Regulation of *Cyclin E1* transcription by the Prox1 homeodomain transcription factor

By

Patricia Bocangel

A Thesis submitted to the Faculty of Graduate Studies of

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Division of Stroke and Vascular Disease

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ABSTRACT

Prox1 is a homeodomain transcription factor shown to be essential for the development of the lymphatic system from pre-existing embryonic veins [1]. Disorders of the lymphatic vessels contribute to the development of diseases such as lymphedema and cancer. A better understanding of how lymphatic vessels are formed will facilitate the development of novel therapies to better treat these diseases.

Previously, it has been shown that the transcription of *Cyclin E1* is up-regulated upon expression of *Prox1* [2]. The objective of this study is to understand the mechanism by which Prox1 activates transcription of this gene. We hypothesised that Prox1 activates *Cyclin E1* transcription in a DNA-binding dependent manner. To test our hypothesis, we examined whether different versions of Prox1, with key domains either deleted or mutated, could activate *Cyclin E1* transcription. Interestingly, our results demonstrated that Prox1 activates *Cyclin E1* transcription in a DNA-binding independent manner. Furthermore, we showed that the *Prospero* Domain of Prox1 is required for full activation of this promoter and for regulating Prox1 subcellular localization. As well, we showed that the Prox1 responsive region of the *Cyclin E1* promoter is located between positions -111 and +95.

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A Hans

En memoria de la cena por la graduación de Fernan

En memoria de los abuelos y las “noches a oscuras como los curas”

I. LITERATURE REVIEW

1. The Lymphatic System

The lymphatic and blood vessels comprise the functional components of the vertebrate circulatory system. The lymphatic system is composed of both lymphatic vessels and lymphoid organs [3]. Lymphatic vessels were first identified in 1622 by the Italian scientist Gasparo Asellli, who during vivisection of a recently fed dog noticed whitish cords in the mesentery [4]. He described these structures for the first time in 1627 as being "milky veins" (for review see [5]). One role of the lymphatic system is to collect protein-rich fluid and macromolecules (lymph) from the extracellular space, and to return it to the blood circulation [6]. Also, lymphatics play a role in fat absorption by means of specialized lymphatic capillaries called lacteals, which are present in the villi of the intestinal mucosa [7]. The lacteals correspond to the "milky veins" described by Aselli. The fatty lymph that the lacteals collect is termed chyle and is also transported back to the blood [7]. Lymphatics also aid in the function of the immune system since they are used by lymphocytes and antigen presenting cells as conduits to reach the lymph nodes and initiate immune responses [8]. The lymph nodes, tonsils, Peyer's patches, spleen and thymus constitute the

lymphoid organs of the lymphatic system and play an important role in the immune response [9].

Lymphatic and blood vessels differ in various aspects. Lymphatic capillaries are blind-ended and are lined by a single lymphatic endothelial cell (LEC) layer which is not extensively invested by pericytes [10]. In constrast, blood vessels are a biport system and the endothelial cells are covered by pericytes. The lumens of lymphatic capillaries are generally wider and more irregularly shaped than those of blood capillaries [10]. Furthermore, lymphatic vessels are more permeable than blood vessels as they have an incomplete basement membrane, which facilitates the uptake of lymph as well as the entry of immune cells [10]. In addition, elastic fibers connected to the LEC attach the capillaries to the extracellular matrix in order to prevent the lymphatic vessels from collapsing when the interstitial pressure changes [8]. A special characteristic of lymphatic capillaries is that the LECs are loosely joined and overlap, forming "mini-valves" that allow fluid to enter the lymphatic capillary [7]. The larger collecting lymphatics are covered with pericytes and smooth muscle cells which spontaneously contract to circulate the lymph through the vessels [8]. The structural and functional differences between lymphatic and blood vessels suggest that they also differ at the molecular level [11]. At the same time, the presence of common markers emphasize the developmental and functional relationship between these two systems [12].

2. Lymphatics and Disease

2.1. Lymphedemas

Insufficient function of the lymphatic system is associated with human diseases such as the lymphedemas. Individuals with these disorders present with accumulation of lymph in the interstitial space, which causes swelling mainly of the limbs [13]. The accumulation of lymph interferes with the delivery of oxygen and other molecules [6] as it prevents the plasma (rich in oxygen and nutrients) from reaching target tissues. Also, this accumulation impaires the immune response and enhances the susceptibility of these patients to infections [6] as the high protein content of the stagnant lymph favours bacterial proliferation. As a result, fibrosis and consequent chronic inflammation and adipose degeneration occurs [6]. Lymphedemas are classified as either primary or secondary lymphedemas according to their origin.

2.1.1. Primary Lymphedemas

Primary lymphedemas have a genetic origin. To date, the genetic causes of three types of inherited lymphedema have been identified. Mutations in the gene that codes for vascular endothelial growth factor receptor-3 (VEGFR-3) have been identified in Milroy's disease, a congenital familial lymphedema (for review see [14]). VEGFR-3 is a receptor tyrosine kinase that is required for embryonic blood vessel formation [15]. Its ligand, VEGF-C, plays an important role in

lymphangiogenesis (the process by which lymphatics develop) since it has been shown to be essential for lymphatic endothelial cell migration [16]. All of the mutations, that have been identified to date, are in the kinase domain of VEGFR-3 [14] and abolish its kinase activity, suggesting that the mutated versions of this receptor act as dominant negative molecules [6].

Lymphedema Distichiasis (LD) syndrome is a condition characterized by the development of edema at or after the onset of puberty and the presence of distichiasis (growth of aberrant eyelashes from the meibomian glands) [17]. In this syndrome, mutations have been identified in the gene that codes for the transcription factor FOXC2 [17, 18]. In most cases, these mutations are a result of small insertions or deletions that led to a premature stop codon [17, 18]. A study using *Foxc2*$^{-/-}$ mice has recently showed that there is an abnormal interaction between LECs and pericytes as well as valve defects in these mice, which cause lymphedema [19].

SOX18 is the gene in which mutations have been identified for dominant and recessive cases of Hypotrichosis-Lymphedema-Telangiactasia syndrome [20]. Individuals with this syndrome develop hypotrichosis (total or partial absence of hair at birth) and lymphedema in childhood, as well as telangiectasia or vascular naevi (dilated superficial blood vessels causing dark red blotches on the skin) which occur mainly on the palms and soles [14, 20]. It is postulated that in the dominant form

of this syndrome, the mutated protein functions as a dominant negative since the activation domain of the protein is truncated and the protein may still be able to bind DNA through its DNA binding domain, thus interfering with the function of the wild type protein [20]. The fact that most familial lymphedema cases do not present with changes in any of these genes indicates that many genes responsible for lymphedema remain to be identified [14].

Lymphedema is a common feature in fetuses with Turner's syndrome (45X karyotype) (TS) [21]. Affected fetuses have a reduced number of lymphatics and enlarged lymphatic channels that end in dilated sacs as a result of a delay in the development of the connections between the lymphatic and the venous systems (for review see [22]) [23]. In severe cases, this lymphedema causes fetal death; however, in milder cases resolution can spontaneously occur and the fetus can be born [21]. As a consequence of the edema, characteristics such as webbed neck, ptosis (droppy upper eyelids and eyebrows) and low posterior hairline are present [21]. Left sided congenital cardiac anomalies such as bicuspid aortic valve and coarctation of the aorta are common in TS [22, 24]. Fetal lymphedema has been suggested as a cause, since it is thought that the edema as well as the enlarged lymphatics exerts pressure and damages the developing heart [22, 24]. The gene(s) responsible for the presence of lymphedema in TS have not been identified yet. Lymphedema is also present in Noonan syndrome (NS), a multiple congenital anomalies

syndrome, and it has been proposed as the cause for the observed webbed neck phenotype observed [25]. Pulmonary and peribronchial lymphatic dilatation is thought to be the cause for the right sided cardiac defects typically present in individuals with NS [25]. Mutations in the gene *Ptpn11*, which encodes the non-transmembrane protein-tyrosine phosphatase Shp2, occur in approximately 50% of individuals with NS. In most of the cases, these mutations alter residues involved in the regulation of the catalytic activity of this protein, suggesting that in individuals with NS this protein is constitutively active (for review see [26]) Recently, the creation of a transgenic mouse expressing a *Ptpn11* gain-of-function mutation demonstrated that defects in this gene are responsible for the Noonan Syndrome phenotype as these animals exhibit all the major features of this syndrome, including edema [26].

