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Biologia Molecolare e Applicata

Produzione e caratterizzazione biochimica/funzionale della proteina Pin1 umana per l'identificazione di nuovi partner molecolari.

Biochemical and functional characterization of human Pin1 for protein-protein interaction studies.

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A mia madre, che mi ha insegnato ad essere forte nel rialzarmi dopo ogni caduta e che ha sempre creduto in me nonostante io non lo facevo.

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Infine a me stessa, che nonostante tutte le difficoltà incontrate sono giunta alla fine di questo lungo percorso.

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1 INTRODUCTION

Peptidyl-prolyl Isomerases (PPIase) catalyse the cis-trans isomerization of peptidyl-prolyl bonds, which affects the conformation of the protein backbone. Proline exists in two different conformations, *cis* and *trans*, and the spontaneous interconversion between both conformations is slow. Three phylogenetically conserved families of PPIases are known: cyclophilins, FK506-binding proteins and parvulins. To this latter subgroup belongs the peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (Pin1)[1].

Pin1 catalyses the cis–trans isomerization of peptide bonds between phosphorylated serine-proline or threonine-proline motifs (pSer/Thr-Pro) in proteins[2], different from the other two PPIase families that target unphosphorylated substrates[3]. Pin1 is ubiquitously expressed; its structure comprises two domains connected by a flexible linker (Figure 1.1). In details, Pin1 consists of an N-terminal domain named "WW" domain, which targets pSer/Thr-Pro motifs in a sequence-dependent manner, and a C-terminal PPIase domain, which exerts the catalytic activity[4].

[1] Prolyl isomerase Pin1 in cancer, Zhimin Lu and Tony Hunter, 2014.
[2] Prolyl isomerase Pin1: a promoter of cancer and a target for therapy, Yang Chen et al, 2018.
[3] Pin1 in Alzheimer's disease, D. Allan Butterfield et al, 2006.
[4] A guide to Pin1 function and mutations across cancers, Maguie El Boustani et al, 2019.

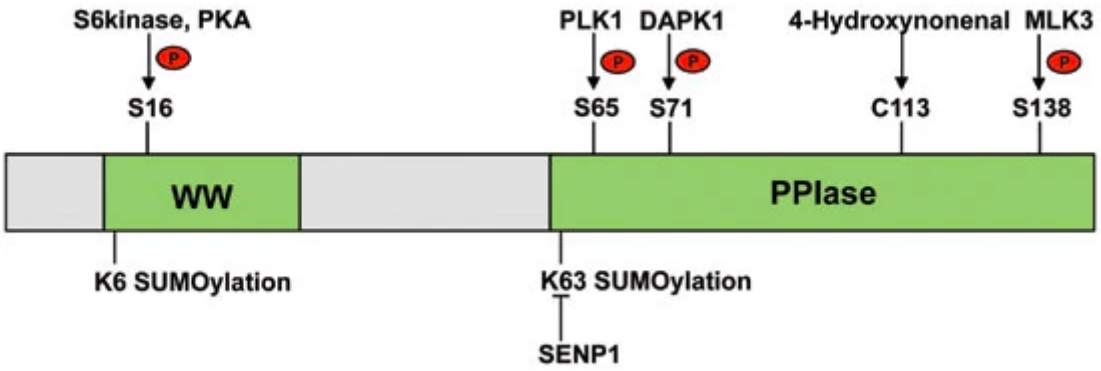


Figure 1.1[5]: Structure of Pin1

Pin1 is involved in a large number of pathways and as a consequence it has been implicated in the pathogenesis and progressions of several diseases. Indeed, protein phosphorylation is responsible for the activation of signalling cascades that control cell differentiation and survival. In particular, proline-directed protein phosphorylation is implicated in mechanisms that mainly control cell proliferation and transformation, and its dysregulation is thus responsible for the onset of many human cancers[6].

1.1 Key role of Pin1 in different cellular pathways

The function of Pin1 has shown a pleiotropic and context-dependent nature, depending on the phosphorylation patterns of its heterogeneous cellular targets. Pin1 is involved in both physiological and pathological conditions; it guarantees a homeostatic equilibrium essential to maintain the ability of cells to transduce stimuli and to trigger subsequent responses. On the other hand, a number of

[5] Prolyl isomerase Pin1: a promoter of cancer and a target for therapy, Yang Chen et al, cit.
[6] Prolyl isomerase Pin1 in cancer, Zhimin Lu and Tony Hunter, cit.

pathological conditions are exacerbated by the dysregulation of Pin1, which redirects cellular processes towards sustaining the pathological mechanisms[7].

In particular, Pin1 is regarded as a mitotic regulator of checkpoint mechanisms during the cell cycle, but it also interacts with non-nuclear targets, thereby participating in other cellular mechanisms like apoptosis, endocytosis, protein translation, maintenance of the cytoskeleton, and neuronal functions[8].

Pin1 is expressed in a variety of human tissues, such as the central and peripheral nervous system. In particular, it is found in mitochondrial membranes, neurons cytosol, dendrites, and distal axons. The expression of Pin1 is mainly detected during neurodevelopmental stages regulating the cortical differentiation of Neuron Progenitor Cells (NPC). At the base of this mechanism seems to be an interplay with β-catenin, which is identified as the major Pin1 substrate in NPC. Here, Pin1 binds and stabilizes the β-catenin conformation at late stage during brain development[9]. In fact, the Wnt/β-catenin pathway exerts a well-established role in neurogenesis, and it depends on the developmental stage during brain development. This pathway promotes the differentiation of NPC during the early stage (neurogenic phase), and induces their differentiation during the late neurogenic phase, determining the size of final cell population and also the timing of differentiation[10].

[7] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, 2020.
[8] Ivi.
[9] Ivi.
[10] Prolyl isomerase Pin1 regulates neuronal differentiation via β-catenin, Kazuhiro Nakamura et al, 2012.

It has been demonstrated that Pin1 triggers both pro-survival and pro-apoptotic pathways, in adult and developing neurons, respectively[11].

Neuronal apoptosis is a fundamental process of the nervous system development. In the beginning, an excess of neurons initiate development, to which follows a consistent apoptosis stage that guarantees the formation of correct neural circuits. At the same time, apoptosis is also involved in the onset and pathogenesis of several common diseases, like ischemia and motor neuron diseases. In these settings, the BH3-only protein BIMel exerts a crucial role in the regulation of neuronal apoptosis following trophic factor deprivation, and also the activation of p75 cell death receptor. Moreover, BIMel is also implicated in Alzheimer's Disease and ischemia. Pin1 interacts with the phosphorylated serine 65 of BIMel via the WW domain, protecting BIMel from the proteasomal degradation by avoiding the link with E3 ubiquitinase. This way, BIMel is stabilized and protected against proteasomal degradation, and the mitochondrial cell death pathways is activated[12].

[11] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, cit.
[12] Pin1 in neuronal apoptosis, Esther B.E. Becker and Azad Bonni, 2007.

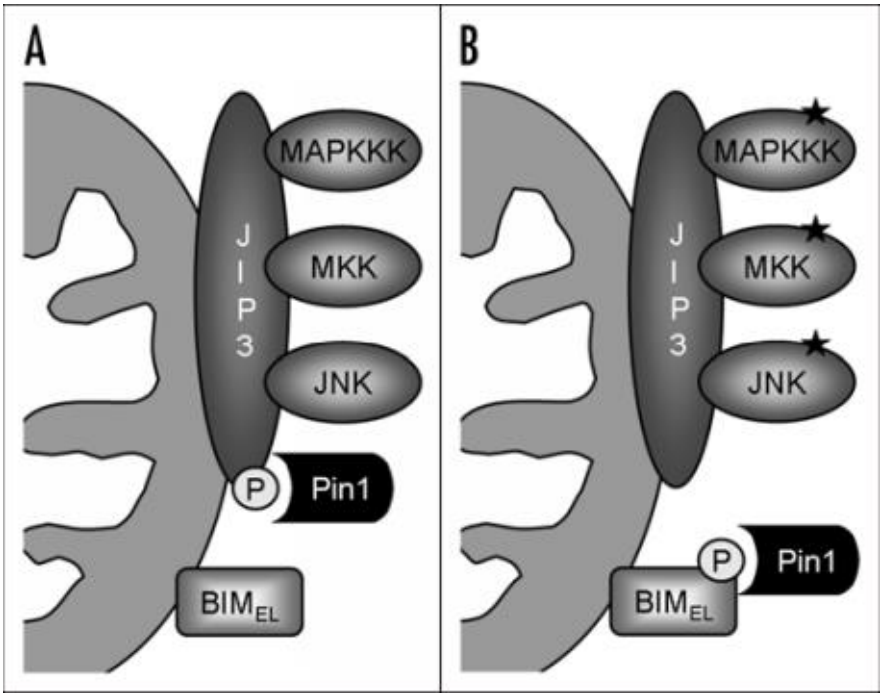


Figure 1.2[13]: Neural-specific Pin1-mediated activation of BIMEL following JNK-induced phosphorylation of BIMEL at serine 65.

Pin1 also interacts with the p53 tumor suppressor protein, activating its active pro-apoptotic conformation and thereby promoting cell death[14]. p53 has a transcriptional role in the nucleus, but it can also induce cell death in the cytosol in a transcription-independent manner. This latter function is mediated by the activation of the apoptotic effector proteins BAK and BAX, which then oligomerize in the outer mitochondrial membrane, modify the membrane integrity, and induce apoptosis by promoting the release of cytochrome c. Pin1 promotes the p53-dependent BAX activation by catalysing the cis-trans

[13] Ivi.
[14] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, cit.

interconversion of p53 Pro47. This cooperation between p53 and Pin1 seems to integrate the cell stress signal, inducing a direct apoptosis response[15].

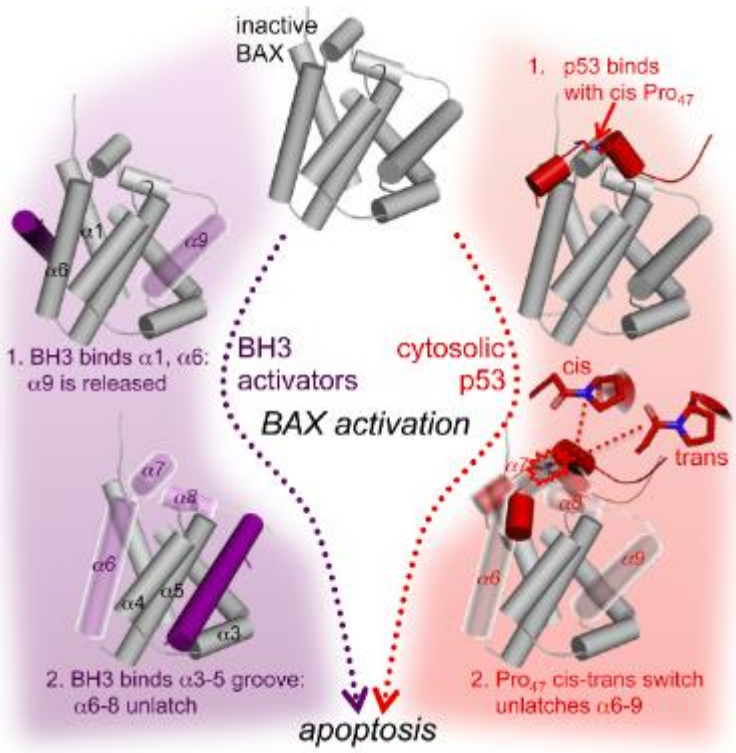


Figure 1.3[16]: Activation of BAX; Isomerization of p53Pro47

In the nervous system, Pin1 seems to be involved in the regulation of both excitatory and inhibitory synaptic transmission. It is located in the dendrites of glutamatergic synapses, where it is catalytically active, and inhibits protein synthesis under physiological conditions. The synthesis of new proteins is

[15] Pin1-induced proline isomerization in cytosolic p53 mediates BAX activation and apoptosis, Ariele Viacava Follis et al, 2015.
[16] Ivi.

essential for the maintenance of long-term synaptic plasticity, as the Long-Term Potentiation (LTP).

It has been shown that Pin1 interacts with PSD-95 at the post-synaptic terminal. PSD-95 shows a multimodular structure constituted by three PDZ domains, a Src homoly 3 domain, and a catalytically inactive guanylate kinase domain. PSD-95 is able to bind both NMDA and AMPA glutamatergic receptors at the post-synaptic site[17]. This protein acts as a scaffold anchoring NMDA receptor via GluN2-type receptor subunit. The interaction between Pin1 and PSD-95 at the level of a specific Ser/Thr-Pro consensus motif, which is located at the linker region between PDZ2 and PDZ3 domains induces conformational changes, so that the interaction with the NMDA receptor is prevented, and these receptors are downregulated[18].

