clear what protein of the RZZ complex interacts with Spindly. This data indicates that Zwilch might mediate the interaction between the RZZ complex and Spindly. Knowing that, I decided to test the interaction of Zwilch with full length Spindly as well as N-Terminal and C-terminal Spindly fragments. Since RZZ complex is a dimmer(Civril, Wehenkel et al. 2010), and suspecting that spindly might dimerize as well, I tested the interaction between Spindly with itself and between N and C-terminal fragments. (Supplement table 2)

To decide what Spindly fragments I should create, I used a program that predicts the tertiary structure of the protein (Figure 35). The program used was ELM. This program uses the programs listed on the Figure 35: GlobPlot, SMART/Pfam and IUPRED. Knowing the structure, I decided to incorporate the coiled-coil part of Spindly in one fragment (comprising amino acids 1 to 361) and the C-terminal part of it (comprising amino acids 362 to 479) in other fragment.

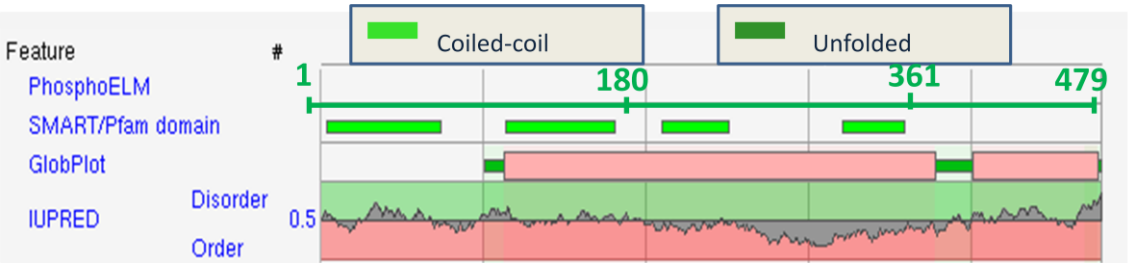


Figure 35 – Prediction of Spincly struture by ELM.

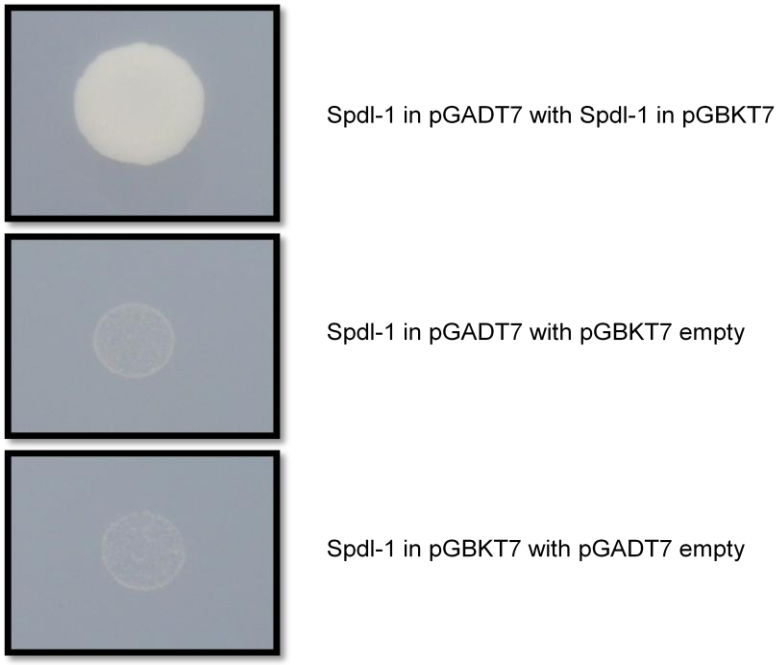


Figure 36 - Result of the interaction between Spdl-1.

It was not possible to see any interaction between Spindly and Zwilch. However, by this assay we were capable of seeing the interaction between spindly and itself. This result means that probably, as well as the RZZ complex, spindly function as a dimmer in the cell, as I suspected.

4.2. Recombinant Protein Expression and Purification

Yeast two Hybrid assays gave us important data regarding the interactions between the RZZ complex proteins but inconclusive information about the interaction between Spindly and RZZ complex and what region of Spindly is responsible for the it dimerization. In this part of the work I will focus on expressing and purifying recombinant proteins and use these proteins to test interactions between them with different tags to be able to try different protein/fragment combinations.

4.2.1. GST tagged Spindly Protein

Following the protocol described above, I was able to express, from bacteria, the *C. elegans* protein Spindly tagged with GST (expected size 88,4 kD) in the vector pGEX6P1 (Figure 37). Is possible to see on the gel a band appearing at around the expected size in the induced lane (Figure 37 Lane 2). The protein was soluble – appears in the lysate (Figure 37 Lane 3) – and is successfully bound to the agarose beads (Figure 37 Lane 4). After elution from the beads, is clear an increase in the purity of the solution.

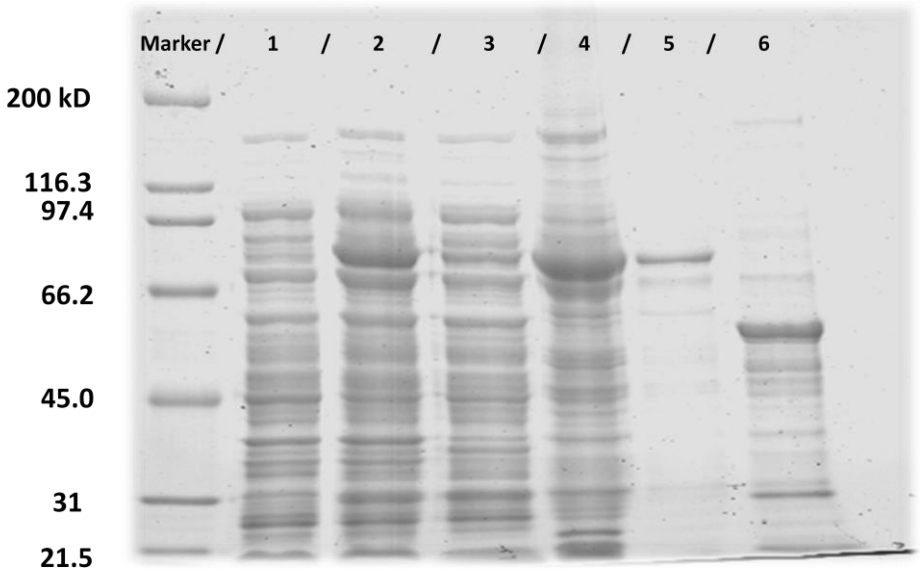


Figure 37 – Gel of the expression of full length Spindly in pGEX6P1. Lanes : 1 – Uninduced; 2 – Induced ; 3 – Lysate (1/16000 of the total); 4 – Glutathione Agarose Beads (1/7500 of the total); 5 – Elution (1/9600 of the total); 6 – Cleaved (1/12000 of the total).

After elution from the Agarose Beads, the protein was successfully cleaved, and still soluble, using PreScission™ Protease, to the expected size of 62,4 kD (Figure 37 lane 6). The amount of protein in the elution and in the cleaved fraction is approximately the same. Is possible to notice more contaminant in the cleaved fraction than in the elution, possible due to degradation of the cleaved protein.

The next step is loading the eluted protein in a gel filtration column with the aim of trying to better purify the protein and to have a clue of what is its behavior in solution, since gel filtration chromatography is a technique that allows this perdition. Before loading the protein in the gel filtration column, I did two different controls that will allow us to know what the void volume is and to estimate the molecular weight of the proteins that came out of the column. In the first one, I used Blue Dextran. Blue Dextran is a large molecule of around 2,000 kD that give us the void volum (V_0) of a gel filtration column. Is then possible to know that the void volume of this column is 7,5 mL. The second control is a mix of 10 mg/mL of each protein: Albumin, bovine serum, of 66 kD; Catalase ; of 50 kD and Chymiotripsin, of 25 kD (Figure 38).