2.1.2. Secondary Lymphedemas

Secondary or acquired lymphedemas occur when lymphatic vessel function is impaired as a consequence of obstruction or destruction of lymphatic vessels and lymph nodes. In developed countries, secondary lymphedemas appear as a frequent consequence of radical surgery or radiotherapy for cancer treatment [27, 28]. An example is breast cancer related lymphedema (BCRL), which is the swelling of the arm or hand that occurs in some breast cancer survivors as a consequence of the removal of axillary lymph nodes and damage of lymphatic vessels by surgery and

irradiation [29]. The incidence of BCRL after mastectomy has been reported to vary from 27% to 49% (for review see [3]). In developing countries, the main cause for secondary lymphedemas is filariasis, an infectious disease caused by the parasitic filarial worms *Wuchereria bancrofti* and *Burgia malayi* [6]. This condition can lead to massive edema of the limbs and genitals, and result in permanent disability [30]. The World Health Organization has estimated that more than 120 million people suffer from filarial induced lymphedema [6].

2.2. Cancer

Lymphatics have been proposed to contribute to the dissemination (metastasis) of tumour cells to secondary sites [31]. Many solid tumours appear to spread to regional lymph nodes via the lymphatic vessels [32, 33]. It is thought that the absence of pericytes, smooth muscle cells and extensive extracellular matrix make the lymphatic capillaries more susceptible to tumour cell invasion [34]. The mechanism by which tumour cells access the lymphatic system remains unclear. A traditional model suggests that lymphatics play a passive role in tumour metastasis [28]. This model proposes that tumour cells access the lymphatic system by invading pre-existing peri-tumoural lymphatics [28, 35]. Now that orthotopic models for tumour dissemination have been developed and lymphatic markers, there is growing evidence that supports an active role of lymphatics in the process of cancer metastasis [28]. According to this

new model, the access of tumour cells to the lymphatic system is facilitated by the formation of new lymphatic vessels inside the tumour (intratumoural lymphangiogenesis) and in the surrounding area (peritumoural lymphangiogenesis) [9, 28]. Whether tumour cells access the lymphatic system through pre-existing peritumoural lymphatics or whether they do so by inducing lymphangiogenesis is still under debate. Studies using xenotransplants and orthotopic transplants have shown an association between the degree of intratumoural lymphangiogenesis and the extent of lymphatic metastasis [32, 33]. Moreover, studies on biopsies from human gastric, gallbladder and head and neck carcinomas have shown a positive correlation between the degree of intratumoural lymphangiogenesis and the amount of tumour cell spread [36-38]. However, microlymphography studies have shown that intratumoural lymphatics are disorganized and non-functional, probably as a result of the high mechanical pressure generated by the growing tumour cells [39, 40]. In addition, enlarged peri-tumoural lymphatics have been reported to facilitate tumour spread and to be sufficient for metastasis [35, 40, 41].

3. Molecular mechanisms of lymphangiogenesis

Unlike blood vessel formation (angiogenesis), lymphangiogenesis is less well understood, mainly due to the lack of specific markers that

define the lymphatic system and differentiate it from the blood vessels [42]. The study of lymphangiogenesis has been facilitated by the identification of lymphatic endothelium markers such as podoplanin, lymphatic vascular endothelium-specific marker (LYVE-1), secondary lymphoid chemokine (SLC), VEGFR-3 and Prox1 [1, 43-46]. Interestingly, studies using these markers have supported Florence Sabin's hypothesis about the development of the lymphatic system (for review see [9]), [1]. Approximately 100 years ago, she proposed that the lymphatic system develops by the sprouting of endothelial cells from the embryonic veins, which then coalesce to form the primitive lymph sacs and subsequently, the mature lymphatic networks (for review see [9]), [1].

Podoplanin is a plasma membrane protein that was first identified in glomerular epithelial cells (podocytes) [47] and which is also expressed by lymphatic endothelial cells but not by blood endothelial cells [46]. It has been shown to have an important role in the latter stages of lymphatic development [48]. Podoplanin knockout mice present with mispatterned lymphatics, enlarged cutaneous and intestinal lymphatic vessels as well as congenital lymphedema [48].

LYVE-1 is a plasma membrane protein identified as a receptor for hyaluronan, an extracellular matrix glycosaminoglycan [43]. It is expressed on the surface of lymphatic vessels and is involved in the uptake of hyaluronan from the extracellular space [49]. LYVE-1 is not specific for

LECs, as it is also expressed by normal hepatic blood sinusoidal endothelial cells [50].

3.1. Prox1 in the context of lymphangiogenesis

Prox1 is a homeodomain transcription factor recently identified as being essential for the development of the lymphatic system from pre-existing embryonic veins [1]. During mouse development, Prox1 is expressed in a subpopulation of venous endothelial cells of the embryonic veins [1]. After budding and sprouting, these cells form primitive lymphatic sacs and eventually, the entire lymphatic network [1]. In *Prox1* null mice, the budding and sprouting of these cells stops prematurely and as a result these mice do not develop a lymphatic vasculature [1]. These mice are embryonic lethal and die around embryonic day 14.5 –15 [1]. *Prox1* heterozygous mice, from all but one of the backgrounds evaluated, died within 2 or 3 days after birth and their intestines were filled with chyle [1]. This neonatal lethality suggests a haploinsufficiency effect of *Prox1* for enteric lymphatic development [1]. Apart from being required for the budding of venous endothelial cells from the embryonic veins, Prox1 has also been shown to be required by these cells to differentiate into lymphatic endothelial cells [42]. Upon expression of Prox1, these cells switch their fate and become lymphatic endothelial cells by down-regulating blood endothelial cell (BEC) markers such as laminin and CD34 while simultaneously up-regulating lymphatic endothelial cell markers such

as VEGFR-3, LYVE-1 and SLC [42] **(Figure 1).** In *Prox1* null mice, the cells that have started, but prematurely stop budding, do not express lymphatic endothelial cell markers and instead still express markers of venous endothelial cells, further supporting the hypothesis that Prox1 functions as a cell fate switch [42]. Interestingly, in *Drosophila*, prospero (the fly ortholog of Prox1) determines the fate of different neuroblasts lineages *via* its asymmetrical distribution during cell division [51, 52]. The role of Prox1 as a cell fate determinant for lymphatic endothelial cells was further bolstered by *in vitro* experiments which showed that overexpression of Prox1 was sufficient to induce BECs to express lymphatic specific markers [2, 53].

A recent study has shown that Prox1 is also required for later stages of lymphatic development [54]. A role of Prox1 in the regulation of the structure and patterning of the lymphatic vasculature has been proposed as these mice present with enlarged lymphatics in the skin of the ear and with hypoplastic, disordered and leaky lymphatic vessels in the mesenteric area [54]. Interestingly, these mice were obese, apparently as a consequence of lymphatic leakage, which was shown to stimulate adipocyte hypertrophy as well as preadipocyte differentiation [54].

3.2. A model of lymphangiogenesis

At this time, only some of the events that take place during lymphangiogenesis have been identified. Wigle *et al.* [42] proposed a