On the other hand, the impact of Pin1 on GABAergic transmission is related to the conformational alterations of the adhesion molecule Neuroligin2 (NL2). NL2 is phosphorylated at the Ser714-Pro715, thus avoiding the interaction with Gephyrin and subsequently downregulating the GABAergic transmission[19].

[17] Pin1 modulates the Synaptic Content of NMDA receptors via Prolyl-isomerization of PSD-95, Roberta Antonelli et al, 2016.
[18] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, cit.
[19] The role of prolyl-isomerase PIN1 in GABAergic and glutamatergic synaptic transmission, Roberta Antonelli, 2015.

1.2 Role of Pin1 in neurodegenerative diseases

Pin1 has an important role in the onset of neurodegenerative diseases. In Alzheimer's disease, the processing and phosphorylation of Amyloid Precursor Protein (APP) and the hyperphosphorylation of Tau protein are increased. These two conditions lead to the production of β-amyloid peptides and neurofibrillary tangles, respectively substrates[20]. In the brain of AD patients, Pin1 is oxidated and mainly located at neuronal cytoplasm and perikaryal NFTs[21]. In these patients, Pin1 binds the phosphorylated Thr231 residue of Tau protein, being redirected to the cytoplasm of NFT-containing neurons and subsequently promoting the decreased availability of soluble Pin1 protein. This depletion of nuclear Pin1, together with the up-regulation of phosphoprotein targets (p53 and Bcl-2) can be considered at the base of neurodegeneration and neuronal apoptosis in AD patients[22].

[20] Pin1 in Alzheimer's disease, D. Allan Butterfield et al, cit.
[21] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, cit.
[22] Pin1 in Alzheimer's disease, D. Allan Butterfield et al, cit.

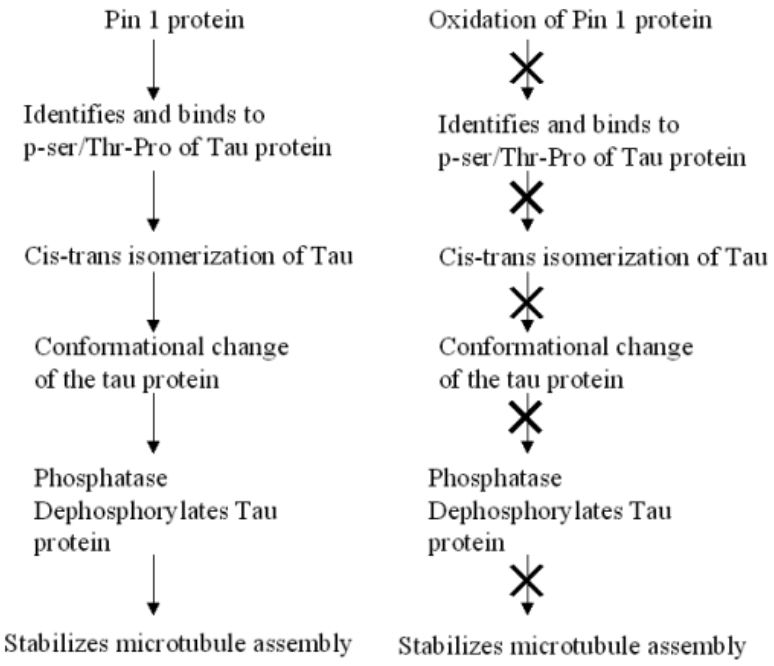


Figure 1.4: Putative roles of Pin1 (dx) and effects of oxidation on its functionality.

A putative role of Pin1 in Parkinson Disease is being investigated. This neurodegenerative disease is characterized by the loss of dopaminergic neurons caused by the formation of Lewy Bodies, cytoplasmic inclusions constituted by α-synuclein[23]. Ryo and collaborators demonstrated that in PD patients, Pin1 accumulates into Lewy Bodies and also colocalizes with α-synuclein inclusions. In particular, Pin1 overexpression seems to enhance the half-life and the insolubility of α-synuclein. Moreover, Pin1 binds the synphilin-1 precisely at Ser-211-Pro and Ser-215-Pro motifs, increasing its interaction with α-synuclein and thus promoting the formation of inclusions.

[23] Prolyl-isomerase Pin1 accumulates in Lewy bodies of Parkinson disease and facilitates formation of α-synuclein inclusions, Ryo, Akihide, et al, 2006.

In addition, Pin1 seems to be also implicated in a toxic feedback loop which promotes the expression of mutated huntingtin protein in Huntington Disease. The disorder is characterized by the CAG repeat expansion in the gene coding for the huntingtin protein, as well as by the massive loss of medium spiny neurons in striatum. The loop at the base of the disease involves the Pin1-activated p53, which under severe or persistent stress is phosphorylated at Ser46. This way, the p53 apoptotic potential is activated increasing the production of the mutated protein, in response to which the Pin1/p53 pathways induces neuronal apoptosis[24].

These findings suggest that, under normal conditions, Pin1 is responsible for the regulation of a number of signalling pathways, maintaining the homeostasis and providing for the response to different stimuli. On the other hand, when dysregulated or imbalanced, it is involved in different pathological mechanisms.

1.3 Pin1 and the hallmarks of cancer

Cancer is characterized by some distinctive features that are considered hallmarks because they are found in virtually every cancer type. There are mainly ten hallmarks, and Pin 1 seems to be involved in all of them (Figure 1.5):

1. Sustaining proliferative signalling
2. Evading growth suppressors
3. Activating invasion and metastasis

[24] The peptidyl-prolyl isomerase Pin1 in neuronal signaling: from neurodevelopment to neurodegeneration, Francesca Fagiani et al, cit.

4. Enabling replicative immortality
5. Inducing angiogenesis
6. Resisting cell death
7. Evading immune destruction
8. Tumour-promoting inflammation
9. Reprogramming of energy metabolism
10. Genome instability and mutation

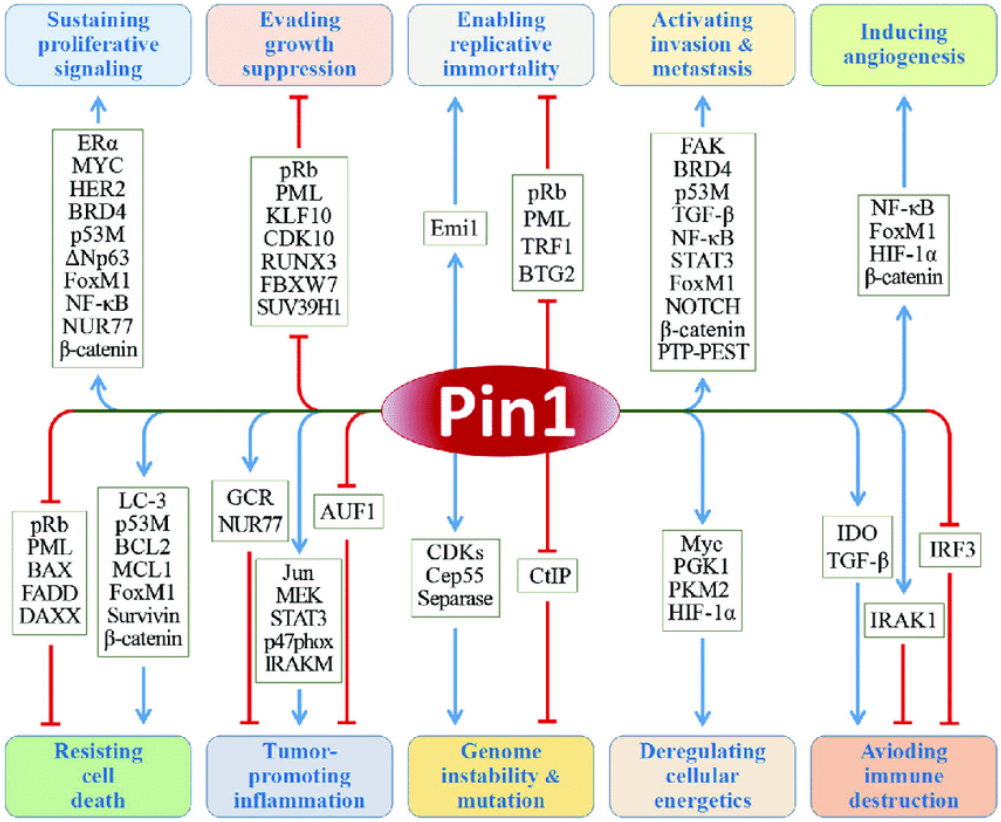


Figure 1.5[25]: Role of Pin1 in different hallmarks of cancer.

[25] Prolyl isomerase Pin1: a promoter of cancer and a target for therapy, Yang Chen et al, cit.

Cancer cells are characterized by unregulated proliferation. Pin1 interacts with Cyclin D1, which is responsible for the cell cycle progression and also allows the proliferation of cancer cells. The interaction, and subsequent Pin1-mediated isomerization, lead to an accumulation of this cyclin, promoting the cell cycle progression and thus cell proliferation. Pin1 also interacts with the Estrogen Receptor α (Erα) that promotes the progression of cancer by stimulating the expression of estrogen response element (ERE)-containing genes. The transcription activity is amplified by the activity of Pin1, as well as the ERE binding affinity, and the inhibition of E3 ligase E6AP-induced degradation of Erα in breast cancer. Moreover, Pin1 activates the NfKB pathways, increasing the cell proliferation. This pathway is activated by enhancing the nuclear accumulation of RelA/p65, c-Rel and v-Rel. Finally, Pin1 inhibits the E3 ligase WWP1-induced ubiquitination of ΔNp63, an isoform of p63 that lack the N-terminal transactivation domain. This way, it increases the proliferation of human oral squamous cell carcinoma[26].

On the other hand, Pin1 inhibits the activity of different tumour suppressors, which normally limit cell growth and proliferation, but are generally bypassed in cancer cells. The tumour suppressor Promyelocytic Leukemia (PML), involved in apoptosis and DNA damage repair, is bound by Pin1 at Ser403 and Ser505, inducing breast cancer. Pin1 also stabilizes the oncogenic fusion PML-RARα, decreasing the anti-proliferative activity of ATRA in AML[27]. Somewhat, also the

[26] A guide to Pin1 function and mutations across cancers, Maguie El Boustani et al, cit.
[27] Prolyl isomerase Pin1 in human cancer: function, mechanisms, and significance, Wenchen Pu et al, 2020.

inactivation of Rb is attributed to Pin1: it promotes the CDK-induced phosphorylation and inhibits the PP2A-mediated dephosphorylation of Rb, which then induces the release of E2F and finally the transition to S phase[28].

Metastatic cancers show a higher level of Pin1, compared with primary tumours. In particular, the SMAD proteins are the major downstream adapters of TGF-β signal, and they are well recognized by WW domain-containing proteins, like Pin1. In the same way, Pin1 induces the activation of the NOTCH pathway, starting a loop mechanism: NOTCH1 induces the expression of Pin1, that enhances cell transformation. Pin1 also activates NOTCH3 signal via its cleavage, thus stabilizing its intracellular domain[29]. Metastases are largely influenced by Ras and STAT3 signalling. Ras-induced cell migration, invasion, and metastasis are promoted by the interaction between Pin1-isomerized PTP-PEST and FAK Tyr397, which is then dephosphorylated. In addition, Pin1 interact with STAT3 to promote its transcriptional activity and target gene expression, as well as the recruitment of p300 inducing the epithelial-mesenchymal transition of MCF-7 cells[30].

Angiogenesis is that process that occurs both naturally and pathologically, as occurs in cancers. This process leads to the formation of new blood vessels, that have the main role of providing nutrients and oxygen to tumour cells, and at the same time discharging waste products and carbon dioxide. Pin1 interacts with the

[28] A guide to Pin1 function and mutations across cancers, Maguie El Boustani et al, cit.
[29] Ivi.
[30] Prolyl isomerase Pin1 in human cancer: function, mechanisms, and significance, Wenchen Pu et al, cit.

Hypoxia-inducible factor 1α, which is the responsible for the expression of genes involved in angiogenesis. The interaction occurs both at endogenous and exogenous level, stabilizing the protein in colon cancer cells, also thanks to the ubiquitin-dependent degradation of its inhibitor. Pin1 also induces the upregulation of the VEGF factor, which is the major player in the angiogenesis process[31].

Cells proliferation is generally characterized by the Hayflick Limit, which represent the maximum number of possible replication cycles. Once overpassed this limit, cells can undergo fatal crisis and death, or can become immortal, as occurs in cancer cells. This mechanism is controlled by telomere shortening, DNA and mitochondrial damage, and in these settings Pin1 showed an antisenescence function in cancer cells. In detail, Pin1 promotes the degradation of telomeric repeat-binding factors, responsible for the telomere elongation. In addition, it prevents the degradation of Early Mitotic Inhibitors 1, which normally induces the senescence induced by DNA damages. Finally, it suppresses the senescence-inducing factors pRb and PML[32].