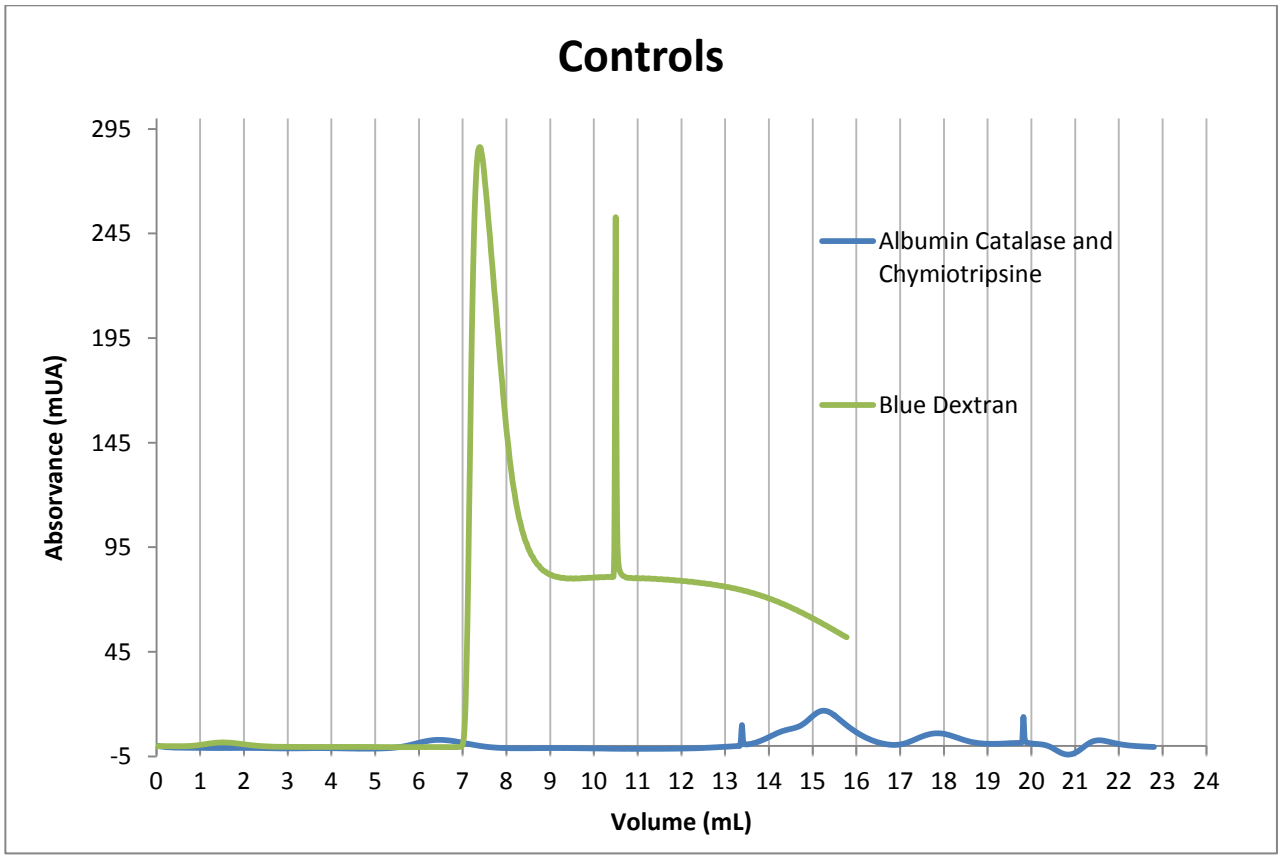


Figure 38 - Graphic of the elution patterns of the two different controls: Blue dextran and Albumin/Catalase/Chymiotripsine.

Knowing the elution volume of each protein and the void volume, is possible to calculate the partition coefficient (Kav) of each protein and, with that value, construct a standard curve that will allow the possibility of calculate the molecular weight at with our protein is eluted. In a gel filtration column, big proteins are eluted first and smaller proteins are eluted last. So, albumin is the first protein to come out of the column and chymiotripsine the last. Table 1 indicates the Kav of each protein. The standard curve is represented in Figure 39.

Protein	Ve	Kav	Molecular Weight (kD)
Albumin	15,3	2,19	66
Catalase	17,8	2,54	50
Chymiotripsine	21,5	3,07	25

Table 2 – Elution volume, partition coefficient and molecular weight of each protein of the control run.

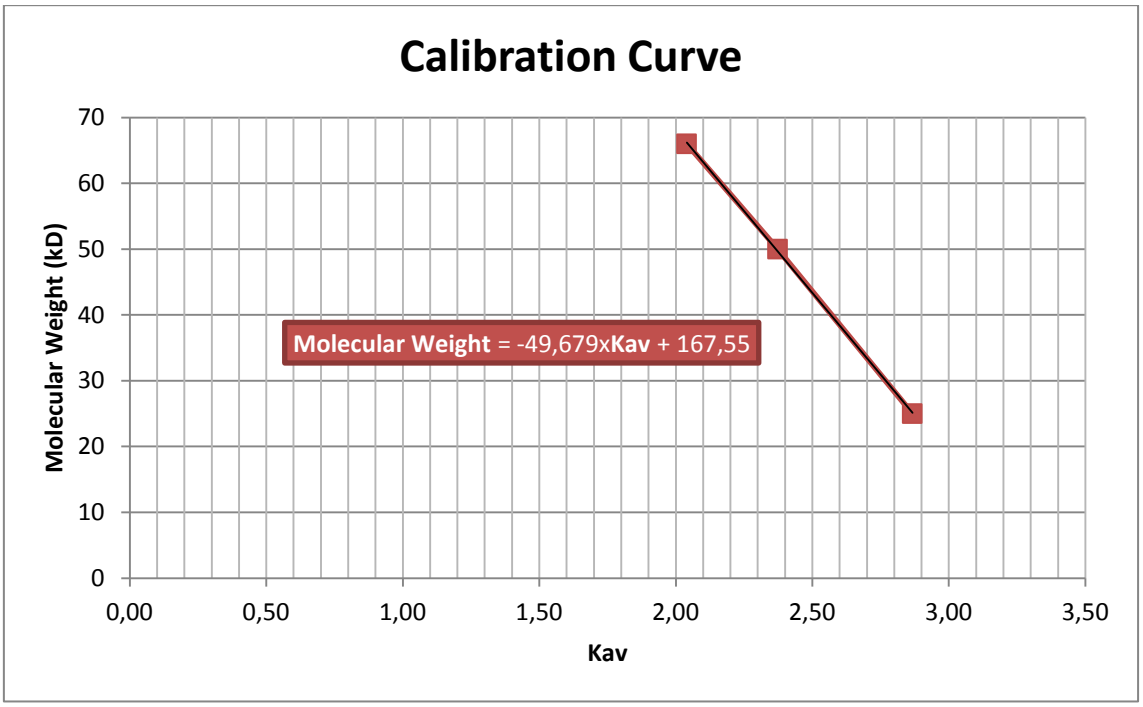


Figure 39 - Calibration curve obtained with Albumin, Catalase and Chymiotripsine.

The eluted and cleaved Spindly was then loaded in the gel filtration column (Figure 40).

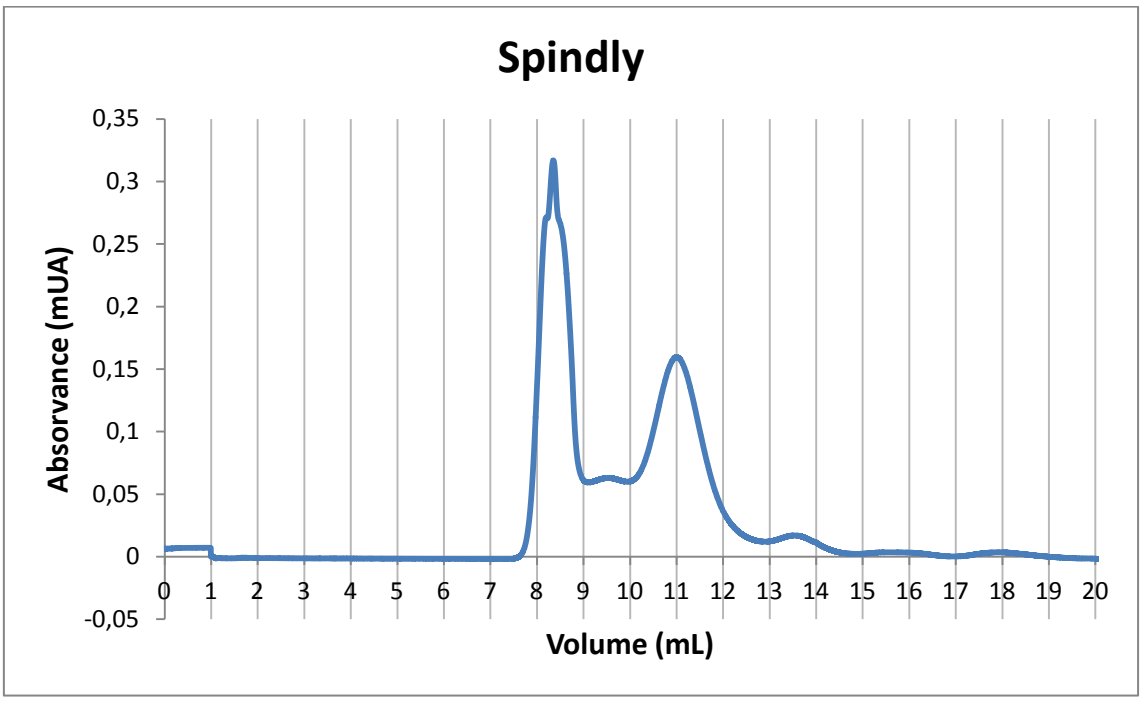


Figure 40 – Graphic of the purification of Spindly by Gel Filtration Column.

It is possible to distinguish two different peaks, the first one corresponding to the void volume. In Figure 41 are the fractions collected after the first peak (Volume 9 mL). The fractions collected were the fractions between lane 4 and lane 7. Is possible to notice the presence of a bigger protein in the gel, at around 70 kD, that may correspond to a heat shock protein. By Bradford assay it was possible to calculate the amount of protein, that is 3,5 mg/mL.

By analysis of the Figure 39, is possible to see that our protein of interest (62,4 kD) as it major pick at Lane 5 of Figure 40, that corresponds to the volume of 11 mL in Figure 17. The Kav of this protein is then 1,47. Using the equation given on Figure 39, I extimated the molecular weight in which this protein comes out of the column, that is 94,5 kD. This size is higher than the actual size of the protein, that is 62,4 kD, and this is possibly due to the shape of the protein: elongate molecules come out of the column at higher size.