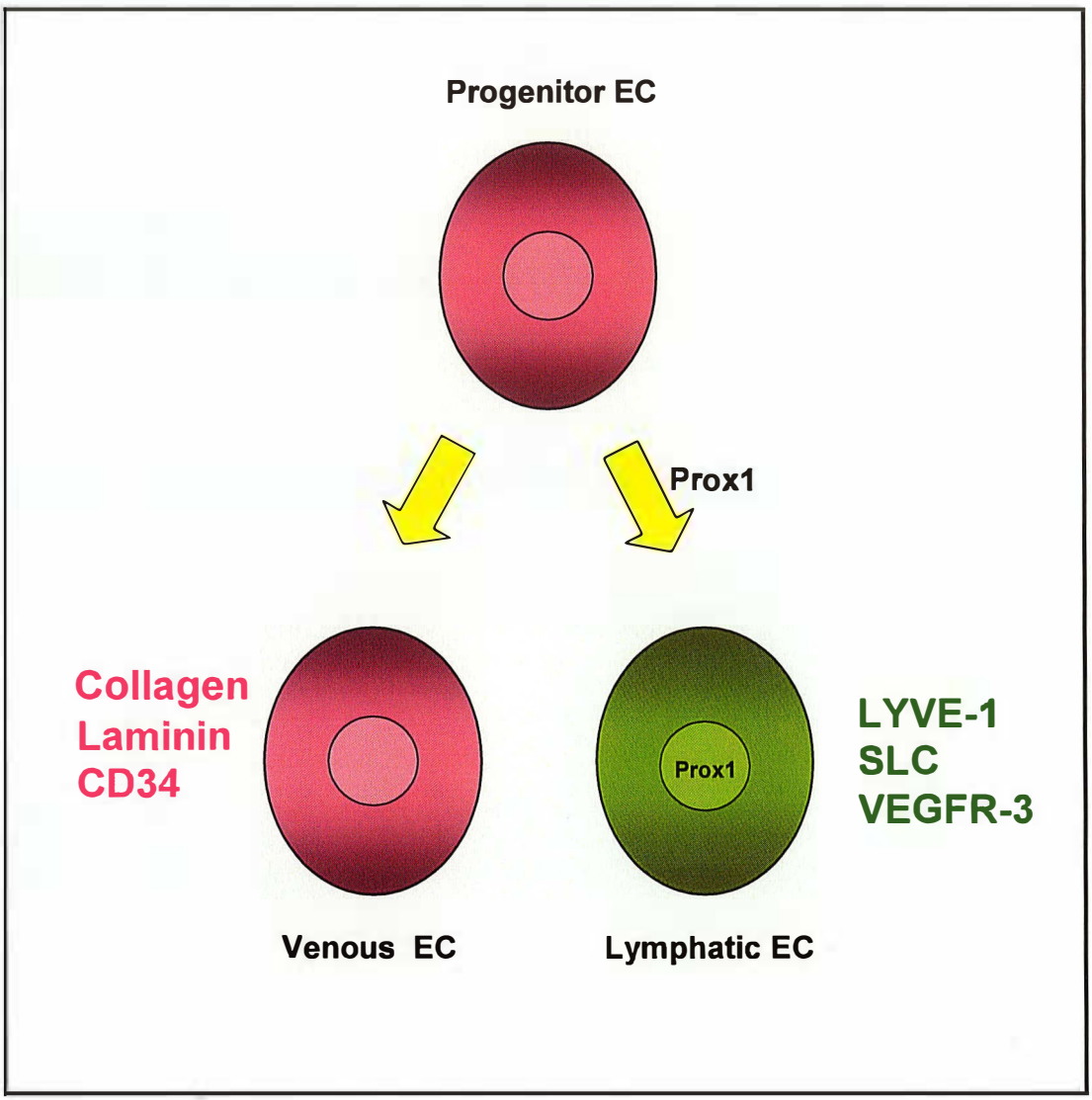


Figure 1. Potential for *Prox1 as a lymphatic endothelial cell fate switch*
During lymphangiogenesis, a subpopulation of cells in the cardinal vein start expressing Prox1. Upon expression of this homeobox gene, these venous cells switch their fate and become lymphatic endothelial cells, up-regulating the expression of lymphatic endothelial markers (green) and, down-regulating the expression of blood endothelial markers (pink) [42]. EC=Endothelial cell.

model for the development of the mammalian lymphatic vasculature. The venous endothelial cells of the cardinal vein start expressing LYVE-1 around E9.5-E10 [42]. At the same time, Prox1 starts being expressed only by a subpopulation of these cells [42]. According to this model, initially all of the blood endothelial cells in the vein are bipotent, and it is upon the expression of Prox1 that some cells become committed to the lymphatic endothelial phenotype [42]. As development continues, this subset of endothelial cells expressing LYVE-1 and Prox1 start budding from the veins in a Prox1-independent manner [42]. At this point in development, the marker VEGFR-3 is expressed equally in both types of endothelial cells [42]. Prox1 expression is required for the maintenance of the budding and migration [42]. As cells bud and migrate in an ordered manner, they start expressing other lymphatic endothelial markers such as SLC, which is detected at around E11.5 [42]. While VEGFR-3 expression remains up-regulated in lymphatic endothelial cells, its expression is decreased in blood endothelial cells [42]. Prox1 is also required to maintain the lymphatic phenotype of these cells [42]. According to this model, blood endothelial cells adopt a lymphatic phenotype when LYVE-1, Prox1, SLC and VEGFR-3 are expressed [42]. Recently, VEGF-C, a VEGFR-3 ligand, was shown to be essential for the migration of Prox1 positive endothelial cells from the cardinal vein and for the formation of the primitive lymph sacs [16]. The signals that initiate the expression of Prox1

in this subset of blood endothelial cells in the embryonic cardinal vein are still unknown [16].

4. Prox1

The homeobox gene *Prox1*, an ortholog of the *Drosophila* gene *prospero*, was first cloned in 1993 [55]. Homeobox genes are transcription factors involved in the regulation of cell growth and fate, tissue differentiation and morphogenesis during development and disease [56]. These genes are characterized by the presence of an evolutionary conserved sequence which encodes a 60 amino acid domain (homeodomain) that functions as a DNA binding and protein-protein interaction domain [57-61]. In vertebrates, according to the arrangement that these genes have in the chromosomes, they are divided into a) clustered homeobox genes (HOX genes) and b) non-clustered (or divergent) genes [62]. HOX genes are physically linked in a chromosome and their physical order on the chromosome reflects the order of their expression along the anterior-posterior body axis. However, non-clustered or divergent genes are scattered throughout the genome [63]. The *prospero/Prox1* family members belong to the non-clustered homeobox gene family [64].

The homeodomain consists of three alpha helices (α_1, α_2 and α_3) which fold to form a globular structure with an N- terminal extension [65-68]. During DNA binding, the α_3 helix (recognition helix) makes specific contacts with nucleotides located in the major groove of the DNA, while the N-terminal extension makes additional specific contacts in the adjacent minor groove [69, 70]. Apart from its function in DNA binding, the homeodomain has been reported to play a role in mediating protein-protein interactions [58, 59, 61, 71]. Although in many cases homeodomain proteins regulate the transcription of their target genes through directly binding DNA, in some instances they perform this regulation in a DNA-binding independent manner [72-76].

In *Drosophila*, prospero functions as a cell fate determinant for neuroblasts as it is essential for the correct differentiation of neuronal lineages [51, 52, 72]. As a consequence of *prospero* loss of function mutations, neuronal specific genes such as *fushi tarazu* and *engrailed* have been deregulated, leading to defects in axonal outgrowth and glia differentiation [51, 52, 77]. Prospero has also been reported to control the fate of colour photoreceptor cells in the fly [78].

Prospero orthologs, have been identified in a variety of animals including *C.elegans*, *X. laevis*, zebrafish, chicken, mouse and human [55, 79-83]. In vertebrates, Prox1 is expressed in several tissues including the lens, retina, liver, pancreas and the lymphatic endothelium [1, 55, 80, 81, 83]. In the lens, Prox1 is essential for lens-fiber differentiation and

elongation during development [84]. In the absence of Prox1, the expression of the cell cycle inhibitors $p27^{KIP1}$ and $p57^{KIP2}$ is down-regulated and as a consequence, a subset of epithelial cells that normally undergo terminal differentiation proliferate, disturbing the process of lens-fiber elongation [84]. Prox1 has a role in the control of cell cycle function in the mammalian retina as it is required for the cell cycle exit of a subset of retinal progenitor cells [85]. In the liver, Prox1 has a role in the differentiation of hepatoblasts into hepatocytes, as well as in the control of hepatocyte migration during development [86, 87]. A recent study has reported that Prox1 plays a role in the regulation of bile acid synthesis [73].

Few Prox1 target genes have been currently identified. Prox1 has been reported to regulate the transcription of the mouse *six3* and *γ-crystallin* promoters in the lens [88, 89]. However is still unknown if these genes are direct or indirect Prox1 targets. In a study of the regulation of the mouse *βB1*-crystallin promoter, Prox1 was proposed to activate this promoter by binding to a specific DNA element [90]. However, this study did not provide sufficient evidence to fully support the hypothesis that Prox1 activates this promoter in a DNA-binding dependent manner. For example, the authors did not confirm their results by using a version of Prox1 with the DNA binding domain mutated and showing that it fails to activate this promoter. Recently, Prox1 was shown to up-regulate the transcription of fibroblast growth factor receptor-3 (FGFR-3) in LECs [91].

This activation was shown to be DNA-binding dependent, through a newly identified Prox1 response element [91].

Prox1 has also been reported to act as a co-repressor of the nuclear receptors Steroidogenic Factor 1 (SF-1) and Liver Receptor Homologue 1 (LRH-1) [73, 75, 76]. SF-1 and LRH-1 belong to the Fushi tarazu factor 1 superfamily and have been shown to have an active conformation in the absence of a ligand [92]. In zebrafish, Prox1 has been shown to act as a co-repressor of Ff1b, a homolog of SF-1, during interrenal organogenesis [76]. In mammals, Prox1 represses the transcriptional activity of LRH-1, acting as a co-repressor for the transcription of Cholesterol-7-alpha hydroxylase, a rate limiting enzyme for the synthesis of bile acid [73, 75]. Therefore, Prox1 is a homeodomain transcription factor which is able to behave as an activator and a co-repressor depending on the particular gene it regulates and its binding partners.