Cancer cells are also characterized by the "Warburg effect", a particular form of metabolism which provides a rapid biosynthesis through an increased glucose import, aberrant hypoxia response system, and the incapacitation of oxidative phosphorylation. All these mechanisms are under control of aberrant signals as

[31] Prolyl isomerase Pin1 in human cancer: function, mechanisms, and significance, Wenchen Pu et al, cit.
[32] Prolyl isomerase Pin1: a promoter of cancer and a target for therapy, Yang Chen et al, cit.

PI3K/Akt, Wnt/β-catenin, HF-1α, and non-coding RNAs. Regarding Pin1, it is a co-activator of β-catenin increasing the expression of CCDN1 and c-Myc, which in turn induces the expression of glucose transporter 1 and lactate dehydrogenase A, promoting the Warburg effect[33].

Finally, Pin1 also promotes the ubiquitin-dependent degradation of CtIP, a protein involved in the homologous recombination. In this way, the homologous recombination is attenuated, while the non-homologous recombination is facilitated, increasing genome instability. In addition, the overexpression of Pin1 leads to an abnormal centrosome duplication and chromosome instability[34].

1.4 Pin1 in colorectal cancer

A number of molecular mechanisms are at the basis of the initiation and progression of colorectal cancer. A prominent role is played by the Wnt signalling pathway, and one of the key effectors of this route is the β-catenin, which is also involved in intercellular adhesion by linking to the cytoplasmic domain of E-cadherin. Adenomatous polyposis coli (APC) controls levels of β-catenin by both inducing its degradation, and by shuttling it out of the nucleus. In the presence of APC alterations, it cannot bind β-catenin, which starts to accumulate and migrate to nucleus. Here, β-catenin binds to transcription factors, T cells, and the lymphoid enhancer factor (TCF/LEF), leading to the overexpression of some

[33] Ivi.
[34] Ivi.

oncogenes, such as cyclin D1 and c-Myc. APC is mutated in about 80% of colorectal cancers, in which the increased nuclear transcription of cyclin D1 is also associated with a reduced survival of patients affected[35].

In these settings, an overexpression of Pin1 decreases the turnover of β-catenin, which results to be present in high amount. Conversely, the absence of Pin1 leads to a decrease of β-catenin level. This condition results from the binding of Pin1 to the phosphoSer246 of β-catenin, which inhibits the interaction with APC and the subsequent β-catenin degradation [Pin1 Competes with APC for β-Catenin[36].

In addition, Pin1 regulates the stability and localization of cyclin D1, isomerizing its phospho Thr286-Pro site and stabilizing them[37].

1.5 Pin1 as a therapeutic target

A large variety of signalling cascades are at the basis of cancer development, therefore novel therapies are being designed to specifically target crucial factors involved in multiple pathways. Because Pin1 is involved in several signalling pathways, it is regarded as a highly suitable drug target[38].

Pin1 shows high substrate specificity, which facilitates the design of specific inhibitors. Notably, Pin1 is over-expressed in many tumours, and its abundance

[35] High Pin1 Expression Is Associated with Tumor Progression in Colorectal Cancer, Junichi Kuramochi et al, 2006.
[36] https://www.science.org/doi/10.1126/stke.2001.99.tw321
[37] High Pin1 Expression Is Associated with Tumor Progression in Colorectal Cancer, Junichi Kuramochi et al, cit.
[38] Targeting Pin1 for Modulation of Cell Motility and Cancer Therapy, Hsiang-Hao Chung et al, 2021.

correlates with a negative prognosis. Moreover, inhibition of Pin1 via antisense sequences or dominant-negative mutants sequences causes mitotic blockade and apoptosis of transiently transfected cells[39]. Finally, Pin1 knockout mice have shown normal development, suggesting that a therapy targeting Pin1 might have no general toxic effects[40].

Aurora kinase A, Ribosomal S6 kinase 2 and Protein kinase A phosphorylate the Ser16 residue in the WW domain of Pin1, reducing its specificity for the substrate and the subsequent interaction. The death-associated kinase 1 indeed, by phosphorylating the Ser71 in the catalytic site, inactivates Pin1, also inhibiting the centrosome amplification of breast cancer cells. Pin1 is also regulated by miRNAs. In particular, some targeted microRNAs as miR-200b, miR-200c and miR-370, inhibit the human cancer progression decreasing the mRNA level of Pin1. Moreover, a number of small-molecule have been designed as Pin1 inhibitors. An example, is the ATRA: it interacts with the substrate-binding site of Pin1 inhibiting its activation. It is mainly used in Acute Promyelocytic Leukaemia (APL) and in breast cancers. Another compound which interacts with the catalytic core of Pin1 inducing its degradation is the KPT-6566. In addition, the inhibition of Pin1 results to be effective to render many cancer cells more sensitive to the chemotherapy[41].

[39] Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1, Gerburg M. et al, 2001.
[40] Prolyl isomerase Pin1 as a molecular target for cancer diagnostics and therapeutics, Kung Pin Lu, 2003.
[41] Prolyl isomerase Pin1: a promoter of cancer and a target for therapy, Yang Chen et al, cit.

1.6 The circadian rhythm and the molecular clock

The term "circadian rhythm" describes endogenous oscillations with a period of ca. 24-h associated with the earth's daily rotation and light/dark cycle. Such rhythms, observed in organisms ranging from photosynthetic prokaryotes to higher eukaryotes, reflect the existence of an intrinsic circadian clock that temporally orchestrates physiological processes to adapt the internal environment with the external cues[42]. In particular, the daily light/dark cycle represents the primary external synchronizer of circadian rhythms. In mammals, light is processed through the eye and transmitted through the retino-hypothalamic tract to hypothalamic suprachiasmatic nucleus (SCN), the master internal pacemaker.[43] A central role in the regulation of this loop is played by the heterodimeric partnership between two transcription factors, i.e. the brain and muscle Arnt-like protein 1 (ARNTL, also known as BMAL1) and the circadian locomotor output cycles kaput (CLOCK). In particular, transcription of BMAL1 and CLOCK genes, or its related gene NPAS2 (neuronal PAS domain containing protein-2), which is mainly expressed in the forebrain, leads to the heterodimerization in the cytoplasm of the BMAL1:CLOCK complex, which translocates into the nucleus where it binds to canonical Enhancer Box (E-Box)-sequences containing the

[42] Molecular regulations of circadian rhythm and implications for physiology and diseases, Francesca Fagiani,et al,2022.
[43] Molecular regulations of circadian rhythm and implications for physiology and diseases, Francesca Fagiani,et al,cit.

consensus sequence CACGTG or noncanonical E-Boxes of clock-regulated genes.[44]

1.7 Circadian regulation of physiological processes

In addition to their primary role as generator of circadian rhythm, the biological clock serves a key role in controlling physiological functions of almost all tissues and organs in two major ways. First, central outputs from the SCN and/or local outputs from cell autonomous peripheral oscillators drive circadian rhythms in several physiological pathways in a rhythmic fashion. Second, the core clock components have been observed to act as key molecular players in many intracellular pathways, serving additional physiological roles. Examples of circadian-regulated physiological pathways includes cell growth, DNA repair and damage, angiogenesis, apoptosis, metabolism, redox state, as well as immune and inflammatory processes.[45]

1.7.1 Circadian clock and cell cycle machinery

Accumulating evidence suggests that the circadian clock affect the timing of cell divisions in vivo[46]. Moreover, most of cell-cycle genes involved in G2-M or G1-S transitions contains E-box regulatory elements in their promoters, transcriptionally activated by CLOCK:BMAL1 heterodimer[47]. Accordingly, it has

[44] Ivi.
[45] Control mechanism of the circadian clock for timing of cell division in vivo,Matsuo, et al,2003.
[46] Ivi.
[47] Riding tandem: circadian clocks and the cell cycle,Hunt et al,2007.

been demonstrated that the cell-cycle-related gene Wee-1, which contains three B-boxes in its promoter and encodes a protein kinase that inactivates the CDC2/Cyclin B1 complex, thereby delaying or preventing entry into mitosis, is under the direct transcriptional control of CLOCK:BMAL1 (figure 1.6).[48]

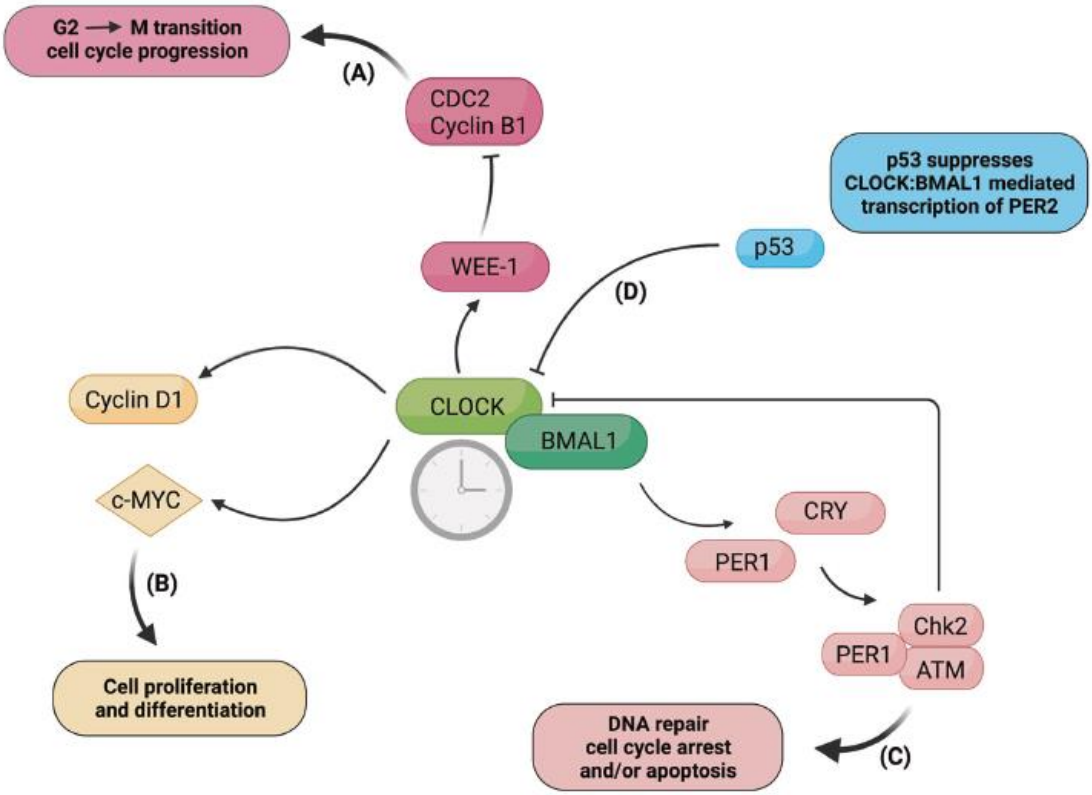


Figure 1.6: Molecular interaction between the circadian core clock and cell cycle components.[49]

Within this context, the circadian clock has been implicated in DNA damage response (DDR), including cell cycle checkpoints and DNA repair. DNA damage-induced cell cycle checkpoints are transduction pathways driven by DNA damage,

[48] Control mechanism of the circadian clock for timing of cell division in vivo,Matsuo, et al,cit.
[49] Molecular regulations of circadian rhythm and implications for physiology and diseases, Francesca Fagiani,et al,cit.

acting as a genome surveillance mechanism to ensure genomic [50]. Moreover, it has been demonstrated that, upon UV damage, Cryptochrome-1 (CRY1) modulates the ATR (ATM- and Rad3-Related)-mediated DNA damage checkpoint response by interacting with TIMELESS (TIM) in a time-of-day-dependent manner, thereby generating circadian oscillation of ATR activity[51]. Interestingly, it has been recently demonstrated that androgen receptor-induced CRY1 was stabilized by genotoxic insult and regulated DDR by directly binding to promoters of homologous recombination factors, thereby modulating genome integrity and promoting castration resistant prostate cancer growth[52]. As such, CRY1 emerges as a pro-tumorigenic factor that rhythmically controls DNA repair mechanisms and cell survival through temporal transcriptional regulation. Therefore, one can speculate that failure in such a crosstalk between clock and DNA damage response factors may cause genomic instability and tumorigenesis[53].