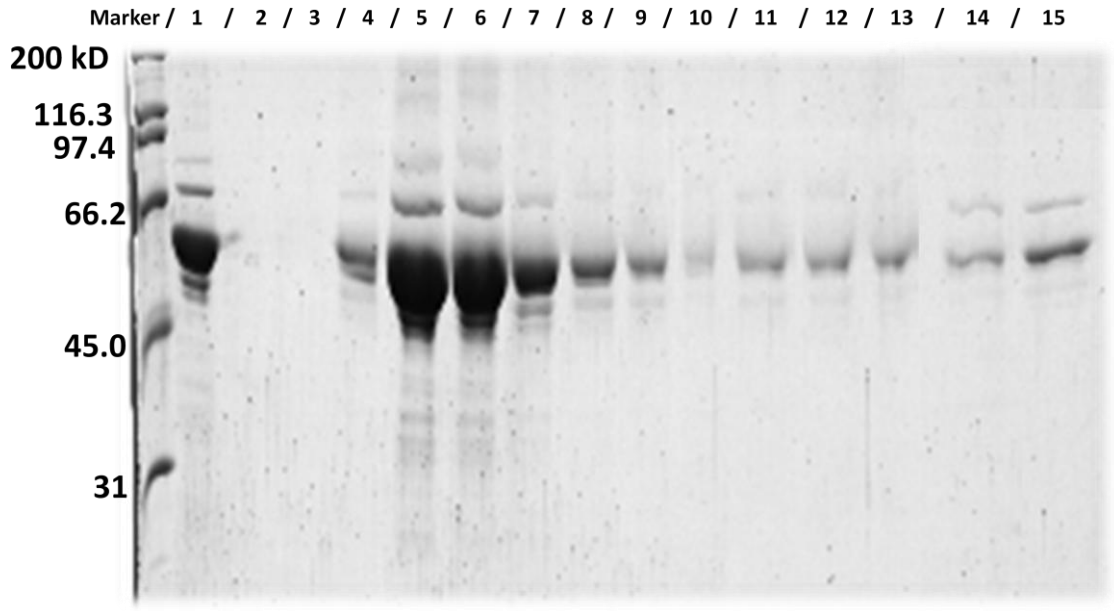


Figure 41 - Fractions of Spdl-1 collected in the gel filtration column. Lanes: 1 – Input (1/6000 of the total); 2-15 – Factions collected after 9 mL, 0,5 mL Factions. (1/70 of the total)

GST-Spindly from amino acid 1 to 361 (expected size 68.9 kD) was successfully expressed in pGEX6P1 (Figure 42 lane 2). The protein is soluble, given that it appears in the lysate and in the supernatant (Figure 42 lane 3 and 4). The protein as bound to the beads (Figure 42 lane 5) and a significant amount of protein is eluted (Figure 42 lane 7). There are still protein bounded to the beads after elution (Figure 42 lane 6). The protein was concentrated to the respective volume (Figure 42 lane 8) and loaded in the gel filtration column. Is possible to see that after concentration a significant amount of protein as precipitated in the eppendorf tube (Figure 42 lane 9).

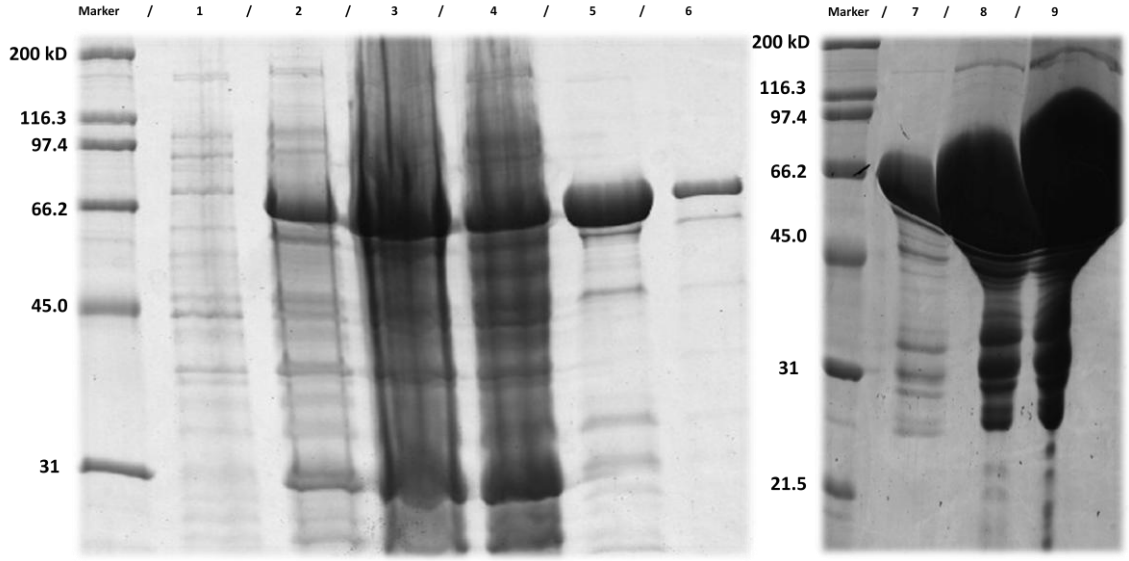


Figure 42 - Gel of the expression of Spindly fragment from amino acids 1 – 361 in pGEX6P1. Lanes : 1 – Uninduced; 2 – Induced ; 3 – Lysate (1/16000 of the total); 4 – Supernatant (1/16000 of the total); 5 – Glutathione Agarose Beads before elution (1/7500 of the total) ; 6 – Glutathione Agarose Beads After Elution (1/7500 of the total); 7 – Elution (1/9600 of the total); 8 – Concetrated sample before loading in the gel filtration column; 9 – Pellet after spinning.

After elution, desalting and concentration the sample was loaded in the gel filtration column. In the graph of the absorbance of the sample ft. volume of sample (Figure 43) is possible to see a lot of different peaks in different volumes corresponding to different proteins or fragments. To distinguish witch peak corresponds to our protein I run the collected fractions of 1 mL in a gel (Figure 44), starting on the volume 10 mL.

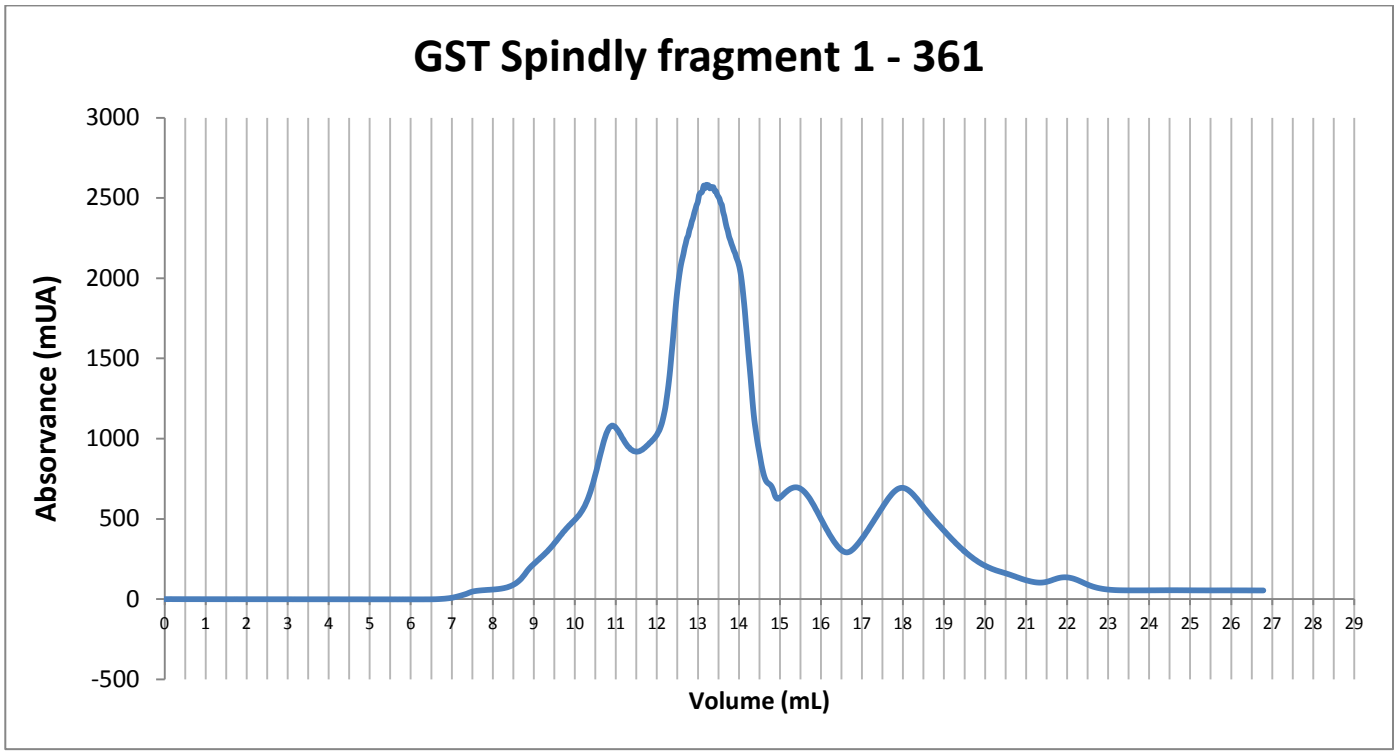


Figure 43 – Graphic of the Purification of GST Spindly fragment 1 -361 by Gel Filtration Column.

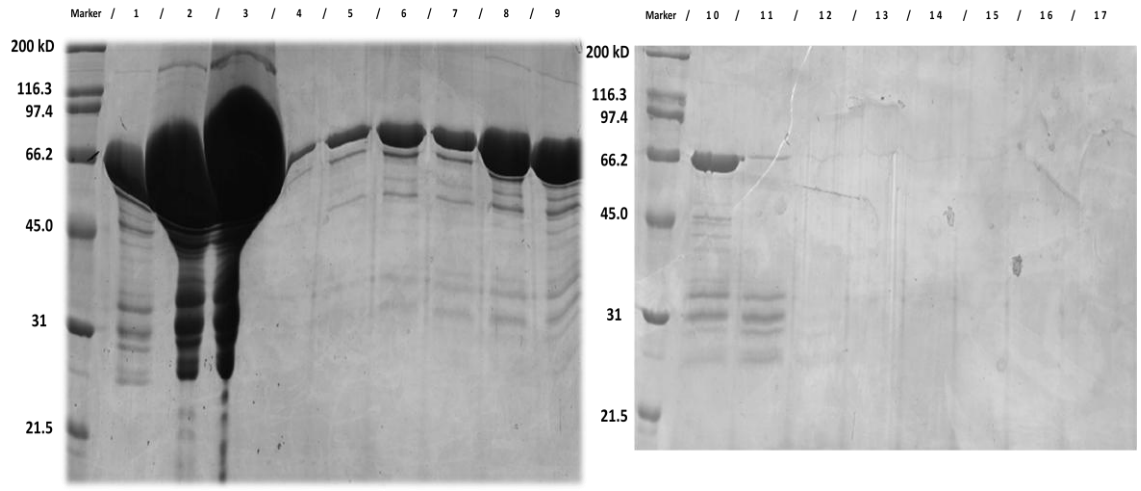


Figure 44 - Fractions of GST Spdl-1 fragment 1 .361 collected in the gel filtration column. Lanes: 1 – Input (1/6000 of the total); 2 - Concentrated Sample; 3 – Pellet; 4-17 – Factions collected after 10 mL, 1 mL Factions (1/140 of the total).