Prospero/Prox1 family members have been classified as atypical homeodomain proteins since their homeodomain has a low level of sequence identity to other homeodomain proteins [93]. In fact, Prospero/ Prox1 family members have three extra amino acids inserted between α_2 and α_3 of the homeodomain [93]. Another exclusive feature of the Prospero family of proteins is the presence of a conserved 100 amino acid domain, the *Prospero* Domain. This domain is composed of four alpha helices (α_3-α_6) and is located 3' to the homeodomain [94]. A recent

structural study revealed that the α_3 helix connects the homeodomain and the *Prospero* Domain and that these 6 helixes together form a single structural unit, the homeo-prospero domain [94]. This study also showed that the *Prospero* Domain appears to be able to contact the DNA through residues located within or close to the N- terminus of the α_6 helix, which suggests that this domain may modulate the DNA binding specificity of the Prox1 homeodomain [94]. The *Prospero* Domain has also been proposed to regulate prospero subcellular localization by masking a nuclear export signal (NES) present in the homeodomain and thereby preventing it from being exported to the cytoplasm [95, 96]. This hypothesis has been supported by one of the findings from the prospero structure analysis study, which indicates that the C-terminus of the Prospero Domain creates a steric effect on the NES in the homeodomain, preventing access to this nuclear export signal [94].

5. Cyclin E1

Cell-cycle checkpoint controls constitute a set of regulatory pathways that regulate the efficient progression of the cell through the cell-cycle by sensing extracellular as well as intracellular growth regulatory signals and preventing the cell from proceeding to the next cell-cycle phase until the successful completion of the preceding one [97, 98] **(Figure 2).** Cyclin

dependent kinases (Cdks) are molecules required for cell-cycle control [99]. In mammals, Cdks are present throughout the cell-cycle; however, their activity is regulated according to the cell cycle-stage [97]. Cyclins are the main positive regulators of Cdks activity; each cyclin binds to and activates specific Cdks enabling them to phosphorylate their target proteins [99, 100].

Cyclin E1 (previously known as Cyclin E) is initially expressed during the late G_1 phase of the cell cycle, peaks near the G_1-S phase transition and decreases by the end of the S phase [101, 102]. Cyclin E1 regulates the activity of cdk2 and is thought to function at the G_1 checkpoint, allowing cells to proceed to S phase [102-105]. The Cyclin E1/cdk2 complex regulates the transition to S phase by phosphorylating the protein retinoblastoma (pRb) or other pocket proteins (p107, p130), which leads to the liberation of E2F proteins, allowing them to activate promoters of genes that regulate DNA synthesis [99, 106]. The activity of the Cyclin E1/cdk2 complex is regulated, in part, by the synthesis and degradation of Cyclin E1 and by the binding of cdk inhibitors (CKI) such as $p27^{KIP1}$ and $p21^{Waf1}$ [99].

Recently, a novel cyclin was identified and because of its homology to Cyclin E1 was named Cyclin E2 [107-109]. Cyclin E2 also activates cdk2 and is inhibited in complex with this cyclin dependent kinase by $p27^{KIP1}$ and $p21^{Waf1}$ [107-109]. As Cyclin E1 and Cyclin E2 are quite homologous, similar functions have been suggested for them [110].