1.7.2 Circadian disruption: implication for human health

The molecular clock tightly regulates crucial cancer-related pathways, ranging from cell cycle progression to p53-mediated apoptosis. However, clock dysfunction has been either associated with pro- or anti-tumorigenic effects

[50]Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints, Sancar, et al,2004.
[51] Ivi.
[52] The circadian cryptochrome, CRY1, is a pro-tumorigenic factor that rhythmically modulates DNA repair Shafi, et al, 2021.
[53] Ivi.

depending in a context-dependent manner[54]. Despite the molecular mechanisms are not fully elucidated, clinical data provide evidence of a link between circadian misalignment and increased incidence of specific cancers. Notably, studies on carcinogenic risk factors suggested that disruptions in circadian rhythms play a central role in tumour progression. Recent studies have revealed alterations in post-translational modifications, such as phosphorylation and methylation, in the promoters of the core clock genes in cancerous tissues, leading to the deregulation of clock gene expression. [55]Accordingly, preclinical data correlate the environmental disruption of the biological clock, due to shift work and light exposure at night, with hormone-dependent cancers, such as breast and prostate cancer[56]. Compared to normal tissues, the expression of clock genes is altered in many solid human tumours and it has been identified an altered pattern in cyclic genes in human breast cancer cell lines compared to normal breast epithelial cell line. These observations indicate that a global reprogramming of circadian gene expression may confer a physiological advantage to tumour cells and suggest that clock dysfunction could be considered a pro-tumoral event, thus indicating clock dysfunction as a potential hallmark of cancer[57].

[54] Cancer clocks in tumourigenesis: the p53 pathway and beyond,Stephenson, et al,2021.
[55] Epigenetic basis of circadian rhythm disruption in cancer,Reszka, et al,2018.
[56] Night work and breast cancer risk among Norwegian nurses: assessment by different exposure metrics Lie, et al, 2011.
[57] The genomic landscape and pharmacogenomic interactions of clock genes in cancer chronotherapy, Ye et al,2018.

2 AIM OF THE STUDY

Pin1 is the only known peptidyl-prolyl cis–trans isomerase (PPIase) that specifically binds and isomerizes the pSer/Thr-Pro motif, thereby mediating posttranslational structural rearrangements that regulate the activity of a large number of protein targets. Pin1 is crucial in several cellular processes, and its aberrant regulation leads to degenerative and neoplastic diseases.

The role of Pin1 in the regulation of the cellular circadian clock is so far largely unexplored, despite several of its factors being targets for phosphorylation.

The structure of Pin1 bound to several target peptides of known specific interactors has been recently solved; combined with the rapid improvement of computational techniques, this makes it possible to identify potential target proteins via bioinformatic tools.

The purpose of the present study is thus to computationally search for possible Pin1 binding site in the main components of the molecular clock and then to apply molecular biology techniques to verify the putative interaction in vitro.

3 MATERIALS AND METHODS

3.1 Bioinformatic analisys of putative Pin1 targets

From the human PIN1 Uniprot page of (code Q13526), several 3D structures of the protein bound to known target peptides, obtained via NMR or X-ray, were traced.

Structures encompassing the whole protein were considered, as well as the structures of the isolated WW domain because this domain makes contact with the substrates and in particular with the conserved pSer/Thr-Pro motif. The residues of the contact site in the target proteins are those that undergo isomerization.

The sequence of each target peptide was aligned over the sequence of a list of candidate proteins involved in the regulation of the circadian clock.

A Pairwise Sequence Alignment was performed using the web tools Needle (EMBOSS) for the global alignment and Matcher (EMBOSS) for the local alignment, both from the European Bioinformatics Institute EMBL-EBI.

The candidate proteins were thus ranked according to the number of matches and the goodness of fit, prioritizing the matches that involved known phosphorylation sites.

3.2 Pin1 expression vector

After a literature search on the Addgene site, a vector containing the Pin1 open reading frame has been ordered.

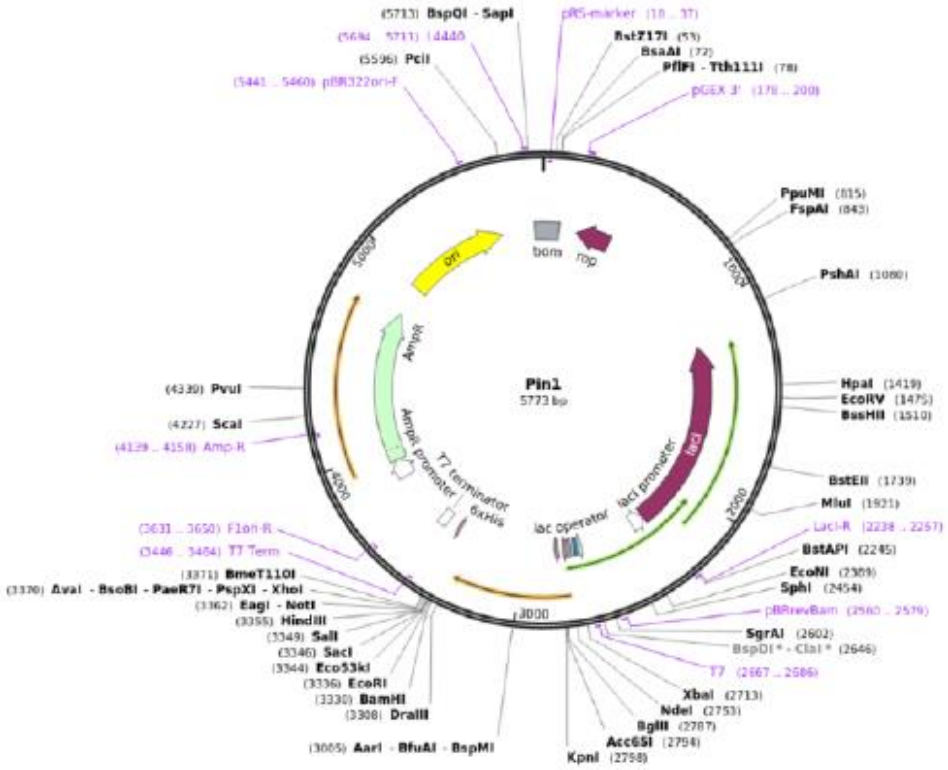


Figure 3.1: Pin1 plasmid 1

The plasmid of interest bears the following features:

- A strong inducible T7 promoter, *i.e.* the promoter of bacteriophage T7 gene 10;
- A Lac operator, to control inducible the production and induction of the target gene;

- A ribosome binding site (RBS), which determines the site of protein synthesis initiation;
- A 6x His-tag, to facilitate the purification of the protein of interest;
- A selection marker, ampicillin resistance;
- A T7 terminator;

3.3 Plasmid digestion with restriction enzymes

Restriction enzymes (ER) are endonucleases that cause internal double-strand breaks in DNA at particular nucleotide sequences.

Restriction enzymes are named after the organism from which they are isolated.

ER are distinguished into type I and type ER II. Type I ER recognise a specific nucleotide sequence, but cut the DNA at non-specific positions at some distance from the recognised sequence. This is because the cut site does not correspond to a specific base pair.

Type II ER also recognise specific nucleotide sequences, but cut the DNA within those specific sequences, named cut sites. The recognition sequence for most type II ER is 4-6 nucleotides long.

Cloning and expression vectors usually contain multiple unique restriction sites that can be exploited for cloning and restriction analyses.

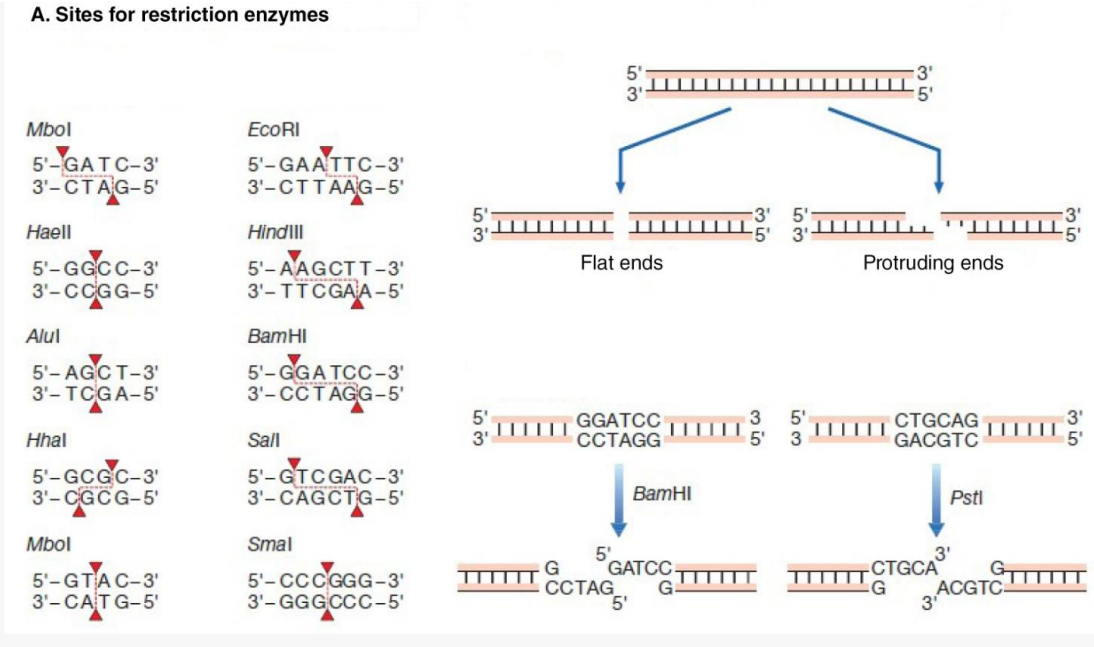


Figura 3.2[58]: Restriction enzymes

Operating procedure

O verify the correct length of the insert in the Pin1 expression vector, a restriction analysis has been set up using restriction enzymes with unique cutting sites flanking the protein-coding insert.

The expected insert length was calculated from the plasmid map downloaded from the Addgene website using Snapgene Viewer; this way restriction sites were selected on either side of the sequence of Pin1.

58

From the sequence analysis, the following unique restriction sites were found:

5' cutting enzymes + cutting site	3' cutting enzymes + cutting site
XbaI (2714)	DraIII (3309)
NdeI (2754)	BamHI (3331)
BglII (2788)	EcoRI (3337)
Acc65I (2795)	Eco53kI (3345)
KpnI (2799)	SacI (3347)
	SalI (3350)
	HindIII (3356)
	NotI,EagI (3363)
	AvaI,XhoI,PspXI,BsoBI,PaeR71 (3371)
	Bmet110I (3372)

Figure3.3: restriction sites

The enzymes BamHI and KpnI have been chosen; the length of the insert cut with must therefore be:

3331 (BamHI) - 2799 (KpnI) = 542 nt long.

To check whether the plasmid contains the correct size insert, we are going to cut it with restriction enzymes.

Restriction enzymes are given in units equal to the amount of DNA cut (in μg) in 1 hour.

The reactions were prepared as shown in the table. (Figures 3.4)

	Reaction 1	Reaction 2	Reaction 3
H20	to 10 ul	to 10 ul	to 10 ul
10x cutsmart buffer	1 ul	1 ul	1 ul
DNA-plasmid	400 ng	400 ng	400 ng
Enzyme 10units	BamHI 0,5ul	KpnI 0,5ul	BamHI,KpnI tot 1ul

Figura3.4: Reaction 1,2 and 3

We loaded the prepared solutions onto an agarose gel.

3.4 DNA electrophoresis on agarose gel

DNA molecules of differing sizes can be separated by gel electrophoresis on an agarose gel.

The gel is a solid matrix, mostly consisting of agarose

-Agarose is a water-soluble sugar at boiling temperature that becomes solid when it cools, forming a matrix through hydrogen bonds between linear chains;

-polyacrylamide gels, on the other hand, are formed by copolymerisation of acrylamide, a monomer soluble in water-soluble monomer, and a cross-linking agent so as to form a three-dimensional lattice.

The two types of gels differ in the size of the pores of the pore size of the lattice: gels with large pores allow the separation oflarge molecules, gels with small pores separate molecules of limited size.

If a gel is subjected to the action of an electric field, molecules of DNA and RNA migrate in the gel depending on their charge, size and structure.

The electrophoresis apparatus is connected to a power supply of electric current and the DNA and RNA molecules that are charged negatively charged due to the presence of phosphate groups, migrate from the negative pole (cathode) to the positive pole (anode).

These fragments that separate by electrophoresis can be easily visualised by dipping the gel in a solution containing ethidium bromide. This substance intercalates between the two strands and emits a fluorescence when exposed to UV light, allowing the various fragments to be visualised.