By analysis of the Figures 43 and 44, is possible to see that our protein of interest (68.9 kD) as it major peak at fraction 8 and 9, that corresponds to the peak with a maximum at volume 13.2 mL in Figure 43. The Kav of this protein is then 1,76. Using

the equation given on Figure 39, I estimated the molecular weight in which this protein comes out of the column, that is 86 kD. This size is higher than the actual size of the protein, that is 68.9 kD, and this is possibly due to the shape of the protein: elongate molecules come out of the column at higher size. Nevertheless, is possible to conclude that this fragment doesn't dimerize.

After collecting the selected fractions (Lanes 6 to 9, Figure 44) I estimate the protein amount using a BSA standard assay. I obtained 10 mg/mL of Gst spindly Fragment 1-361. A small amount of this total (100 µL) were successfully cleaved of Precission Protease to a soluble protein of the expected size of 43 kD (Figure 45). The amount of protein cleaved was a little less than the uncleaved (Figure 45).

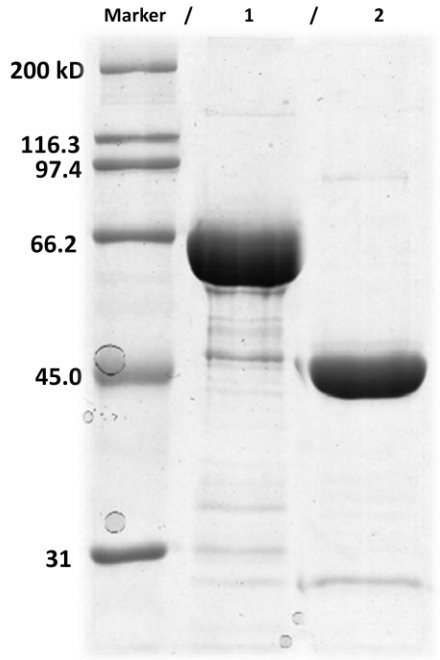


Figure 45 - Gel of the cleavage of GST from GST-Spindly fragment 1-361. Lanes: 1 – GST-Spindly fragment 1-361 uncleaved (1:30 of the total 100µL); 2 – Spindly fragment 1-361 after cleavage (1:30 of the total).

Gst Spindly from amino acid 362 to 476 (expected size 40 kD) was successfully expressed in pGEX6P1 (Figure 46 Lane 2), demonstrated by the appearance of a band expressing after addition of IPTG at around the expected size. The protein is soluble, given that it appears in the lysate (Figure 46 lane 3). The protein as bound to the beads

(Figure 46 lane 5) and a significant amount of protein is eluted (Figure 46 lane 7). In Figure 46, lane 5, the elution fraction still as proteins other than the protein of interest, namely at around 70 kD, that I suspect that is a heat shock protein. After elution, desalting and concentration the sample was loaded in the gel filtration column. In the graph of the absorbance of the sample ft. volume of sample (Figure 47) is possible to see a big peak with maximum at 14,4 mL. To understand the features of the fractions collected, I run a gel from fraction collected in volume 10 mL to volume 16,5 mL (0,5 mL fractions- Figure 48).

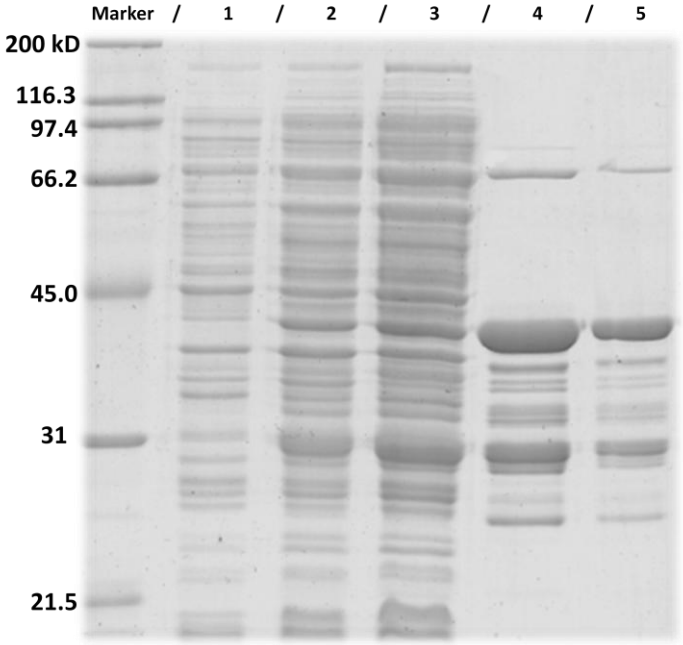


Figure 46 - Gel of the expression of Spindly fragment from amino acids 362 - 479 in pGEX6P1. Lanes : 1 – Uninduced; 2 – Induced ; 3 – Lysate (1/16000 of the total); 4 - Glutathione Agarose Beads before elution (1/7500 of the total) ; 5 – Elution (1/9600 of the total);

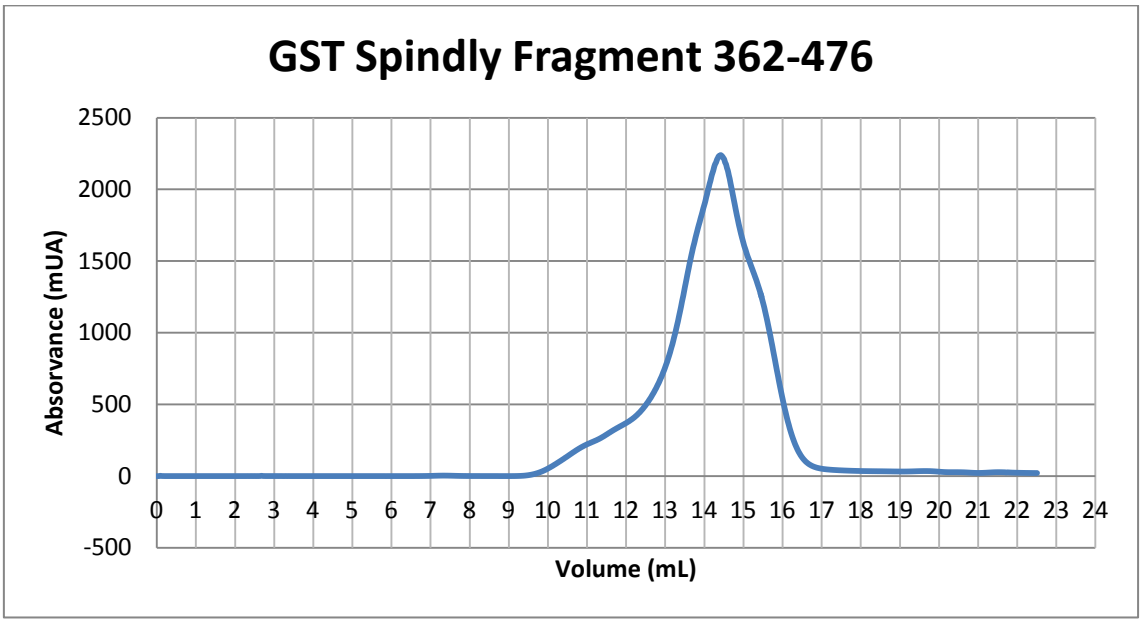


Figure 47 – Graphic of the purification of GST Spindly fragment 362 - 479 by Gel Filtration Column.

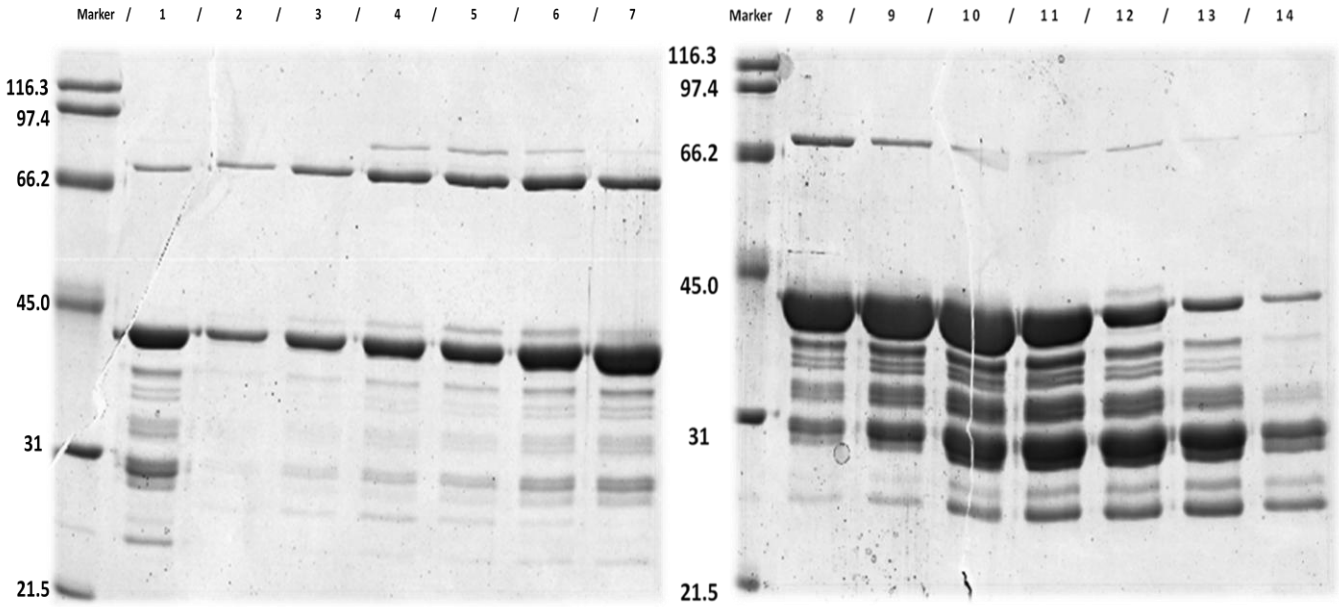


Figure 48 - Fractions of GST Spdl-1 fraction 362 – 479 collected in the gel filtration column.
Lanes: 1 – Input (1/6000 of the total); 2-14 – Factions collected after 9 mL, 0,5 mL Factions (1/70 of the total).