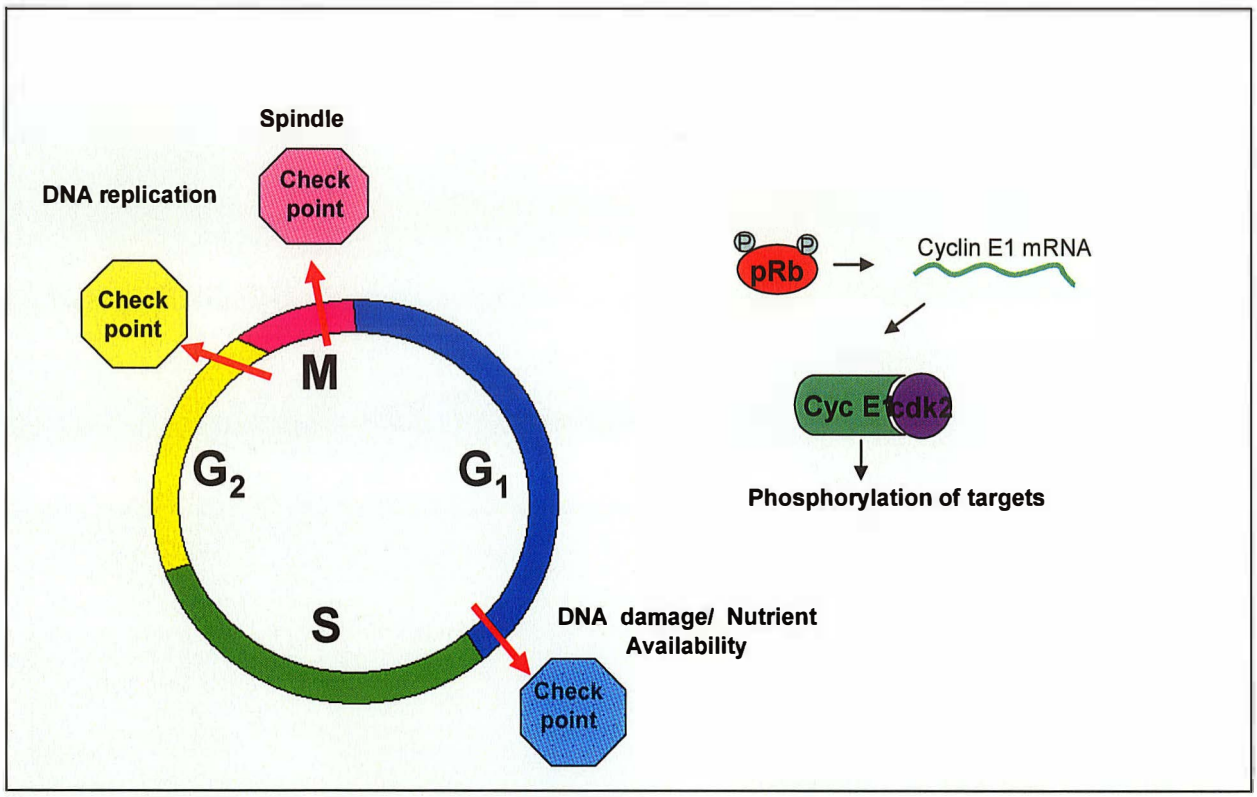


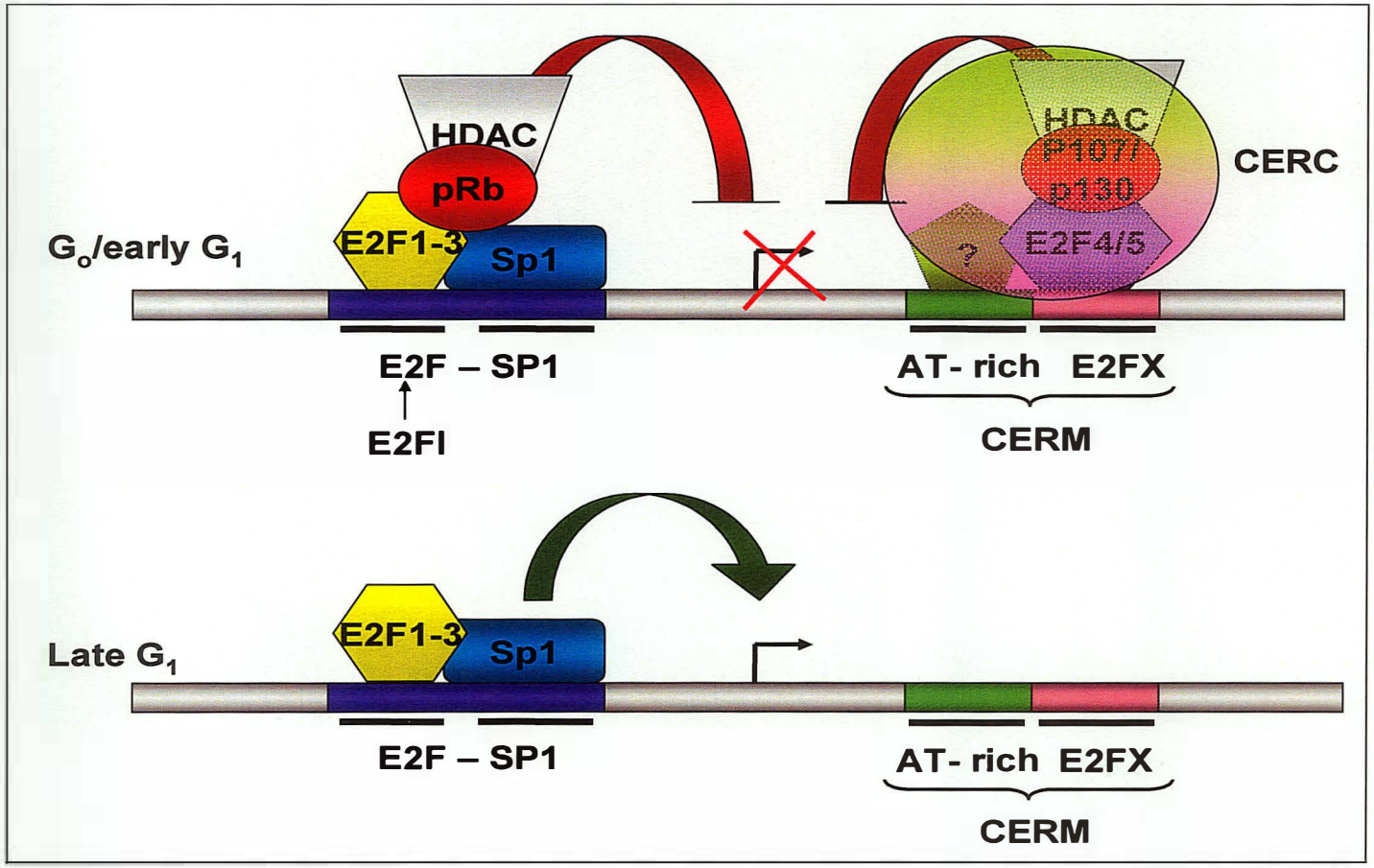
Figure 2. ***Cell-cycle and checkpoints***
The cell cycle is divided in two main phases: Interphase (which includes G_1, S and G_2) and mitosis (cell division). G_1 is a stage of cellular growth where new organelles are synthesized preparing the cell for division. During S phase the cells replicates its DNA. During G_2, the cell continues growing and synthesizes proteins required for mitosis. Cell-cycle progression is controlled by checkpoints. The G_1 checkpoint senses damaged DNA and if present, the cell cycle is arrested and the DNA repaired before entering S phase. It also monitors that the cell is large enough to divide and that the environment provides enough nutrients for the daughter cells. The G_2 check point senses unreplicated and damaged DNA which prevents the cell from entering M phase until the DNA is completely replicated and repaired. The mitosis checkpoint verifies the alignment of the chromosomes in the mitotic spindle and ensures equal chromosomal distribution to the daughter cells [98]. *Cyclin E1* activation of transcription is mediated by the phosphorylation of pRb. Once expressed, Cyclin E1 associates with and activates cyclin dependant kinase 2 allowing for the phosphorylation of targets involved in DNA synthesis [118].

Previously, it has been a dogma that E type Cyclins are essential for cells to enter S phase. However, the nearly normal development of the *Cyclin E1* + *Cyclin E2* double knockout mouse has shown that this concept is inadequate [111]. Studies using cells deficient in E-type Cyclins have revealed that these Cyclins are dispensable for S phase transition of cycling cells but are essential for cells cycle re-entry from the G_0 state [111]. While *Cyclin E1/E2* null embryos are normal except for cardiac anomalies of variable severity (wild type as well as knockout embryos die at birth as a consequence of the tetraploid complementation approach), mice lacking cdk2 are viable but present with meiotic failure, gonadal hypertrophy and sterility [112]. The fact that cdk2 is the only known partner of E-type cyclins in mice, and that the phenotypes of the Cyclin E1/E2- null mice and the mice lacking cdk2 are different suggest that E-type cyclins also function independently of cdk2 and/or that a novel cdk partner for this type of cyclins has not yet been identified [99, 112].

Recently, *Cyclin E1* and *Cyclin E2* were identified as being two genes which were up-regulated upon the over-expression of Prox1 in blood endothelial cells [2]. In this same study, transient transfection of Prox1 was shown to stimulate transcription of *Cyclin E1* [2]. However, the mechanism by which Prox1 induces *Cyclin E1* transcription has still not been elucidated. During lymphangiogenesis, the number of endothelial cells budding from the cardinal vein increases with time [1]. In Prox1 null embryos, however, only a few endothelial cells are seen budding and as a

consequence of loss of Prox1 function, these cells have not differentiated towards a lymphatic endothelial phenotype [1]. In *Drosophila*, *prospero* up-regulates *Cyclin E* thereby promoting proliferation of glial cells during growth cone guidance [113]. As well, Cyclin E has been shown to be critical for the correct specification of segment-specific neural lineages in the fly [114]. This role has been suggested to be independent of Cyclin E's role as a cell-cycle regulator [114]. It is possible that during lymphangiogenesis, Prox1 mediated up-regulation of *Cyclin E1* is required for the proliferation of the cells budding from the cardinal vein and thus for the maintenance of budding, as well as for the differentiation of these cells towards a lymphatic endothelial phenotype.

The periodic regulation of *Cyclin E1* transcription is controlled by two variant E2F elements which form part of two regulatory modules located proximal to the transcription start site [115, 116] **(Figure 3).** E2F-Sp1 is a bipartite module located upstream of the transcription start site and has been shown to be constitutively occupied throughout the cell cycle [115]. This bipartite module encompasses many overlapping Sp1 binding sites and a variant E2F site named E2FI [115]. As Sp1 has been shown to bind E2F1-3 but not E2F4-5, it is believed that Sp1 and E2F1-3 bind to the E2F-Sp1 module [117, 118]. During repression, pRb binds to the E2F complex and recruits Histone Deacetylase 1 (HDAC1), which causes the deacetylation of a single nucleosome located at the transcription start site, thus preventing transcription [119]. In late G_1, pRb and HDAC1 dissociate



Modified from [118]

Figure 3. ***A model for the regulation of Cyclin E1 transcription.***
The regulation of transcription of the *Cyclin E1* promoter is regulated by two main regulatory modules, E2F-Sp1 and CERM. During repression (G_0/early G_1), pRb binds to E2F1-3 and Sp1 in the E2F-Sp1 module, and recruits HDAC1. In addition, CERC binds to CERM. For transcription to take place (late G_1), CERC is released from CERM, and pRb and HDAC1 are released from the E2F-Sp1 module and E2F1-3 and Sp1 are able to activate transcription [118,119].

from the E2F complex [119]. As the E2F-Sp1 site has been shown to be occupied regardless of the cell cycle stage, it is believed that after pRb and HDAC1 dissociate, the Sp1-E2F1-3 complex remains bound and activates transcription [118] **(Figure 3).** The E2FI site in the Sp1-E2F module has been shown to be required for *Cyclin E1* activation of transcription in late G_1 [115]. The second E2F element (E2FX) is located downstream of the transcription start site and together with an AT-rich region forms part of a repressor module termed CERM (*Cyclin E* Repressor Module) [116]. In contrast to the E2F-Sp1 module, CERM is occupied only during *Cyclin E* repression, therefore it is occupied in G_0 and early G_1, is not occupied in late G_1 and M phase and is occupied again in the next G_1 phase [116] **(Figure 3)**. The complex that binds to CERM is known as CERC (*Cyclin E* Repressor Complex) and consists of a pocket protein, E2F4, DP-1 (E2F Dimerization Partner 1), an HDAC activity and other as yet unidentified proteins [101, 116]. Prox1 has been reported to activate transcription of *Cyclin E1* in an apparently DNA-binding dependent manner [2]. However, the mechanism by which Prox1 activates *Cyclin E1* is currently unknown.

II. RATIONALE

Disorders of lymphatic vessels contribute to the progression of diseases such as lymphedema and cancer. Lymphedemas develop when lymphatic function is inadequate and as consequence, lymph accumulates in the interstitial space, leading to swelling that mainly occurs in the limbs [13]. Lymphatics have been proposed to play an important role in the dissemination of tumour cells to secondary sites [31]. In the case of many solid tumours, tumour cells spread to regional lymph nodes through the lymphatic vessels [32]. However, the mechanism by which these cells initially enter the lymphatic system remains unclear. A current model suggests that tumour cell entry into the lymphatic vessels is facilitated by the creation of new lymphatics (lymphangiogenesis) both inside the tumour and in the surrounding tissue [9, 28]. The process of lymphangiogenesis has been poorly understood due to the lack of specific lymphatic markers [42]. The recent discovery of such markers has facilitated the study of lymphatic development and has shown that the lymphatic system develops by the sprouting of endothelial cells from embryonic veins [1]. A better understanding of the events involved in this process will facilitate the development of improved approaches to either promote or arrest lymphangiogenesis to more effectively treat lymphedemas and tumour metastasis, respectively.