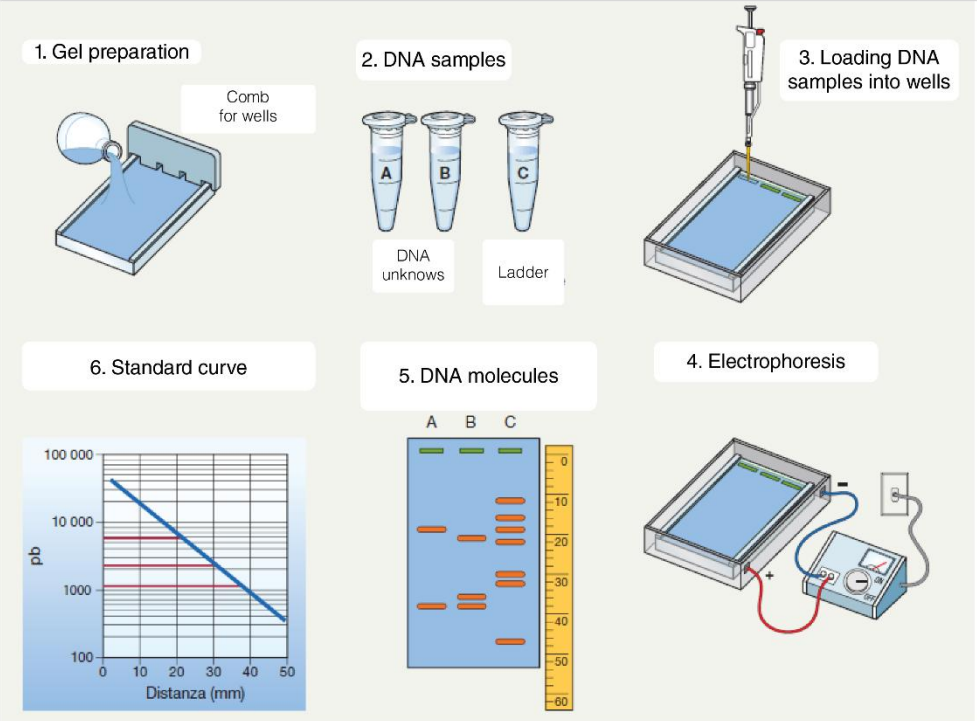


Figura 3.5[59]: Electrophoresis

3.5 E. coli BL21(DE3) + pLysS cell transformation

The technique that allows to introduce exogenous DNA molecules into bacteria is called transformation.

Some bacteria are naturally capable of absorb exogenous DNA molecules, while others, such as *E. coli*, acquire this capacity upon specific treatments that alter the permeability of the bacterial membrane.

The permeability of the bacterial membrane can be increased by chemical transformation, based on the treatment of the bacteria with CaCl2, or by

[59] Biologia Molecolare, terza edizione, Francesco Amaldi et al, Zanichelli, 2018.

electroporation, which consists of a short, intense electrical discharge that causes pores in the membrane to open[60].

The competent *E. coli* cells obtained are mixed with the plasmid of interest and must be subjected to a short heat shock for transformation to take place, after which they are allowed to grow in rich medium before being plated on solid medium.

Operating procedure

The *E. coli BL21 (DE3) pLysS* strain was used for the expression of Pin1. This strains expresses the T7 polymerase gene, upon induction by the allolactose non-hydrolysable analogue isopropyl-β-D-thiogalactoside (IPTG), which binds the lacUV5 promoter. Thus, upon induction with IPTG, large amounts of the protein of interest can be produced.

50 ng of the plasmid were added to a 50-µl aliquot of competent cells and incubated on ice for 30 minutes. Cells were then subjected to heat-shock transformation: the cells were first incubated for 45 seconds at 42°C using a thermostatically controlled bath and immediately placed back on ice for two minutes. Next, 1 ml of LB medium was added to the mixture and the cells were incubated for 1 hour at 37° with gentle stirring. 200 µl of the cell suspension were plated on solid LB medium containing 100 µg/ml Ampicillin and 34 µg/ml Chloramphenicol; the plates were then incubated overnight at 37°.

[60] Ivi.

3.6 Large-scale Pin1 expression

A large-scale expression of a protein of interest requires the gradual propagation of a single bacterial colony in liquid medium in order to obtain a sizeable amount of cells.

Culture scale-up

One colony was selected from the agar plate and inoculated in 5 ml of liquid LB medium supplemented with Ampicillin and Chloramphenicol (LB-Amp+Chlo). The inoculum was incubated for 6 hours at 37° at 180 rpm in a *Shel Lab* incubator. The 5-ml culture was then transferred to an *Erlenmeyer* flask containing 100 ml of liquid LB-Amp+Chlo and was then incubated *overnight* at 37°, 180 rpm to reach saturation.

The following day, the optical density (OD) of the culture was checked at 600 nm using a spectrophotometer. For the large-scale expression, a suitable volume of saturated culture was transferred to an *Erlenmeyer* flask containing 500 ml LB-Amp+Chlo to a starting OD of 0.1/ml. The culture was incubated at 37°C, 180 rpm; spectrophotometer readings were taken every hour until the culture entered the logarithmic growth phase and reached 0.6 OD/ml.1 ml of culture was taken as a pre-induction control: the cells were centrifuged at 12000 x g for 3 minutes and resuspended in 120 µl/OD 2X SDS-PAGE loading buffer (0.125 M Tris-HCl pH 6.8, 25% glycerol, 4% SDS, 5% β-Mercaptoethanol, 0.001% Bromophenol Blue).

Time (minutes)	Measure (600 OD)
0	0,27
45	0,24
75	0,36
105	0,59
120	0,762
240*	2,25

*after 2 hours of induction with IPTG

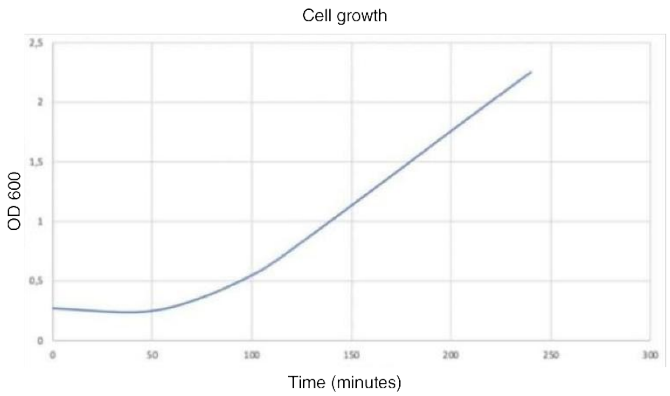


Figure 3.6:time-dependent cell growth 1

Induction of Pin1 expression

When the culture reached 0.6 OD/ml, Pin1 expression was induced by addition of 0.5 mM IPTG, a non-hydrolysable allolactose analogue that induces gene expression under the control of the Lac operon.

After 3h, 1 ml of the culture taken as a post-induction control: cells were resuspended in 120 µl/OD 2X SDS-PAGE loading buffer; the remaining culture was centrifuged at 4000 rpm in a Beckman JLA8.100 rotor at 4°C and the cells pellet was stored at -80°C.

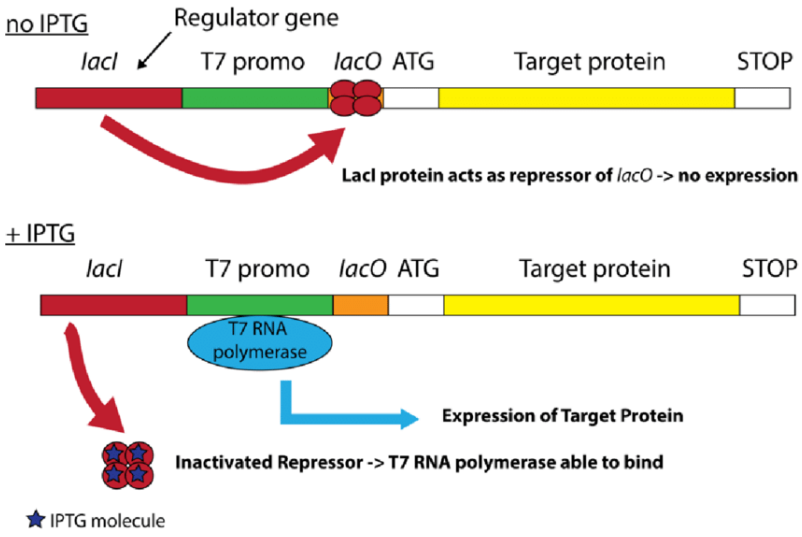


Figure 3.7[61]: Induction with IPTG

3.7 Protein electrophoresis on SDS-PAGE

Electrophoresis in an electric field is the most widely used technique also for separating mixtures of proteins of different sizes.

The separation of the various protein components is usually on vertical polyacrylamide gels, with the porosity best suited to the size of the proteins to be separated.

The rate of gel migration of a protein depends on on the intensity of the applied electric field, the size and the charge. The net charge of a protein depends, in turn, on the pH of the buffer used for the electrophoretic run. The most commonly used technique in the laboratory is the separation of proteins under denaturing conditions in the presence of sodium dodecyl sulphate (SDS): this is referred to

[61] Ingegneria Genetica, principi e tecniche, Sandy Primrose et al, Zanichelli, 2004.

as the SDS-PAGE technique (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis). In the presence of SDS and other specific experimental conditions, the secondary structures of proteins are denatured and the molecules are actually covered by negative charges allowing the migration of proteins to the anode with a speed essentially dependent on the size (molecular weight) of the different protein species in the mixture. After the electrophoretic run using various dyes, the most common of which is Coomassie blue.

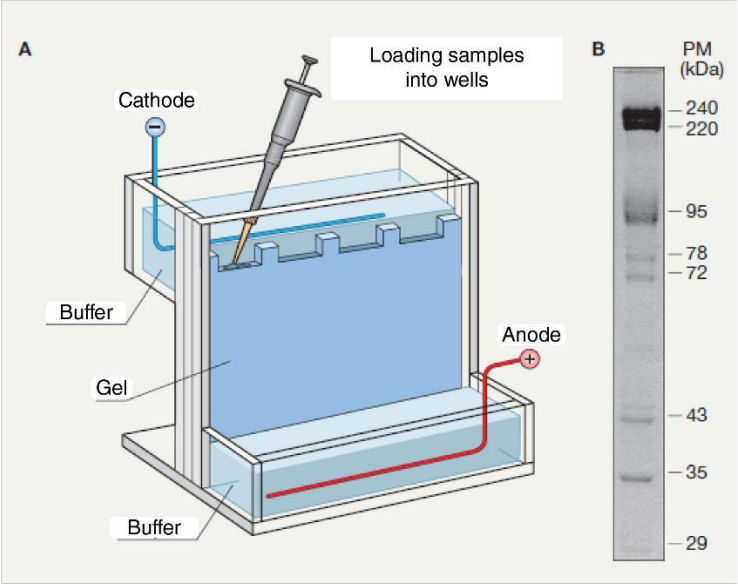


Figura 3.8[62]:SDS-PAGE

[62] Biologia Molecolare, terza edizione, Francesco Amaldi et al, Zanichelli, 2018.

3.8 Pin1 affinity purification

The purpose of protein purification from heterogeneous systems (*E.Coli*) is to isolate a protein of interest from a heterogeneous mixture containing other proteins, nucleic acids, lipids and other molecules.

To this end, a number of features can be exploited to facilitate the purification of the protein, such as the differences in chemical and physical properties between the protein to be purified and the others in the mixture.

Pin1 was expressed with a N-terminal tail of 6 histidines, which is known as His-tag.

As can be seen from the figure(3.9), the His-tag fused to the recombinant protein has a strong affinity for a nitriltriacetic acid (Nta) resin complexed with bivalent nickel ions. A His-tag-containing protein thus binds specifically to the resin via the specific interaction between the histidine residues and the nickel ions. Elution of the tagged protein under specific conditions can lead to substantial purification of the recombinant protein in a single step[63].

[63] Biologia Molecolare, terza edizione, Francesco Amaldi et al, cit.

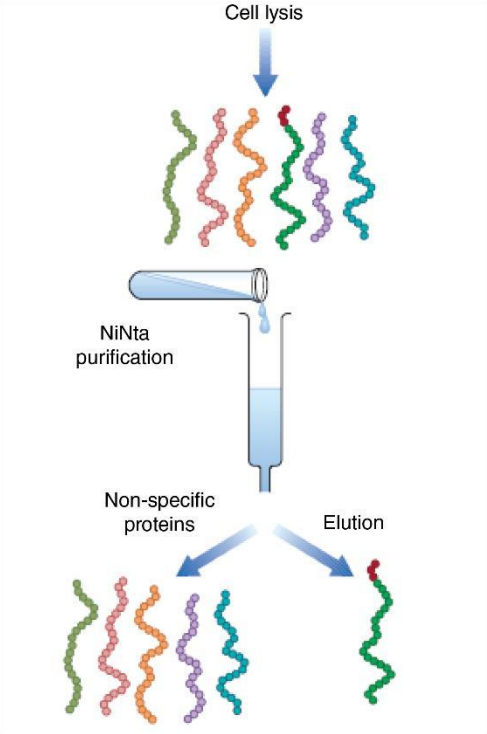


Figure 3.9[64]: Purification of a recombinant His-tagged protein

[64] Ivi.