By analyzing figures 47 and 48, it is possible to see that our protein (40 kD) is coming out along the hole peak, but in a great amount in lanes 8 to 10. The protein at 70 kD is probably a heat shock protein. After lane 9, is possible to notice an increase in a contaminant at molecular weight of 30 kD, so, to avoid that contaminant, I pulled

down the fractions 6 to 9, and measured the concentration by a Bradford assay: 1,5 mg/mL.

The major peak in Figure 47, is at 14,4 mL. Using this value, and knowing that the void volume is 7,5, the Kav of this protein is 1,92. Using the equation given on Figure 39, I estimated the molecular weight in which this protein comes out of the column, that is 72 kD. This size may be indicating that this protein is a dimmer, since two times 40 kD equals 80 kD, that is a molecular weight near of 72 kD. Nevertheless, this dimerization may be due to the presence of the GST in the protein, that occurs as a dimmer in the cell.

In the next step, I used 100 µL of the collected sample and successfully cleaved this fragment of the GST (25 kD), as seen in Figure 49, comparing the sizes of the bands before (lane 1) and after addition of Prescission Protease (lane 2). The cleaved fragment is higher than the expected size, that is 15 kD.

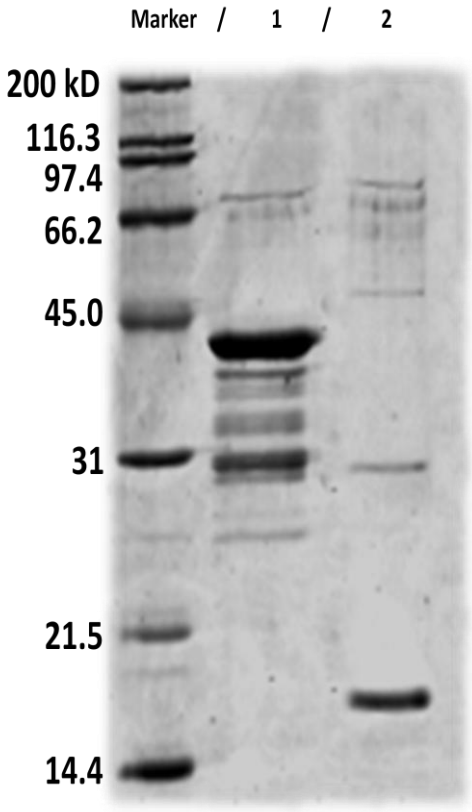


Figure 49 – Gel of the cleavage of GST from GST-Spindly fragment 362-479. Lanes: 1 – GST-Spindly fragment 362-476 uncleaved (1:30 of the total 100µL); 2 – Spindly fragment 362-479 after cleavage (1:30 of the total).

4.3. 6xHis tagged Spindly

Based on Figure 35, I decided two add extra Spindly fragments for expressions try outs in pRSET A vectors: fragment 1 -180 and 181-361, to see if cutting the coiled-coil domain would have any effect on the protein expression/activity, and fragment 401-479, that is the C-terminal fragment without the unfolded region.

By analysis of Figure 50, it is possible to see that none of the fragments 1 to 180 (expected size of 25.8 kD) or 181-361 (expected size of 26 kD) is expressing after addition of IPTG, given that any band is appearing in the gel at the expected sizes of the proteins. The band appearing in the lysate (lanes 3 and 6 of figure 50) is the lysozime.

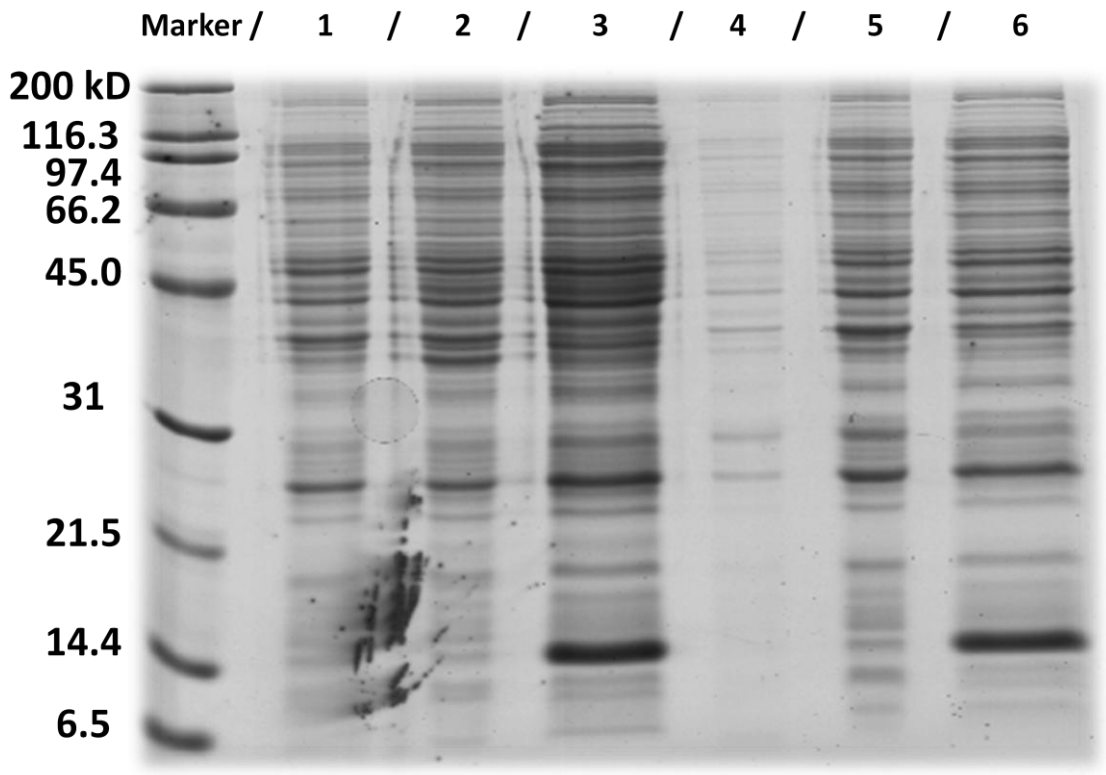


Figure 50 - Gel of the expression try out of Spindly fragments from amino acids 1 – 180 and 181 – 361. Lanes: 1 – Uninduced Fragment 1 – 180; 2 – Induced Fragment 1 – 180; 3 - Lysate Fragment 1 -180; 4 – Uninduced Fragment 181 – 361; 5 – Induced Fragment 181 – 361; 6 - Lysate Fragment 181 -361;

By analysis of Figure 51, it is possible to see that fragment 401 to 479 (expected size of 13,6 kD) is not expressing, but fragment 362 to 476 (expected size of

18 kD) is expressing, given that is possible to see a band with a higher intensity in the induced lane (lane 2) and in the lysate (lane 3), at around the expected size (a little higher, the band appears at 21,5 kD) of our protein.

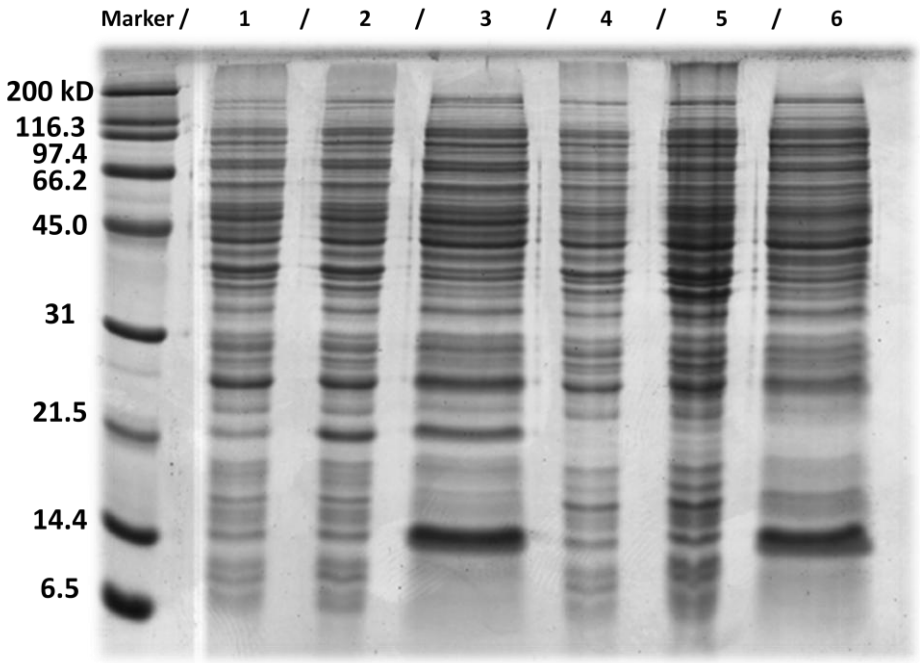


Figure 51 - Gel of the expression try out of Spindly fragments from amino acids 362 - 479 and 401 - 479. Lanes: 1 – Uninduced Fragment 362 - 479; 2 – Induced Fragment 362 - 479; 3 - Lysate Fragment 362 - 479; 4 – Uninduced Fragment 401 - 479; 5 – Induced Fragment 401 - 479; 6 - Lysate Fragment 401 - 479;

I continued with the purification of the 6xHis tagged Spindly fragment 362-479. By analyzing Figure 52, is possible to see that the protein is successfully bound to the Ni-NTA resin, as well as a protein of around 100 kD (lane 1 of figure 52). The protein was eluted from the resin (lane 3 of figure 29), but not totally (lane 2 of figure 52). In all the lanes is possible to see a faint band a little bit above the band of our protein. After desalting and concentration, the protein was loaded in the gel filtration column.