Prox1 plays an essential role in lymphangiogenesis. It has been shown to be required for the budding of venous endothelial cells and for maintaining their

differentiation towards a lymphatic phenotype [42]. Upon expression of *Prox1*, the venous endothelial cells that start to bud from the cardinal vein switch their fate and become lymphatic endothelial cells, down-regulating the expression of blood vascular genes and up-regulating the expression of lymphatic markers [42]. The subset of genes directly regulated by Prox1 in this context has not been yet identified. *Cyclin E1* transcription has been shown to be up-regulated upon expression of *Prox1* in blood endothelial cells [2]. Work by Petrova *et al.* suggested that Prox1-mediated regulation of *Cyclin E1* was *via* a DNA-dependent mechanism [2]. In *Drosphila*, *Cyclin E* is positively regulated by prospero in glial cells during growth cone guidance, thus promoting glia proliferation [113]. Moreover, Cyclin E has been shown to have an essential role in determining the cell fate of the segment-specific neural lineages of *Drosophila* [114]. Therefore, during lymphangiogenesis, Prox1 mediated up-regulation of *Cyclin E1* may be required for the maintenance of the budding of venous endothelial cells and for their differentiation toward the lymphatic phenotype. The identification of the mechanism by which Prox1 activates the transcription of *Cyclin E1* will help to better delineate the process of lymphangiogenesis, facilitating the development of therapies design to regulate this process.

III. HYPOTHESIS

Given that prospero up-regulates *Cyclin E1* expression in the fly [113] and, that Prox1 has been reported to activate the transcription of *Cyclin E1* in blood endothelial cells [2], we hypothesized that Prox1 activates the transcription of *Cyclin E1* by directly binding to a DNA response element in its promoter.

IV. OBJECTIVES

Our overall objective was to identify the mechanism by which Prox1 activates *Cyclin E1* transcription. To achieve this objective, the project was broken down into the following three specific aims:

1. To establish whether Prox1 activates *Cyclin E1* expression in a DNA-binding dependent or DNA-binding independent manner.

2. To determine the domain(s) of Prox1 required to activate transcription of *Cyclin E1.*

3. To identify the region in the *Cyclin E1* promoter necessary for Prox1 mediated activation.

V. MATERIALS AND METHODS

1. Materials

Restriction endonucleases were purchased from either Invitrogen or New England Biolabs. Chemicals were purchased from Fisher, Sigma or EM Science and were of molecular biology grade.

2. Cloning

2.1. Prox1 expression constructs

The full length mouse *Prox1* cDNA (clone 6490801, Invitrogen) was cloned into the *EcoR*I/*Sac*II sites of the pBluescriptII KS (+) vector (Stratagene). This construct was used as the template to amplify all versions of Prox1 that were required for this study (see below). The amplified *Prox1* inserts were then ligated into the pCMV-Tag 4A mammalian expression vector (Stratagene), transformed into DH5α *E. Coli* and sequence verified (University of Calgary). The different Prox1 versions were generated as follows:

2.1.1. Prox1 Wild Type (WT)

Prox1 WT was obtained by PCR amplification using a primer (Mx10) which flanked the 5' untranslated region of *Prox1* and a 3' primer (Mx49) in which a *Xho*I restriction enzyme site was added to allow directional cloning into the pCMV-Tag 4A vector (Table1). The product was then cloned into the *Sac*II/*Xho*I sites of pCMV-Tag 4A, in frame with the carboxyl terminal FLAG epitope that this vector encodes.

2.1.2. Prox1 DNA Binding Domain mutation (Prox1 DBDmut)

Mutagenesis of the Prox1 DNA binding domain was achieved by splice overlap PCR [120] using a primer (Px1) encoding two point mutations that changed the amino acid sequence WF**SN** (aa 623 to 626) present in the third helix of the homeodomain, to WF**EE** [94]. Also, this primer contained a silent mutation that created an *EcoR*I restriction enzyme site which facilitated screening for the mutation. The mutagenesis primer as well as the other primers used to obtain this version of Prox1, are listed in Table 1. The final product was then ligated into the *Sac*II/*Xho*I sites of the pCMV-Tag 4A vector.

2.1.3. Prox1 Homeodomain deletion (Prox1 HDΔ)

Deletion of the Prox1 homeodomain was performed by splice overlap PCR using a primer (Px3) which deletes amino acids 578 to 636. The primers used to obtain this version of Prox1 are listed in Table 1. The final product was then cloned into the *Sac*II/*Xho*I sites of the pCMV-Tag 4A vector.

2.1.4. Prox1 Prospero Domain deletion (Prox1 PDΔ)

Deletion of the Prox1 *Prospero* Domain (aa 648 to 737) was achieved by PCR using the primers listed in Table 1. The product was then inserted into the *Sac*II/*Xho*I sites of the pCMV-Tag 4A vector.

2.1.5. Prox1 Homeodomain + Prospero Domain deletion (Prox1 HDPDΔ)

Truncation of the Prox1 Homeodomain and *Prospero* Domain (aa 578 to 737) was achieved by PCR using the primers listed in Table 1. The product was then cloned into the *Sac*II/*Xho*I sites of the pCMV-Tag 4A vector.

2.1.6. Prox1 NR1 mutation (Prox1 NR1mut)

Prox1 $NR1^{mut}$ was obtained by splice overlap PCR using a primer containing point mutations that changed the NR1 box sequence **L**RK**L**L (aa 70 to 74) to **A**RK**A**L [73] . Also, a silent mutation that created a *Bgl*I

restriction enzyme site was added to allow screening for the mutation. The primers used to obtain this version of Prox1 are listed in Table1. The product was then inserted into the *Sac*II/*Pfm*I sites of pCMV-Tag 4A/ Prox1 WT to replace the *Sac*II/*Pfm*I fragment of Prox1 WT.

2.1.7. Prox1 Q rich region deletion (Prox1 QΔ)

Prox1 QΔ was obtained by splice overlap PCR using a primer containing a deletion for the glutamine (Q) rich region (aa 211 to 259). The primers used to amplify this version of Prox1 are listed in Table 1. The product was then cloned into the *Sac*II/*Xho*I sites of the pCMV-Tag 4A vector.

2.2. Mouse *Cyclin E1* promoter constructs

The BAC genomic clone RP2377J9 (Invitrogen), encompassing the mouse *Cyclin E1* gene was used as a template to amplify the different versions of the *Cyclin E1* promoter used in this study. The different versions of the *Cyclin E1* promoter were cloned into either pCR-Blunt vector (Invitrogen) or pBluescriptII KS (+) vector (Stratagene) and sequence verified (University of Calgary).

2.2.1. 1 Kb *Cyclin E1* promoter (ProCE promoter)

A 1 Kb *BamH*I fragment overlapping the transcription site of the mouse *Cyclin E1* promoter (-905/+95) was amplified using the FailSafe PCR

System (Epicenter) and the primers listed in Table 2. The PCR product was cloned into the pCR-Blunt vector (Invitrogen). The 1 Kb *Cyclin E1* promoter fragment was excised with *BamH*I and then cloned into the *Bgl*II site of the pGL3–Basic reporter vector (Promega). Orientation of the insert was determined by restriction enzyme analysis.

2.2.2. 557 bp *Cyclin E1* promoter

A 557 bp fragment of the ProCE promoter (-462/+95) was amplified by PCR using the FailSafe PCR System (Epicenter) and the primers listed in Table 2. The PCR product was cloned into the pCR-Blunt vector (Invitrogen). The 557 bp *Cyclin E1* promoter fragment was excised with *Kpn*I and *BamH*I and inserted into the *Kpn*I/*Bgl*II sites of the pGL3–Basic reporter vector (Promega).

2.2.3. 206 bp *Cyclin E1* promoter

A 206 bp fragment of the ProCE promoter (-111/+95) was amplified by PCR using the FailSafe PCR System (Epicenter) and the primers listed in Table 2. The PCR product was cloned into the *Kpn*I and *BamH*I sites of the pBluescript II KS (+) vector (Stratagene). The 206 bp fragment was excised with *Kpn*I and *BamH*I and cloned into the *Kpn*I/*Bgl*II sites of the pGL3-Basic reporter vector (Promega).

2.2.4. 206 bp E2FX mutation *Cyclin E1* promoter (206 bp mut E2FX)

Mutagenesis of the E2FX site (nts +29/+36) was accomplished by splice overlap PCR using a primer containing point mutations that changed TGT**CCC**GC to TGT**AGA**GC [116]. This primer also contains a silent mutation that creates a *Sac*I restriction enzyme site to facilitate for screening of the mutation. The primers used to obtain the 206 bp mut E2FX *Cyclin E1* promoter are listed in Table 2. The PCR product was cloned into the *Kpn*I and *BamH*I sites of the pBluescript II KS (+) vector (Stratagene). The 206 bp E2FX mutation *Cyclin E1* promoter fragment was excised with *Kpn*I and *BamH*I and cloned into the *Kpn*I/*Bgl*II sites of the pGL3-Basic reporter vector (Promega).