Operating Procedure

Lysis/wash Buffer	fC	iC	fV	iV
Hepes pH 7.8	50 mM	1000 mM	1000 ml	50 ml
NaCl	300 mM	5000 mM		60 ml
H2O				890,00 ml

Evolution buffer	fC	iC	fV	iV
Hepes pH 7.8	50 mM	1000 mM	40 ml	2 ml
NaCl	300 mM	5000 mM		2,4 ml
Imidazol	200 mM	2000 mM		4 ml
H2O				35,6 ml

Figure 3.10: Buffer used for purification

The cells pellet was thawed directly in Lysis/wash buffer (20 ml) with the addition of DTT (2 mM), PMSF (0.1 mM final), lysozyme (0.1 mg/ml) and benzonase (1 unit/g cells). Cell membranes were ruptured by 5 1-minute cycles of sonication at 60% amplitude and 2s pulses; cell debris and were removed by centrifugation at 12000 x g for 30 min at 4°C in a JLA8.1 rotor.

For protein purification, 4 ml of Ni-NTA resin were poured in an empty column and then equilibrated with 20 ml of Lysis/wash Buffer.

The clarified cell extract was loaded onto the packed column and the flow-through was collected. 20 µl samples were collected at each step for SDS-PAGE analysis.

The resin was washed with 40 ml Lysis/wash Buffer; the bound proteins were eluted step-wise with 15 1-ml Elution Buffer containing 200 mM imidazole, which competes with the His-tag for binding to the resin.

In order to assess the amount and purity level of the resulting protein, samples were collected from each aliquot and loaded on a 15% SDS-PAGE gel, followed by Coomassie staining; all aliquots were kept on ice throughout the SDS-PAGE analysis.

3.9 Buffer exchange and spectrophotometric determination of Pin1 concentration

The recombinant protein is eluted in buffer containing high concentrations of imidazole, which can interfere with downstream purification steps and can hamper the functionality of the protein; therefore, a buffer exchange step is needed to remove imidazole.

The aliquots containing the highest amounts of Pin1 were collected and subjected to multiple centrifugation (3000 x g at 4°C) and dilution steps on a Centricon® Plus-15 Centrifugal Filter Unit (Merck Millipore) with a 3 kD cutoff, using Lysis/wash buffer; the final volume of the protein was reduced to 5 ml.

Each protein is characterised by a specific molar extinction coefficient which is due to the presence of the aromatic aminoacids tryptophan and tyrosine. This allows to calculate the concentration of the protein from the absorbance at 280 nm.

Protein concentration was determined using the Beer-Lambert Law formula:

$$A = \varepsilon b C$$

Where A is the absorbance of the protein at 280 nm, ε is the molar extinction coefficient, b is the path length (1 cm) and C is the concentration of the protein in mg/ml.

The Pin1 molar extinction coefficient ε, 20970 M^{-1} cm^{-1}, was obtained from the ExPASy ProtParam online tool (https://web.expasy.org/protparam/) using the His-tagged Pin1 primary sequence.

3.10 TEV cleavage

The TEV protease is a cysteine protease from the Tobacco Etch Virus (TEV), which is widely used as a tool for the cleavage of fusion proteins and removal of tags from recombinant proteins *in vitro*. This enzyme belongs to the chymotrypsin-like protease family and shows high sequence specificity[65].

The gene coding for Pin1 of interest is fused to a DNA segment coding for the His-tag peptide. These two protein domains are separated from each other by a series of amino acids that are the target of the tobacco virus protease (TEV).

After purification, the recombinant protein is cut using the TEV protease so that the final protein has the native primary sequence[66].

[65] Ingegneria Genetica, principi e tecniche, Sandy Primrose et al, cit.
[66] Biologia Molecolare, terza edizione, Francesco Amaldi et al, cit.

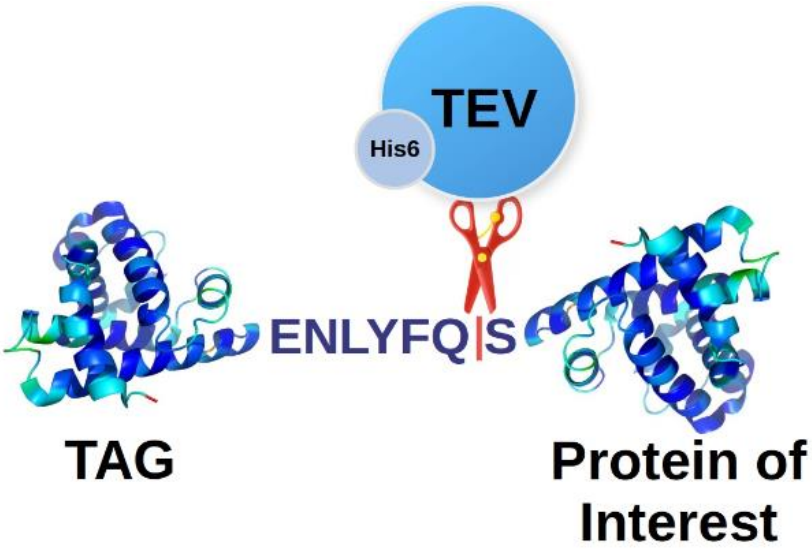


Figure 3.11[67]: TEV cleavage

Operative procedure

1 ml of purified his-tagged Pin1 were incubated with a 1:50 molar ration of TEV enzyme and 0.5 mM EDTA overnight at room temperature.

The cleave extent was determined the following day by loading 10 µg of protein on a 15% SDS-PAGE gel.

[67] Ivi.

3.10.1 NiNTA purification of cleaved Pin1

Operative procedure

The cleaved Pin1 was loaded on the a NiNTA column to remove the free His-tags, the his-tagged TEV and traces of uncleaved Pin1.

The resin was equilibrated with Buffer Exchange (50 mM Hepes pH 7.8, 300 mM NaCl, 10% glycerol). The mixture consisting of Pin1 and TEV was loaded into the column and the flow-through consisting of pure, native Pin1 was collected.

3.11 Western Blot anti-histidine antibody

DNA, RNA and protein molecules of different sizes can be separated by gel electrophoresis. After electrophoresis, both nucleic acids and proteins can be visualised by appropriate staining.

For their visualisation, techniques are used such as blotting techniques that require their transfer onto nitrocellulose membranes and the subsequent incubation with appropriate probes that can hybridise with the molecules of interest.[68]

Protein mixtures of different sizes can be separated by SDS-PAGE electrophoresis and stained with Coomassie blue; to unambiguously highlight a protein of interest in the mixture, specific antibodies against that protein can be used. In the assay known as Western Blot, proteins separated by SDS-PAGE are first electrically transferred onto a nitrocellulose membrane.

[68] Ivi.

The membrane is then incubated in the presence of the specific antibody against the protein of interest and the protein-antibody complex formed on the gel is highlighted using a secondary antibody that is labelled, or capable of conferring a specific colouration, or a chemiluminescence capable of give a signal on a photosensitive plate.[69]

Operative procedure

The following samples collected during Pin1 purification were loaded onto and acrylamide gel: Pin1 pre-cut, Pin1-his, Pin1 after cut with TEV.

After the electrophoretic run on SDS-PAGE, the gel was equilibrated for 20 minutes in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol) and stirred. The membrane (PVDF membranes, Bio-Rad) was equilibrated for a few seconds in methanol. Afterwards, gel and membrane were equilibrated for 10 minutes in transfer buffer. Next, the transfer was performed using Trans-Blot® Turbo™ Transfer System (Bio-Rad) using the standard SD method (1A, 25V, 30 minutes). The membrane was saturated with Tris- and Tween-buffered saline (TBST: 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) supplemented with 3% bovine serum albumin (BSA) . The membrane was incubated with the anti-His primary antibody (diluted 1:10,000 in TBST-BSA) overnight at 4°C. The following day the membrane was washed 3 times in TBST to remove the unbound antibody and then incubated with the secondary anti-mouse antibody (diluted

[69] Genetica, un approccio molecolare, Carla Cicchini et al, Pearson,2014.

1:10000) for 1 hour at 37°C. Finally, after four washes with TBST, the membrane was developed using two substrates capable of reacting with the peroxidase bound to the secondary antibody, for which the Clarity™ Western ECL Substrate kit (Bio-Rad) was used. 500 µl of each solution were added and incubated in the dark for 5 minutes to allow the chemiluminescent reaction to take place. Results and images were then visualised through the ChemiDoc (Bio-Rad).

3.12 Protein-protein interaction studies using the pull-down assay

The pull-down assay is an invaluable tool for studying cellular pathways via protein–protein interactions. The basic principle of pull-down is to utilize a tag fused protein (such as His-tag) immobilized to an affinity resin as the "bait" protein. Proteins binding to the bait (the so called "prey" proteins) can be captured and *pulled down* from a cell lysate flowing through the resin-immobilized bait. The protocol involves the following steps:

1. Immobilization of the "bait" to the affinity resin/beads;

2. Incubation of the immobilized bait with a cellular extract containing the putative binding targets;

3. Analysis of the bound proteins via western blot

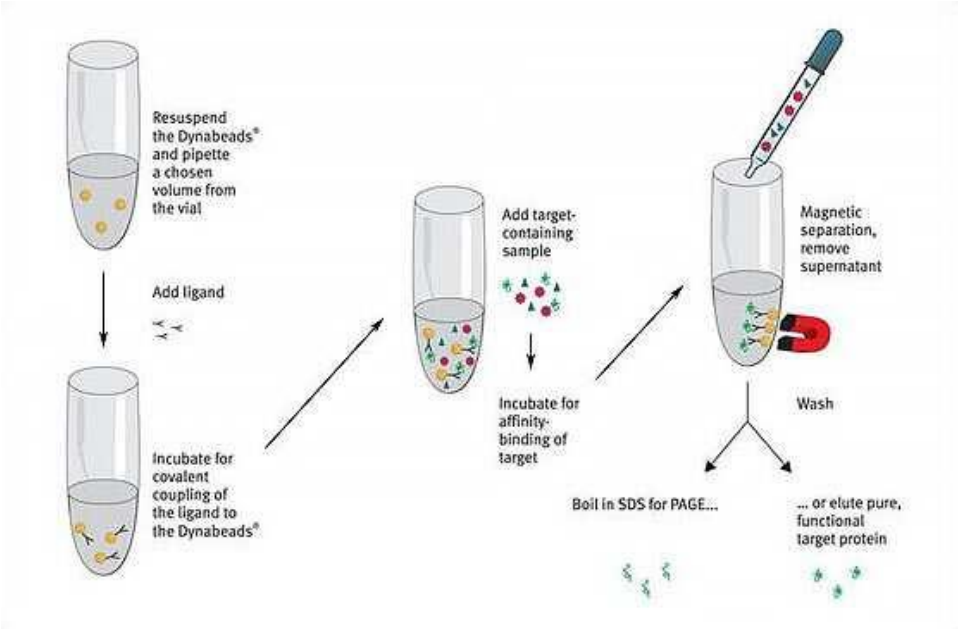


Figura3.12: Pull down assay

For the purpose of this study, magnetic beads with a cobalt-based coating (Dynabeads™ His-Tag, ThermoFisher) were used, that allow the specific binding of his-tagged bait proteins.

Operative procedure

His-tagged Pin1 immobilization on magnetic beads

To test the binding capacity of the Dynabeads, purified His-tagged Pin1 was incubated with in the ratio suggested by the manufacturer (40 µg His-tagged Pin1: 25 µl Dynabeads).

50 µl of Dynabeads slurry, corresponding to 25 µl beads, were washed four times with 400 µl Binding buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl), according to the manufacturer's protocol.

40 µg of purified His-tagged Pin1 were diluted in Binding buffer to 400 µl at room temperature, after which the was incubated with the beads for 30 minutes at 4°C on gentle stirring. The beads were washed 4 times with 400 µl Binding Buffer, after which the bound protein was eluted with 400 µl 2X SDS-PAGE.

The extent of protein binding was determined by Western Blot as described in section 3.11.

For the pull-down experiment, Pin1 immobilization was repeated on a larger scale following the protocol described above.

3.13 Pull-down experiment with Caco-2 cells extract

The cells extracts used in this study were kindly provided by the research group led by Prof. Cristina Lanni (Università di Pavia). Caco-2 are epithelial cells isolated from colorectal adenocarcinoma and represent an excellent model for preclinical colon cancer studies. Briefly, Caco-2 cells were subjected to starvation prior to supplementation with 10% FBS; 1 million cells were collected every 15 minutes up to 4 hours, pelleted by centrifugation and lysed in 1 ml Lysis Buffer (50 mM di Tris-HCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Tryton X-100) and the total protein concentration was determined by Bradford assay.

300 µg total protein from each cell extract were incubated with 25 µl Pin1-immobilized Dynabeads for 30 min at 4°C on gentle stirring. The beads were

washed 4 times with 400 μl Binding Buffer, after which bound proteins were eluted with 60 μl 2X SDS-PAGE loading buffer.