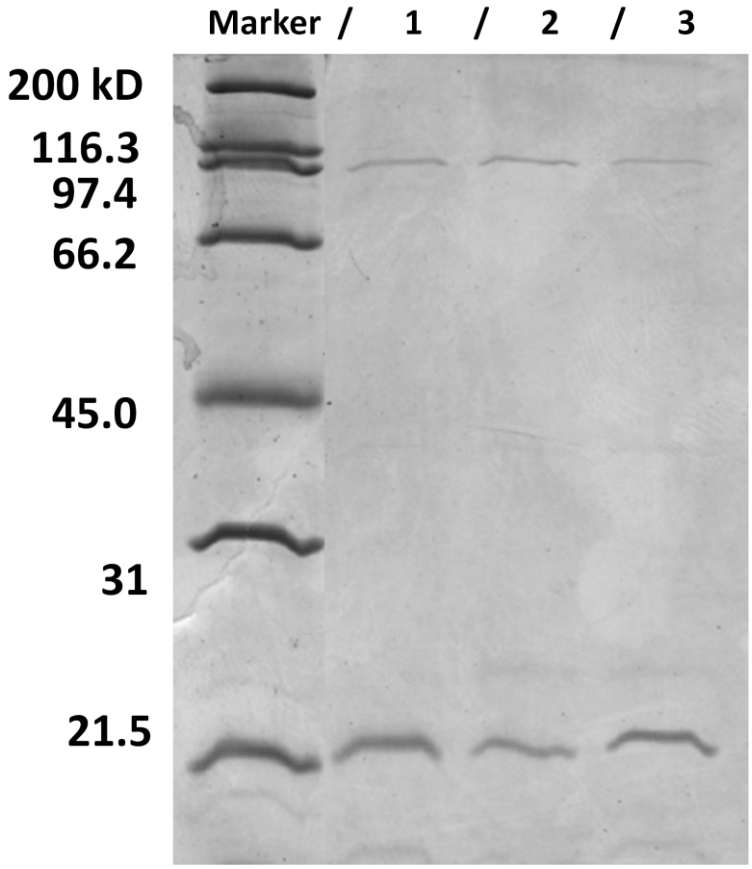


Figure 52 - Gel of the expression of 6xHis tagged Spindly fragment 362-479. Lanes : 1 – Ni-NTA resin before elution (1/16000 of the total); 2 – Ni-NTA resin after elution (1/16000 of the total); 3 – Elution (1/9600 of the total).

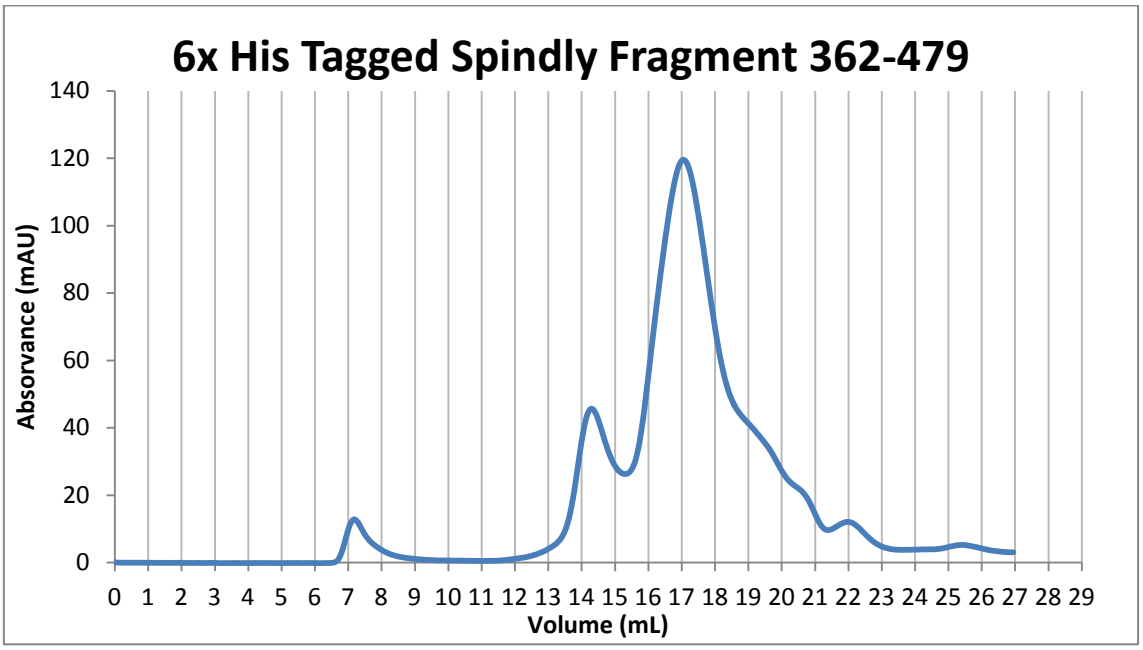


Figure 53 – Graphic of the purification of 6x His Tagged Spdl-1 fragment 362-479 by Gel Filtration Column.

By analyzing figure 53, it is possible to see two different major peaks. To understand what peak correspond to our protein, I runned the 0,5 mL fractions, after volume 14 mL, in a gel (Figure 54). In Figure 54 is possible to see that our protein (18 kD) is coming out of the column after lan 5 until lane 10, that correspond to the peak of volume 17 in Figure 53. I collected fractions from volume 16,5 to 18 and the concentration of the protein, by Bradford assay, is 1,1 mg/mL.

The major peak in Figure 53, is at 17 mL. Using this value, and knowing that the void volume is 7,5, the Kav of this protein is 2,27. Using the equation given on Figure 39, I estimated the molecular weight in which this protein comes out of the column, that is 54,7 kD. This size may be indicating that this protein is a trimer, since three times 18 kD equals 54 kD. So this data may indicate that spindly dimerizes/trimerizes through the C-terminal end.

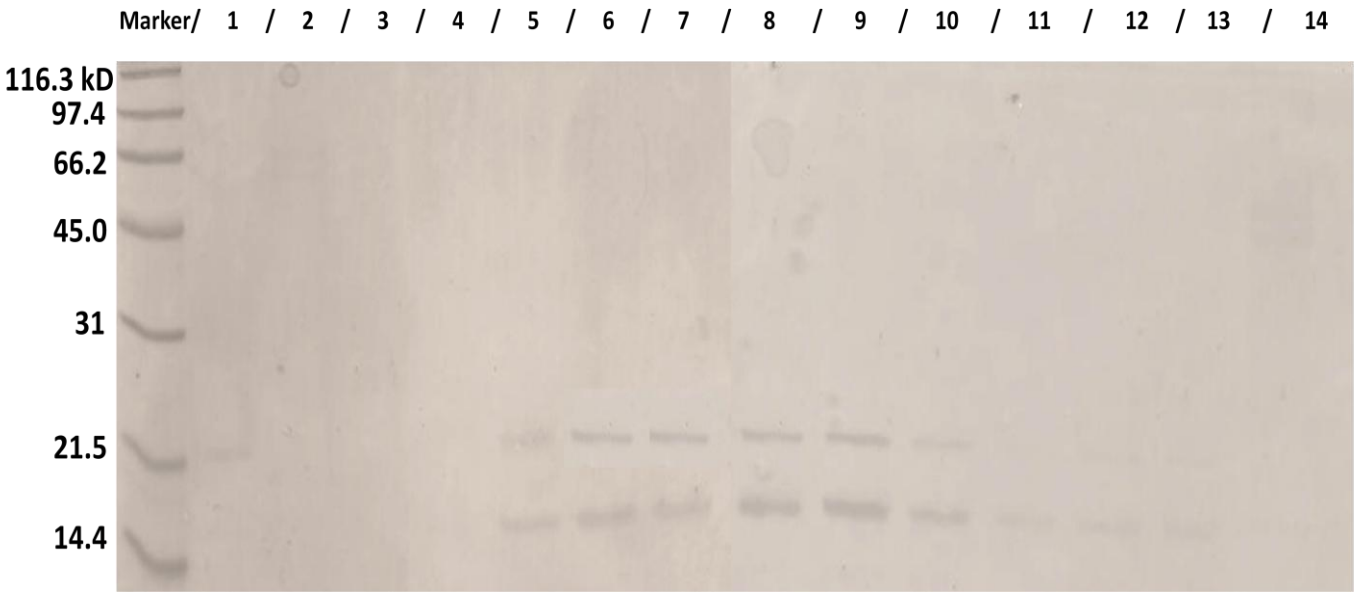


Figure 54 – Fractions of 6xHis tagged Spdl-1 fraction 362 – 479 collected in the gel filtration column. Lanes: 1 – 14 Factions collected after 14 mL, 0,5 mL Factions (1/70 of the total).

His tagged Spindly fragment aminoacids 1 to 361 in pRSET A (expected size 47.3 kD) was not successfully expressed as seen by analyzing Figure 55, given that I cannot see any band appearing at around the expected size after induction with IPTG.