2.2.5. 206 bp E2FI mutation *Cyclin E1* promoter (206 bp mut E2FI)

Mutagenesis of the E2FI site (nts -12/-18) was accomplished by splice overlap PCR using a primer containing point mutations that changed **GC**GC**GA** to **AA**GC**TT** [116]. This primer also contains a silent mutation that creates a *Hind*III restriction enzyme site to facilitate for screening of the mutation. The primers used to obtain the 206 bp mut E2FI *Cyciln E1* promoter are listed in Table 2. Adenine overhangs were added to the 3' of the PCR product according to the manufacturer protocol for the TOPO-TA Cloning kit. (Invitrogen). The PCR product was then cloned into the pCR2.1-TOPO (Invitrogen). The 206 bp E2FI mutation *Cyclin E1* promoter fragment was excised with *Kpn*I and *BamH*I and

cloned into the *Kpn*I/*Bgl*II sites of the pGL3-Basic reporter vector (Promega).

3. Cell Culture and Transient Transfections

Human Embryonic Kidney 293A cells (HEK 293A, Qbiogene) were grown in DMEM (Invitrogen) supplemented with 5% vol/vol fetal bovine serum (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen) at 37°C and 5% CO_2. Human U-2 osteosarcoma cells (U2OS, ATCC) were grown in McCoy's 5A Media (Invitrogen) supplemented with 10% vol/vol fetal bovine serum (Invitrogen) at 37°C and 5% CO_2.

Cells were plated two days before transfection and were transfected when a density of 80-90% confluency was reached. Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction and using a Lipofectamine (μL):DNA (μg) ratio of 2:1. Briefly, prior to transfection, cells were incubated with 10% calf serum in OptiMEM (Invitrogen). For each transfection, the desired amount of DNA was diluted with OptiMEM to a final volume of 250 μL. In a separate tube the corresponding amount of Lipofectamine 2000 was diluted with OptiMEM to a final volume of 250 μL. The DNA and Lipofectamine 2000 dilutions were mixed and incubated for 20-30 minutes at room temperature and then added to the cells in a

drop-wise manner. After 4 hours, the media was removed and changed for DMEM supplemented with 5% FBS and 1% Penicillin/Streptomycin.

4. Western Blotting

Two days prior to transfection, 6 x 10^5 cells were plated onto 10 cm^2 tissue culture plates. Cells were transfected with 12 μg of the pCMV-Tag 4A vector encoding the Prox1 version of interest. Two days after transfection, cells were lysed with new RIPA Buffer, (50mM Tris, 150mM NaCl, 1mM EDTA, 1mM, 1% Triton, 1% Na Deoxycholate, SDS 0.1%, pH=7.4). Protein concentrations of the cell lysates were measured using the DC protein assay (Bio-Rad, [121]) to ensure equal loading. 10-20 μg of protein were electrophoresed in an 8 or 10% SDS-PAGE gel and then immersion transferred onto a nitrocellulose membrane (BioRad). After blocking overnight with 5% skim milk powder in Tris Buffer Saline (TBS) (50mM Tris, 140mM NaCl, 2.7mM KCl, pH=8) at 4°C, immunoblotting was performed with the appropiate primary and secondary antibodies.

To detect the epitope tagged Prox1, the membrane was treated with a mouse anti-FLAG M2 monoclonal antibody (1:10000 dilution, Sigma) for 1 hour at room temperature. After washing the membrane with TBS-T (0.5% Tween in TBS) 3 times for 10 minutes each, the membrane was then treated with a goat anti-mouse IgG (H+L) horseradish

peroxidase conjugate (1:2500 dilution, Molecular Probes) for 1 hour at room temperature. For actin detection, the membranes were treated with a rabbit anti-actin antibody (1:10000 dilution, Sigma), followed by a goat anti-rabbit IgG horseradish peroxidase conjugate (1:2500 dilution, Molecular Probes).

In all the cases, peroxidase activities were detected with the ECL plus Western Blotting Detection System (Amersham Biosciences).

5. Immunocytochemistry

Two days prior to transfection, 15 – 20 x 10^4 cells were plated onto sterile glass coverslips in 6 well plates. Cells were transfected with 4 μg of the pCMV-Tag 4A vector encoding the Prox1 version of interest. Two days after transfection, cells were fixed with 4% paraformaldehyde (EM Chemicals) and permeabilized by treatment with PBS-T [0.3% Triton X-100 (MP Biomedicals) in Phosphate Buffer Saline (PBS) (67mM NaCl, 3mM KCl, 1mM KH_2PO_4, mM Na_2HPO_4)]. After blocking with 5% goat serum (Sigma) in PBS for 1 hour, cells were treated with a mouse anti-FLAG M2 monoclonal antibody (1:1000 dilution, Sigma) overnight at 4 °C. After washing with PBS-T, cells were treated with a goat anti-mouse Texas Red dye-conjugated (1:200 dilution, Jackson Immuno Research Laboratories) for 1 hour. Nuclei were stained using the Slow Fade

Antifade Kit with DAPI (Molecular Probes). Samples were visualized by fluorescence microscopy (Axioskop2 mot plus microscope, Zeiss) and images captured using the Axio Cam color 412-312 camera and AxioVision4 software.

6. Luciferase Assay

Two days before transfection, 8 – 10 x 10^4 cells were plated onto 6 well plates. Cells were transfected with 1 μg of the pCMV-Tag 4A vector encoding the Prox1 version of interest, 1 μg of the appropriate luciferase reporter vector and 1 μg of the β–galactosidase expression plasmid pcDNA3-LacZ (gift from Dr. Mesaeli). Two days after transfection, cells were lysed with NP40 buffer (10% NP40, 0.5 mM DTT) and luciferase as well as β–galactosidase activities were measured as described below. For the luciferase assay, 20 μL of the cell lysates were aliquoted in duplicate into tubes and mixed with 100 μL of luciferase buffer (20mM Tricine, 1.07mM $MgCo_3$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270μM coenzyme A, 470μM luciferin, 530μM ATP) using a Luminometer (Lumat LB 9507). The β–galactosidase assay was performed by aliquoting 18 μL of the cell lysates in duplicate into a 96-well plate and mixing them with 70 μL of water and 30 μL of 2.67 mg/mL ONPG. After one hour of incubation at 37°C, the OD (415 nm) of this mixture was determined using a

microplate reader (Dynex technologies). In order to normalize for differences in transfection efficiency, the luciferase values were divided by the β–galactosidase values (Relative Luciferase Activity). Each experiment was done in triplicate and repeated at least three times. The results are presented as relative luciferase activity or fold activation with respect to the control. For the fold activation values, the relative luciferase activities of the different treatments were divided by the relative luciferase activity obtained for the control.

7. Statistics

Statistical analysis was performed using the graphing and data analysis software Origin 7.5. Statistical differences were obtained using the analysis of variance between groups (ANOVA) test. A P value < 0.05 was considered as being significantly different.

Table1. *Prox1* primers

Prox1 version	Primer Name	Primer sequence	Restriction Enzyme site added
Prox1 WT	Mx 10	F: 5'-GC**ggatcc**TAATACGACTCACTATAGGGC-3'	*BamH*I
	Mx 49	R: 5'-CC**ctcgag**CTCGTGAAGGAGTTCTTGTAG-3'	*Xho*I
Prox1 DBD^{mut}	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 1	R: 5'-GTAAAACTCACG**GAATTC**CTCGAACCACTT-GATGAGCTGCGAGG-3'	*EcoR*I
	Px 2	F: 5'-GATGTGGATCGCTTATGTGATGAGCACC-3'	
	Mx 49	R: 5'-CC**ctcgag**CTCGTGAAGGAGTTCTTGTAG-3'	*Xho*I
Prox1 HDΔ	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 3	R: 5'-**GGCTTGGCGCGCATACTTCTCCTGCATTG-CGCTTCCTGAATAAGGTG**-3'	
	Px 2	F: 5'-GATGTGGATCGCTTATGTGATGAGCACC-3'	
	Mx 49	R: 5'-CC**ctcgag**CTCGTGAAGGAGTTCTTGTAG-3'	*Xho*I
Prox1 PDΔ	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 7	R: 5'-CC**ctcgag**TCCATCATTGATGGCTTGACGCGC-3'	*Xho*I
Prox1 HDPDΔ	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 8	R: 5'-CC**ctcgag**CTGCATTGCGCTTCCTGAATAAGG-3'	*Xho*I
Prox1 NR1^{mut}	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 23	R: 5'-CTCATCACATAAGCGATCCACATCAAAC-3'	
	Px 22	F: 5'-GTCGAACGTA**GCCCGCAAGGC**GCTGAAG-AGGGCGAACTCGTATG-3'	*Bgl*I
	Mx 49	R: 5'-CC**ctcgag**ATGCTTGCCTTCTGGCTGCAAGG-3'	*Xho*I
Prox1 QΔ	Mx 10	F: 5'-GCGGATCCTAATACGACTCACTATAGGGC-3'	
	Px 30	R: 5'-**CGGAGTCTGTGCTGTCATAGACCTTGCG-TTTGTTTTCGCGATAACTTTC**-3'	
	Px 31	F: 5'-**GAAAGTTATCGCGAAAACAAACGCAAGG-**	

	Mx 49	**TCTATGACAGCACAGACTCCG**-3' R: 5'-CC**ctcgag**CTCGTGAAGGAGTTCTTGTAG-3'	*Xho*I

Note: Restriction enzymes sites are showed in **blue**. Mutations are shown in **bold** and are <u>underlined</u>.
Deletion primers are shown in **bold**.