3.13.1 Analysis of bound proteins via Western Blot

The purpose of the pull-down experiments is to assess if the predicted candidate Pin1 targets copurify with the His-tagged Pin1 bait. To this end, western blots were performed according to the protocol described in Section 3.11 using the suitable antibody pairs.

Primary Ab: anti-His(mouse), 1:10.000 in TBST-BSA 3%; anti-Pin1(rabbit), 1:1000 in TBST-BSA 3%.

Secondary Ab: anti-mouse, 1:10.000 in TBST-BSA3%; anti-rabbit, 1:10.000 in TBST-BSA 3%.

4 RESULTS

4.1 Results of computational analysis

To assess whether the main factors involved in the regulation of the cellular circadian rhythm have potential binding sites for the PPIase Pin1, bioinformatics tools were used. The sequence of know Pin1 target peptides were extracted from the available 3D structures of the Pin1-target peptide complexes and searched against the primary sequence of a set of proteins constituting the cell's inner molecular clock. All alignments were analysed for possible interaction sites with Pin1. Sites that did not contain the conserved Ser/Thr-Pro motif were excluded. The remaining matches were then analysed for conserved patterns that were similar to peptides already known to interact with Pin1 (Table 4.1).

The computational analysis yielded several putative Pin1 target proteins, which might interact with Pin1 with a characteristic affinity. To verify which of these candidate proteins bind to Pin1 in the cell, a molecular biology approach was further adopted.

PDB_ID	Protein description	Peptide sequence
1F8A*	Structural basis for the phosphoserine-proline recognition by group IV WW domain	YSPTSPS
1I8G*	Solution structure of PIN1 WW domain complexed with CDC25 phosphothreo-nine peptide	EQPLTPVTDL
2ITK	human Pin1 bound to D-PEPTIDE	XFTXAQX
2Q5A	human Pin1 bound to L-PEPTIDE	XFTXAQX
2LB3*	Structure of the WW domain of PIN1 in complex with a human phosphorylated Smad3 derived peptide	IPETPPPG
2N1O*	PIN1 WW domain in complex with a phosphorylated CPEB1 derived peptide	RISPPLPF
5UY9	Prolyl isomerase Pin1 R14A mutant bound with Brd4 peptide	QASTPRX
6O33	Crystal Structure Analysis of PIN1	XEXARX

* = only WW domain

Figure 4.1: Structures with PDB codes and peptides

Protein	Uniprot code
CRY1-cryptochrome-1	Q16526
CRY2-cryptochrome-2 iso1	Q49AN0
CRY2-cryptochrome-2 iso2	Q49AN0-2l
REV-ERB alfa/NR1D1	P20393
BMAL1	O00327
CLOCK	O15516
PER2 Period circadian protein homolog 2	O15055
PER3 Period circadian protein homolog 3	P56645
ROR2	Q01974
NRF2/NF2L2	Q16236

Figure 4.2: Proteins suggested by the University of Pavia

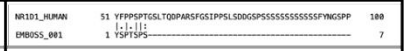
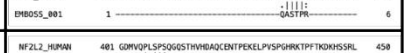
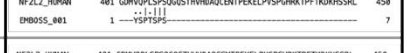
Protein Name	Crystallised peptide	Conserved protein motif	Alignment
CRY1-cryptochrome-1	RISPPLPF	S281P282	
CRY2-cryptochrome-2 iso1	QASTPR	T300P301	
CRY2-cryptochrome-2 iso2	QASTPR	T239P240	
REV-ERB alfa/NR1D1	YSPTSPS	S55P56	
	KVSVVRTPPKSPS	S278T279	
BMAL1	IPETPPPG	T527P528	
CLOCK	YSPTSPS	T429P430	
PER2 Period circadian protein homolog 2	RISPPLPF	S977P978	
PER3 Period circadian protein homolog 3	YSPTSPS	P783T784	
	RISPPLPF Local	S994P995	
ROR2	QASTPR	T438P439	
NRF2/NF2L2	YSPTSPS	S408P409	
	EQPLTPVTDL	S408P409	

Figure 4.3: Alignments between suggested proteins and various peptides

The table in figure 4.3 shows the best alignments for the peptides of the different proteins.

4.2 Restriction enzyme analysis

Digestion of the plasmid with restriction enzymes was performed as described in section 3.2 to verify the correct position of the protein-coding insert and then the results were analysed on agarose gels and shown below in figure (4.4). Two reactions (BamHI in lane B and KpnI in lane C) were set up with the individual restriction enzymes, to verify that each enzyme recognizes a unique site on the vector; the gel confirmed that each enzyme linearizes the vector, as can be seen from the bands corresponding to 6,000 bp DNA fragments.

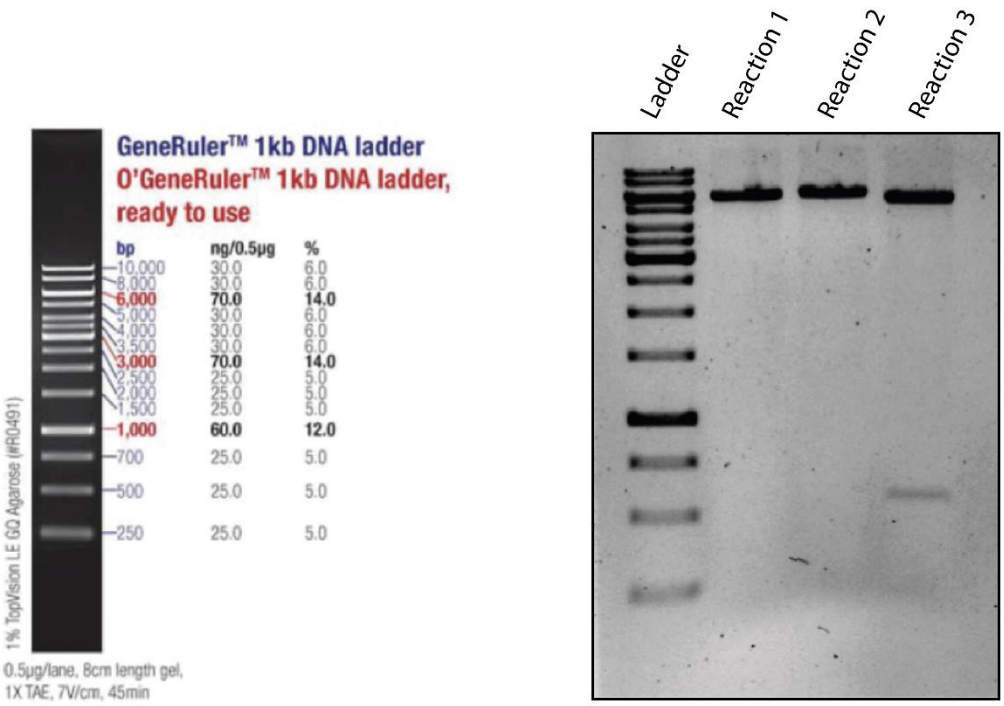


Figure 4.4: Restriction enzyme analysis

In lane D two bands can be seen; afaint one, which corresponds to a DNA fragment a little larger than 500 bp and a larger band that migrates between the 5000 bp and 6000 bp bands of the ladder. This result indicate that the insert matches the expected size of the Pin1 fragment (542 bp).

4.3 Pin1 expression

Pin1 was expressed in BL21 (DE3) + PLysS E. coli cells. The cells were induced with IPTG and to check whether protein overexpression occurred, we loaded the pre and post-induction samples onto the gel.

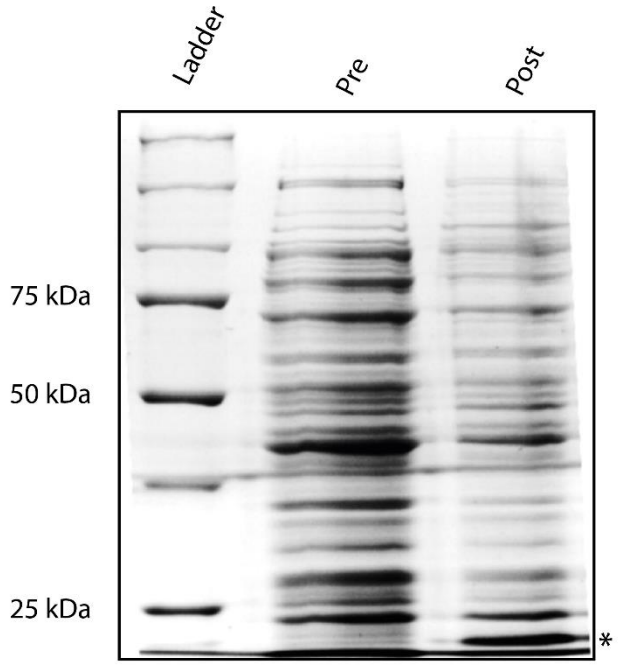


Figure 4.5: Pin1 expression

As can be seen from in the pictures(4.5), we see a darker band in the third column than in the second, indicating that induction has taken place correctly.

4.4 Pin1 Purification

Pin1 was purified from Details of the purification can be found in section 3.5. The bands you see in the pictures (4.6) are the aliquots we obtained from the purification and are 5 microlitres of alternating fractions (1 ml each). Pin1 is about 18 kDa.

The protein still needs to be concentrated and cut with TEV and this should help us improve the results.

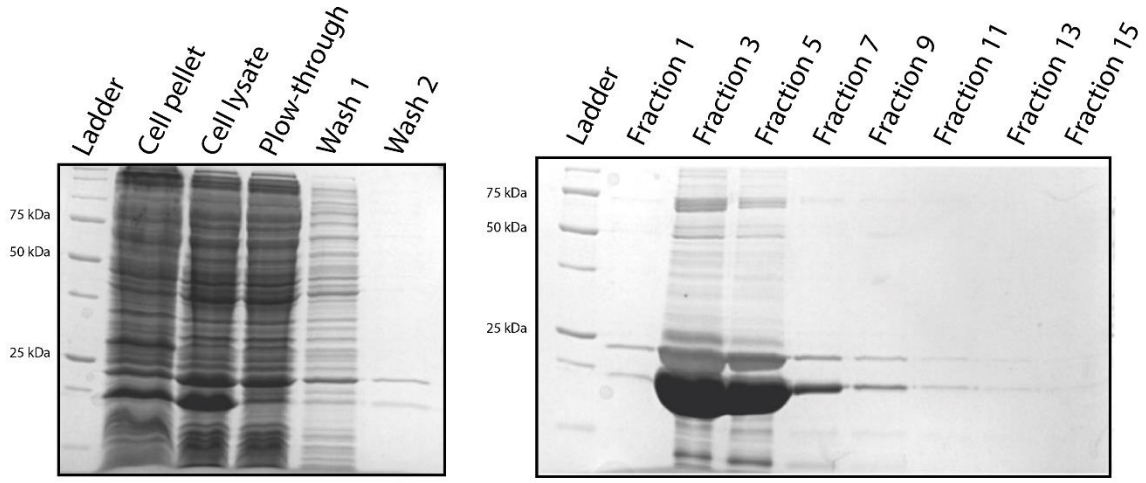


Figure4.6: Results of protein purification

4.5 Spectrophotometric determination of Pin1 concentration

After concentrating the protein as described in section 3.9, its concentration was determined at the spectrophotometer to be 3.72 mg/ml, which corresponds to 205 μM according to Beer-Lambet Law.

To further verify the concentration and purity of the protein, different amounts were loaded on a polyacrylamide gel and compared with a commercial BSA batch.

BSA has a molecular weight of 66.5 kDa, which is significantly larger than PIN1 (18 kDa), so the PIN1 bands should run much faster than those of BSA; however the bands intensityshould not depend on the size of the proteins. (4.7).

The gel bands were quantified using ImageJ software, resulting in a calculated concentration of Pin1 of about 4.4 mg/ml, which is – within the experimental error – in agreement with the concentration obtained from the absorbance.