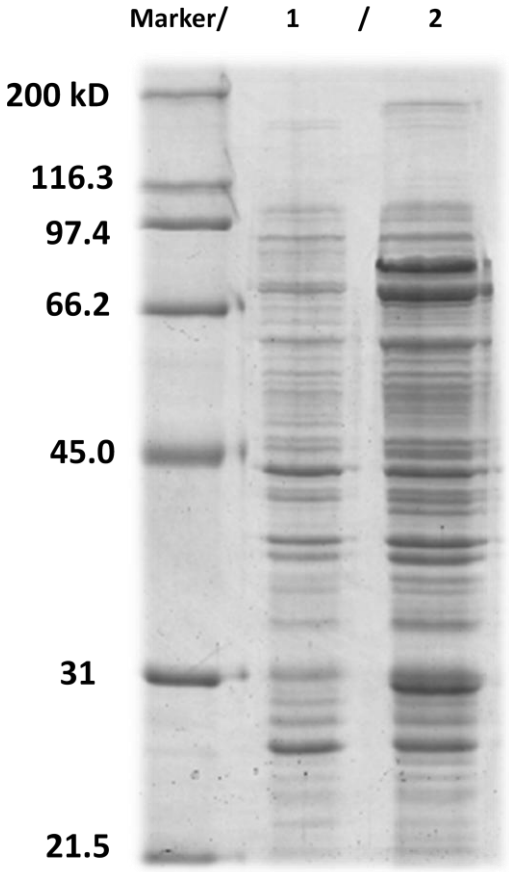


Figure 55 - Gel of the expression try out of Spindly fragment from amino acids 1 – 361 in pRSET A.
Lanes: 1 – Uninduced Fragment 1 – 361; 2 – Induced Fragment 1 – 361

In other approach, by insect cells protein expression, I tried to express and purify 6xhis tagged Spindly protein in pFastBacHta (66 kD). After a small scale induction, I ran a gel, comparing insect cells infected with virus V_0 with cells without virus (Figure 56). Comparing lanes 1 and 4 (Figure 56), is possibly to see a protein expressing at the correspondent size of our protein. To make sure that was in fact our protein that was expressing and not other protein like an heat shock protein, I did a western blot, with a antibody against spindly (Figure 57). By this assay I was able to prove that, in fact, the protein induced was spindly since it was labeled by the antibody, as the control. Nevertheless, by analyzing the gel, I can see that after centrifugation,

the protein appears in the pellet (lane 2) and not in the supernatant (lane 3), meaning that the protein is not soluble in this conditions.

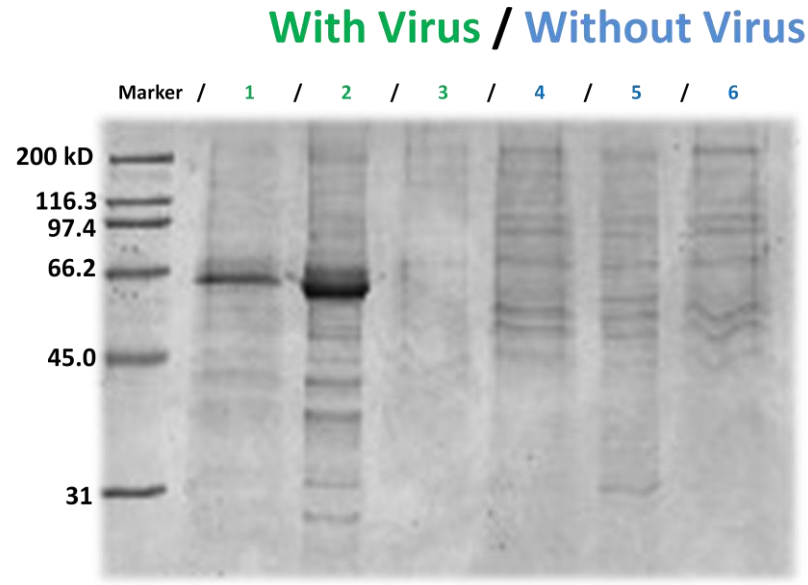


Figure 56 - Gel of the expression of his tagged Spindly in pFastBacHta. Lanes : 1 – Lysate sample with virus; 2 – Pellet sample with virus ; 3 – Supernatant sample with virus ; 4 – – Lysate sample without virus; 5 – Pellet sample without virus; 6 – Supernatant sample without virus. Loaded 1:5000 of the total in all the lanes.

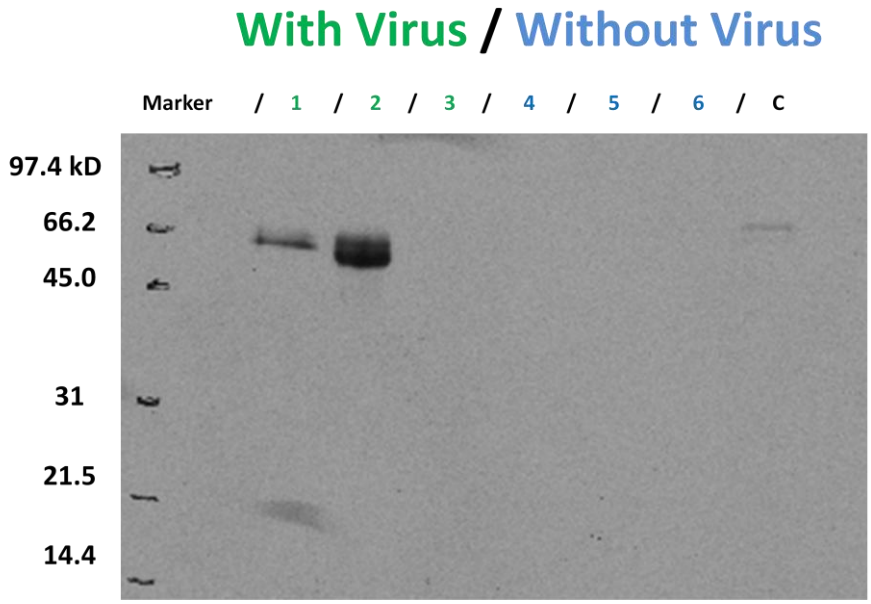


Figure 57 – Western Blot of the expression try out of Spindly in pFastBacHta with an antibody against Spindly. Exposion time of 1 second. Lanes : 1 – Lysate sample with virus; 2 – Pellet sample with virus ; 3 – Supernatant sample with virus ; 4 – – Lysate sample without virus; 5 – Pellet sample without virus; 6 – Supernatant

sample without virus; C – Positive control (Spindly protein, expressed in pGEX6P1). Loaded 1:5000 of the total in all the lanes.

The next step was to trying to solubilize the protein from the pellet to the supernatant. To do that, I lysed the sample in PBS plus 1% of Tween, that is a powerfull detergent capable of, in theory, solubilizing proteins. In Figure 58, the protein was still on the pellet (lane 2), and not on the supernatant (lane 3) or the beads (lane 4). Meaning that this protein, in these conditions, is insoluble, and not able to purify.

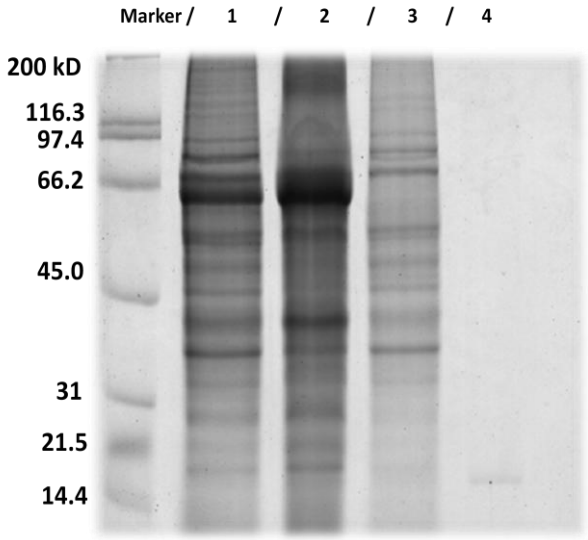


Figure 58 - Gel of the solubility try out of 6xhis tagged Spindly in pFastBacHta. Lanes : 1 – Lysate sample with virus; 2 – Pellet sample with virus ; 3 – Supernatant sample with virus ; 4 – Ni-NTA resin ; Loaded 1:5000 of the total in all the lanes.

4.4. RZ Complex

As indicated in table 1, I made 3 different constructs to express the “RZ complex”: Gst tagged Rod-1 (aa. 1-418), 73,3 kD, plus zwl-1, 71,1 kD, in pST39 and Rod-1 (aa. 1-418), 47 kD, plus 6xHis tagged Zwl-1 (wild type and mutant), 71,4 kD, in pACYDuet-1.

By analyzing Figure 59, it is possible to see that none of this 3 constructs expressed the desire proteins. To take the system to the limit, I did a expression try out by leaving the system expressing proteins, after IPTG addition, at 37 ºC. Even so, it was not possible to see any expression (Data not shown).

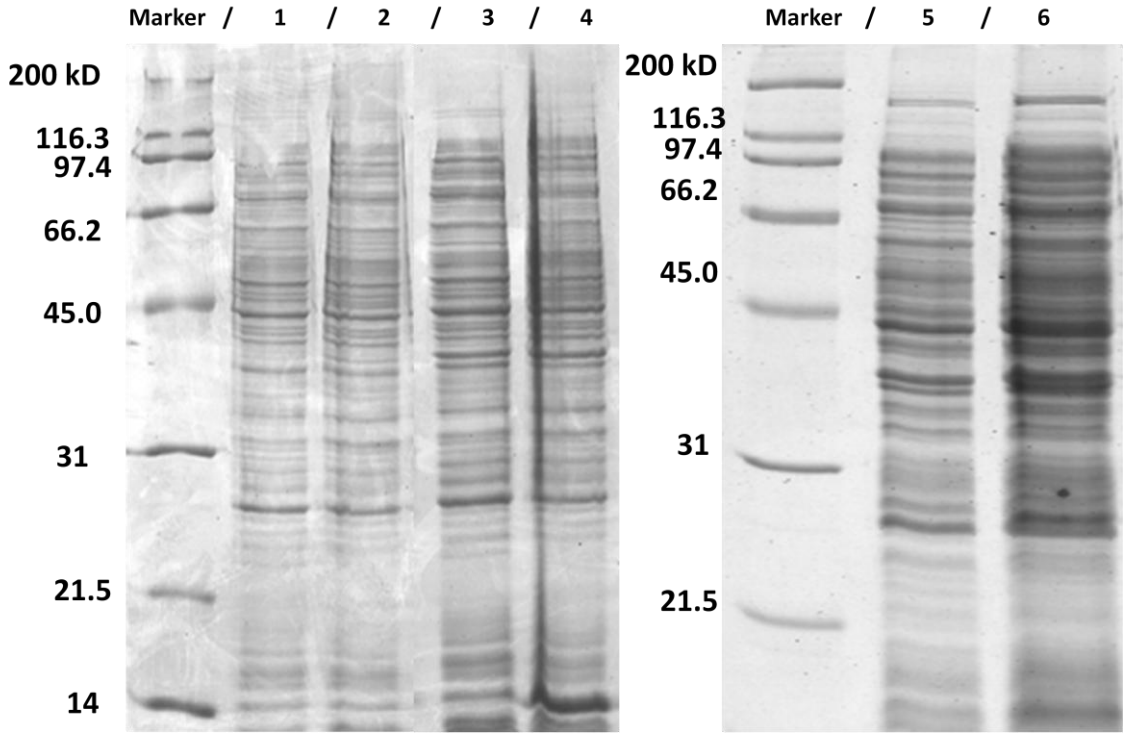


Figure 59 - Gel of the espression try out of the RZ Complex constructs. Lanes: 1 – Uninduced Rod-1 (aa. 1-418) plus 6His::Zwl-1 wild type in pACYDuet-1; 2 – Induced Rod-1 (aa. 1-418) plus 6His::Zwl-1 wild type in pACYDuet-1; 3 – Uninduced Rod-1 (aa. 1-418) plus 6His::Zwl-1 mutant in pACYDuet-1; 4 – Induced Rod-1 (aa. 1-418) plus 6His::Zwl-1 mutant in pACYDuet-1; 5 – Uninduced Gst::Rod-1 (aa. 1-418) plus zwl-1in pST39 ; 6 - Induced Gst::Rod-1 (aa. 1-418) plus zwl-1in pST39.

4.5. GST purification

To obtain GST, important for the pull down assays (see below) we expressed pGEX6P1 empty. In figure 60, in the induced fraction (lane 2) is possible to see a increase of the expression of a protein at around the size of the expected protein (30 kD). In the end, it was possible to elute a pure protein (lane 7) from the agarose beads, with no need to extra purification steps. The concentration of the purified protein, measured by Bradford Assay, is 1,087 mg/mL.

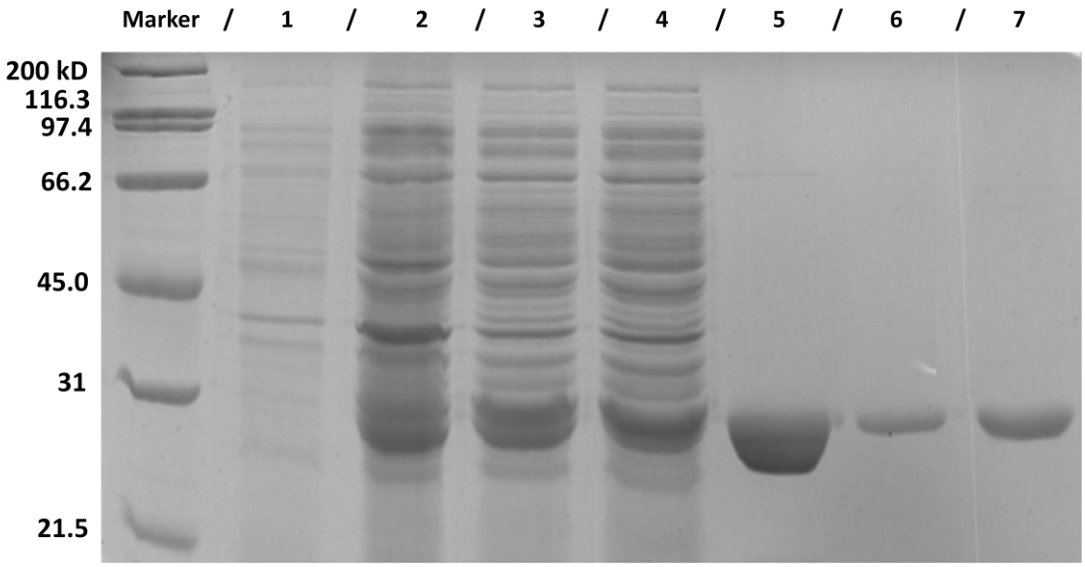


Figure 59 – Gel of the expression of GSTfrom pGEX6P1. Lanes : 1 – Uninduced; 2 – Induced ; 3 – Lysate (1/2700 of the total); 4 - Supernatant (1/2700 of the total); 5 - Glutathione Agarose Beads before elution (1/1250 of the total) ;); 6 - Glutathione Agarose Beads after elution (1/1250 of the total) ; 7 – Elution (1/1600 of the total).

4.6. Pull down assay

In this pull down assay the goal is to identify if in fact I can see an interaction between Spindly and Spindly fragments in order to identify the part of spindly that is responsible of the dimerization of the protein. By gel filtration I already suspect that the part responsible for the dimerization is the C-terminal fragment comprising amino acids 362 to 479. For that I used full length untagged Spindly and GST tagged fragments. The interactions I tested are:

Reaction 1: 1.5 µg GST + 3 µg SPDL-1 (cleaved)

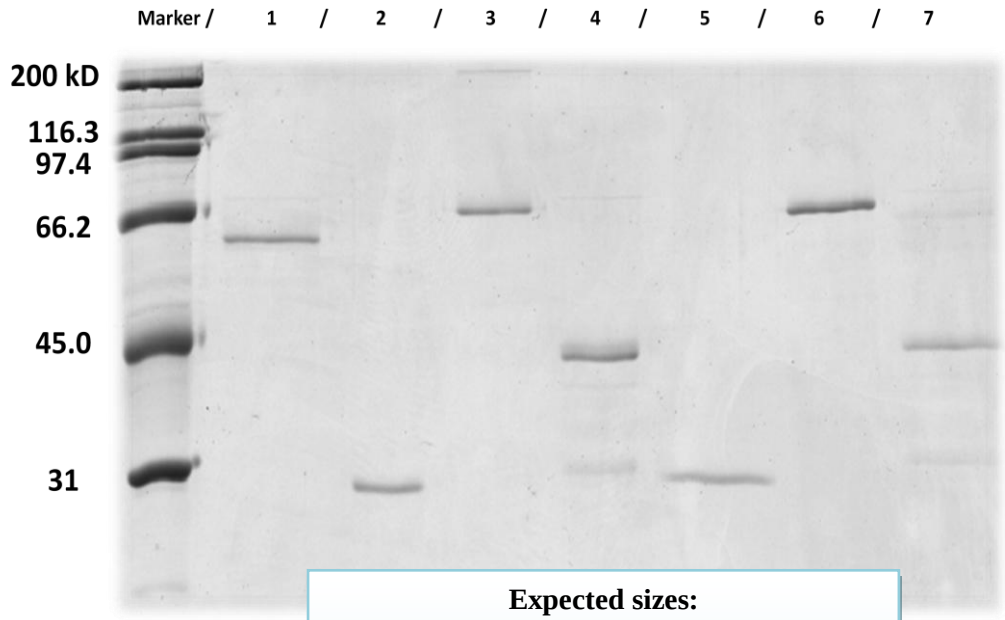
Reaction 2: 3.7 µg GST::SPDL-1(1-361) + 3 µg SPDL-1 (cleaved)

Reaction 3: 2.2 µg GST::SPDL-1(362-479) + 3 µg SPDL-1 (cleaved)

The amounts in µg take into account the relative molecular weights of the recombinant proteins such that both components in a reaction are equimolar. The first reaction is a negative control, to make sure that spindly doesn't interact with

GST alone. The others two reactions will tell us what part of the protein is responsible for the dimerization. If the proteins interact with each other, they should appear both in the gel, indicated that they were eluted together from the agarose beads.

In Figure 60, by analysis of lane 7, is possible to see that spindly full length interacts with GST Spindly fragment 362-479, since it was possible to see a band at around 45 kD that corresponds to GST Spindly fragment 362-479 (and also the faint band at 30, that is also visible in lane 4 - GST Spindly fragment 362-479 alone) and a faint band at 62,4 kD (visible in lane 3) that corresponds to Spindly full length. So, this data, indicates that C-terminal end of Spindly interacts with full length Spindly, meaning that this end is responsible for the possible dimerization of the protein.



Expected sizes:

GST – 30 kD; Spdl-1 – 62,4 kD; GST::Spdl-1 (1-361) – 68,9 kD; GST::Spdl-1 (362-479) – 40 kD.

Figure 60 – Pull down assay with Spindly and it fragments. Lanes : 1 – SPDL-1(1 µg); 2 – GST (0,5 µG); 3 - GST::SPDL-1 fragment 1-361 (1.2 µg); 4 - GST::SPDL-1 fragment 362-479 (0.73 µg); 5 – Elution from Reaction 1 (1/33 of the total); 6 - Elution from Reaction 2 (1/33 of the total); 7 – Elution from Reaction 3 (1/33 of the total).

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5. Conclusion and Future Directions

With this work, by Yeast two Hybrid, I was able to prove that Zwl-1 (*C. elegans* Zwilch) interacts with the first 273 amino acids of Rod-1 (*C. elegans* Rod) and that cZw10 (*C. elegans* Zw10) interacts with Rod-1 between the amino acids 500 and 1203. This was an important discovery that corroborates with the previous discoveries about the RZZ complex(Williams, Karr et al. 1992; Karess 2005; Civril, Wehenkel et al. 2010) and allowed the prediction of what region of the Rod-1 proteins interacts with Zwl-1 and wit Zw10. I was also able to prove that Spindly interacts with itself, to for a dimer. By Yeast two Hybrid I saw that Spdl-1 (*C. elegans* Spindly), interacts with itself, and, by a pull down assay, with purified recombinant proteins, I saw that the fragment of Spdl-1 that is responsible for this interaction in the C-terminal fragment comprising amino acids 362-479. This is a important and novel discovery about this protein, that indicates that Spindly dimerization is parallel and not antiparalel. I was also able to prove that, after mutating Zwilch, spindly localization is lost but RZZ organization remains intact.

One of the goals of my project was to prove, *in vitro*, the existence of an interaction between Zwilch and Spindly. This goal was not succeeded , since I was not able to express and purify Zwilch. For future work it will be important to try to create new constructs that will allow the analysis of this question, of try other approaches like expressing Zwilch in a new host like culture human cells. It will also important to do a pull down assay with the purified C-terminal 6xHis tag spindly. Other techniques, like Sucrose Gradient, may add to this work more accurate information about RZZ behavior in vitro.

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