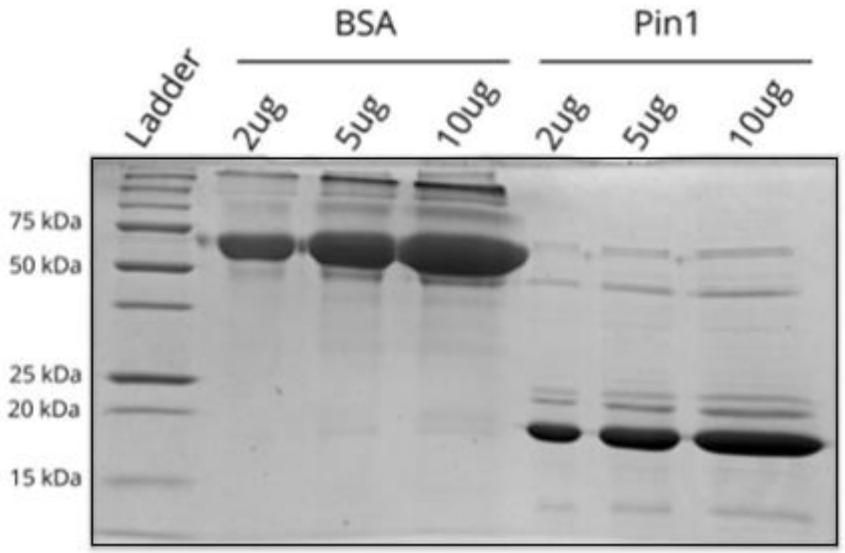


Figure 4.7: Comparison of Pin1 and BSA

4.6 Tev cleavage

The his-tag war removed from Pin1 via cleavage with the TEV enzyme (see section 3.10)

To check the extent of the cleavage, samples were loaded onto a 15% SDS-PAGE gel before and after incubation with the enzyme.

As can be seen from the SDS-PAGE photo (Figure 4.8), the post-cleavage sample is smaller than the his-tagged Pin1; the size difference can be traced back to the size of the His-tag, about 2.5 kDa.

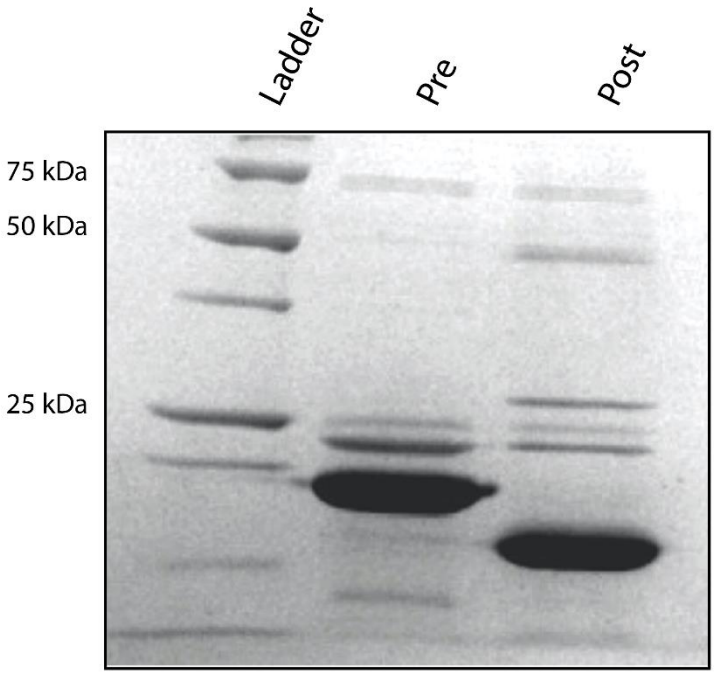


Figure 4.8:Pre and post TEV cleavage

4.6.1 NiNta purification of cleaved Pin1

After cutting the protein with TEV, purification with NiNta was performed to remove the His-tag and the TEV enzyme, which is also his-tagged (section 3.10.1). The results of this procedure were analysed on SDS-PAGE.

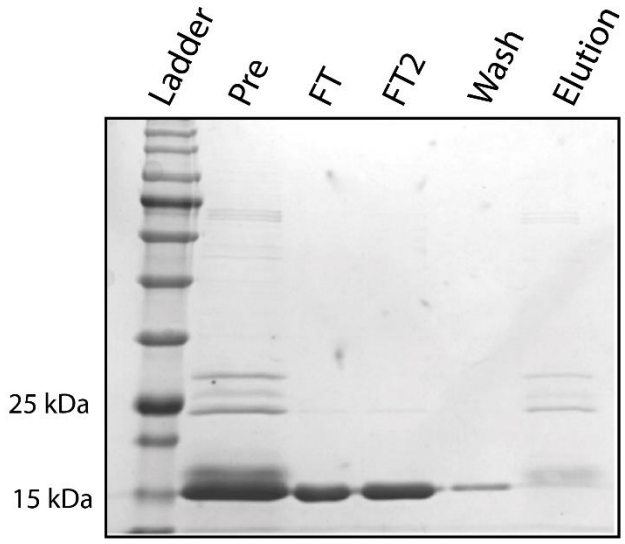


Figure 4.9 :NiNta purification of cleaved Pin1

From the gel we can see that Pin1 is present in the first three wells (excluding the ladder). In well 2 we have the TEV-cut protein, and we had already verified this in the previous gel (Figure 4.9), while in wells 3 and 4 the presence of Pin1 indicates that the protein has not bound to the histidine tail affinity resin because it has undergone the TEV-cut.

We obtained about 3 mg of native Pin1 and about 25 mg of Pin1 with the histidine tail to be cut.

4.7 Western Blot

We run the following samples on the SDS-PAGE gel:

- The gel on the left was stained with comassie blue.

In the first well, Pin1 and TEV are present, in the second well, only Pin1 is present with the histidine tail, and in the last well, the native protein is present.

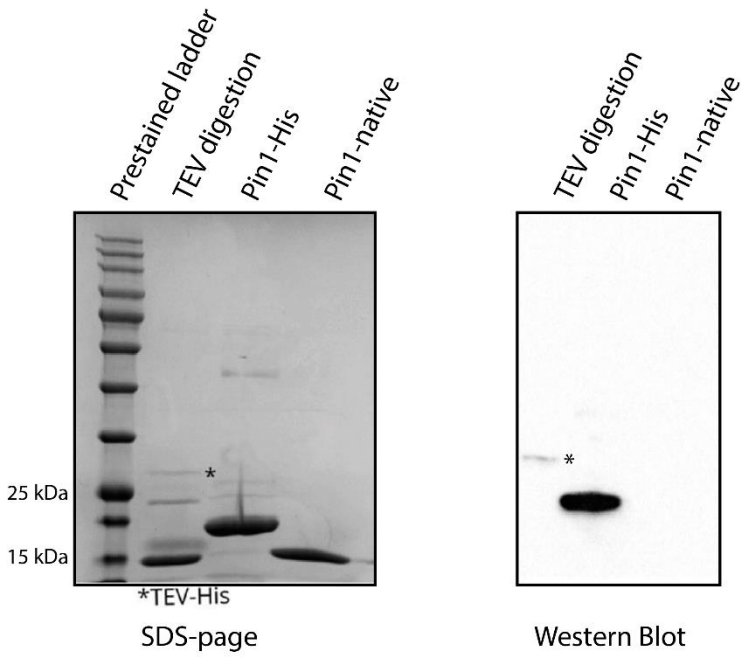


Figure 4.9: Western Blot

- This gel (pictures a sx) was then used to Western blot Pin1 with Ab anti-His (section 3.11).

From the result we obtained, we can see that our anti-his antibody only bound to our Pin1 protein that had a histidine tail without binding anything else.

4.8 Pin1 binding to magnetic beads

The binding of pin1 to the magnetic beads was assessed by Western Blot using anti-Pin1 and anti-histidine antibodies. The results indicate that most of the his-tagged Pin1 bound to the beads.

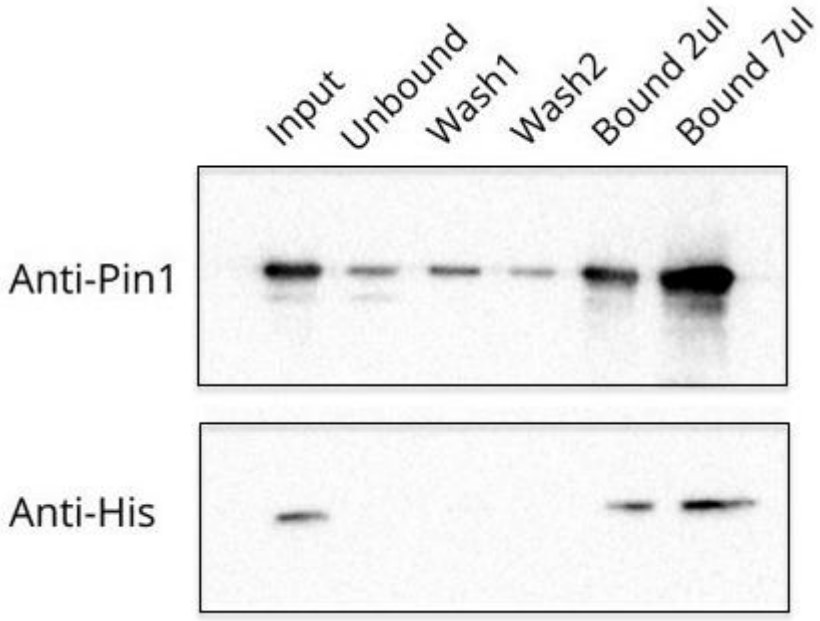


Figure 4.10:Pin1 binding to magnetic beads

small scale immobilization

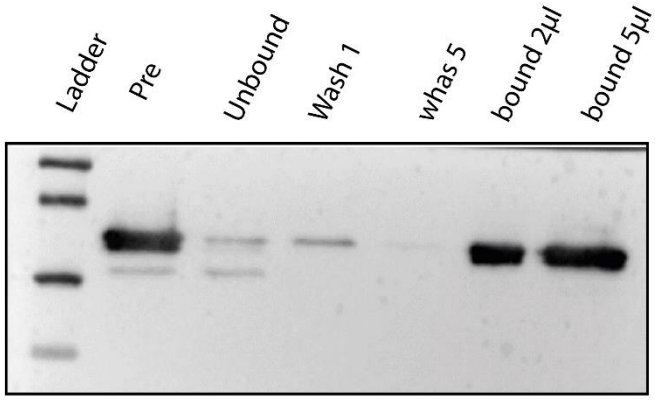


Figure 4.11: Pin1 binding to magnetic beads

Large scale immobilization (only anti-his)

4.9 Pull-down of NRF2 protein from the Caco-2 cells extracts

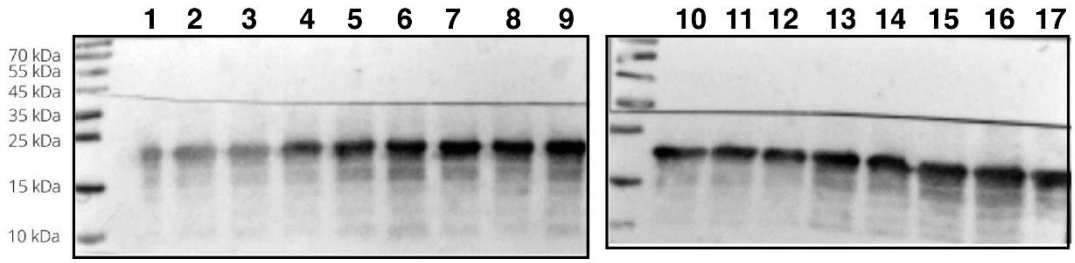


Figure 4.12: Western Blot of NRF2 protein from the Caco-2 cells extracts

Western Blot was performed to verify if the NRF2 protein co-purifies with the His-tagged Pin1 immobilized on the magnetic beads.

The results seem to show that the protein does not bind; however, important controls were left out due to the little amount of cell extract.

5 DISCUSSION

This project was motivated by the lack of studies regarding the role of the PPIase Pin1 in the regulation of the cellular circadian clock.

To fill this important gap in the understanding of molecular clocks, a combination of computational and molecular biology approaches was adopted.

By using bioinformatic tools, a list of putative Pin1 target proteins was generated, which was taken as a starting point for the development of an *in vitro* setup to validate the matches found.

Human recombinant Pin1 was expressed in *E. coli BL21 (DE3) pLysS* cells and purified via affinity chromatography.

The recombinant Pin1 was then used as a "bait" protein in a pull-down assay, where the immobilized Pin1 was incubated with the while cell extract from Caco2 tumoral cells.

The NFE2-like bZIP transcription factor 2 (NRF2) was chosen from the list of candidate proteins obtained from the computational analysis for further Western Blot analysis; unfortunately, we were not able to find NRF2 among the proteins pulled down with Pin1.

This result does not mean that there is absolutely no interaction between the two proteins; indeed, it could just mean that the method used is not sensitive enough to detect transient or sub-stoichiometric interactions. To address this issue, other

methods of analysis of the pulled down proteins, such as mass spectrometry, can be applied.

Mass spectrometry allows to have a higher resolution and therefore identify bound proteins that are too scarse to be detected by antibodies.

An alternative approach would involve using other types of experimental models, be it healthy or tumoral cells, such as triple-negative breast cancer or adenocarcinoma cells, both of which are characterized by dysregulation of several signalling pathways and are available in the laboratory.

In summary, the set up developed in this project represents a versatile tool to understand the protein-protein interactions at the basis of crucial cellular pathways; this approach can also be exploited to test the efficacy of established or novel Pin1 inhibitors in disrupting the interactions with specific target proteins.

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