Table 2. *Cyclin E1* promoter primers

Cyclin E1 promoter version	Primer Name	Primer sequence	Restriction Enzyme site added
1000 bp	Px 18 Px 19	F: 5'-CCTTCAAGTTTTCCGGAAGCACAAACAGCTGGAATGGG-3' R: 5'-GGAGTCCAGGCAGCCCGTACCCGAAGCTGTGTCC-3'	
557 bp	Px 24 Px 19	F: 5'-TGC**ggtacc**GGAGACCGGCGGATGACGGGTTCTTAACTC-3' R: 5'-GGAGTCCAGGCAGCCCGTACCCGAAGCTGTGTCC-3'	*Kpn*I
206 bp	Px 32 Px 19	F: 5' **ggtacc**GCCCCCACCAGAGCTCCTCGCTGGTC-3' R: 5'-GGAGTCCAGGCAGCCCGTACCCGAAGCTGTGTCC-3'	*Kpn*I
206 bp mut E2FX	Px 32 Px 40 Px 38 Px 19	F: 5' **ggtacc**GCCCCCACCAGAGCTCCTCGCTGGTC-3' R: 5'-GGCTTCGAGCTCTACATTTAAAAA-3' F: 5'-TTTTTAAATGT**AGAGCTCG**AAGCC-3' R: 5'-GGAGTCCAGGCAGCCCGTACCCGAAGCTGTGTCC-3'	*Kpn*I *Sac*I
206 bp mut E2FI	Px 32 Px 49 Px 48 Px 19	F: 5'- **ggtacc**GCCCCCACCAGAGCTCCTCGCTGGTC-3' F: 5' CGGCCCCGTCCCGCCC**AAGCTT**CCCGCCCCGAG-3' F: 5-CTCGGGGCGGGAAGCTTGGGCGGGACGGGGCCG-3' R: 5'-GGAGTCCAGGCAGCCCGTACCCGAAGCTGTGTCC-3'	*Hind*III

Note: Restriction enzymes sites are showed in **blue**.
Mutations are shown in **bold** and are underlined.

VI. RESULTS

1. Characterization of the subcellular localization and expression of the different Prox1 proteins

In order to determine the domain(s) of Prox1 necessary for activating *Cyclin E1* transcription, different Prox1 versions were created by either deleting or mutating the different known Prox1 domains **(Figure 4)**. To verify that the different Prox1 versions still localized to the nucleus and that they were stably expressed in comparable amounts, immunocytochemistry and western blots were performed using Human Embryonic Kidney 293 (HEK 293) and Human U-2 osteosarcoma (U2OS) cells transfected with vectors encoding the different Prox1 versions. HEK 293 cells were used because of their ease of transfection. U2OS cells were used since Prox1 was previously shown to activate transcription of *Cyclin E1* in a DNA-binding dependent manner in this cell type [2], and we were interested in corroborating this finding.

Immunocytochemistry results of Prox1 transfected HEK 293 cells showed that all the different Prox1 proteins were localized exclusively to the nucleus except for Prox1 PDΔ, which was also localized to the cytoplasm in a population of cells **(Figure 5)**. Western blot results of transiently transfected HEK 293 cells revealed that all the Prox1 proteins

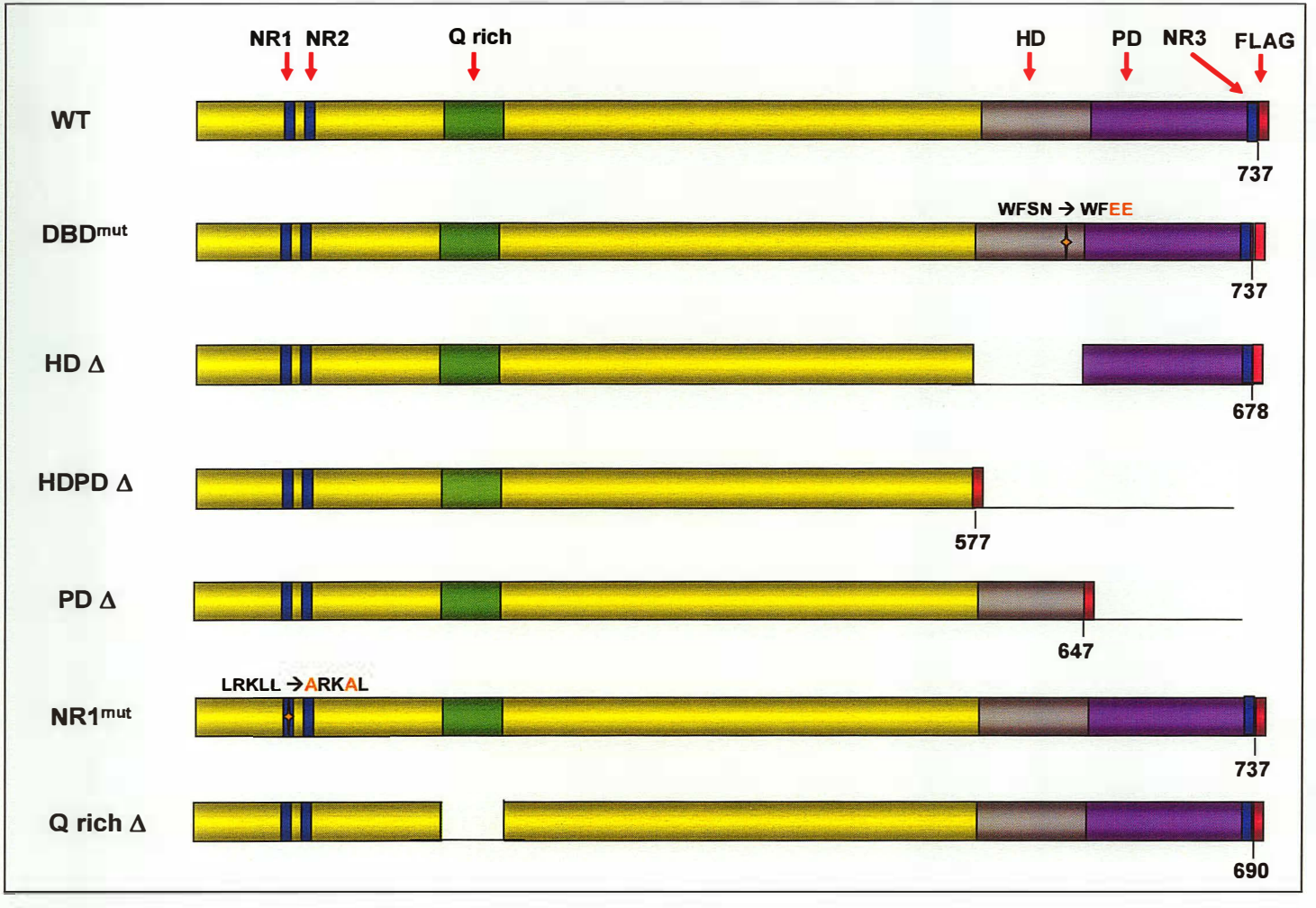


Figure 4. ***Potential Functional Domains of Prox1***
Wild-type and mutant Prox1 versions used in this study. The different Prox1 domains are indicated by red arrows. Deletions performed are indicated as black lines. Mutated amino acids (ϟ) are indicated in orange. All versions of Prox1 were FLAG tagged at the C-terminal end by cloning them into the pCMV-Tag 4A vector. NR1=Nuclear box 1. NR2 =Nuclear box 2. NR3=Nuclear box 3. Glutamine rich region=Q rich. HD=Homeodomain. PD=*Prospero* Domain.

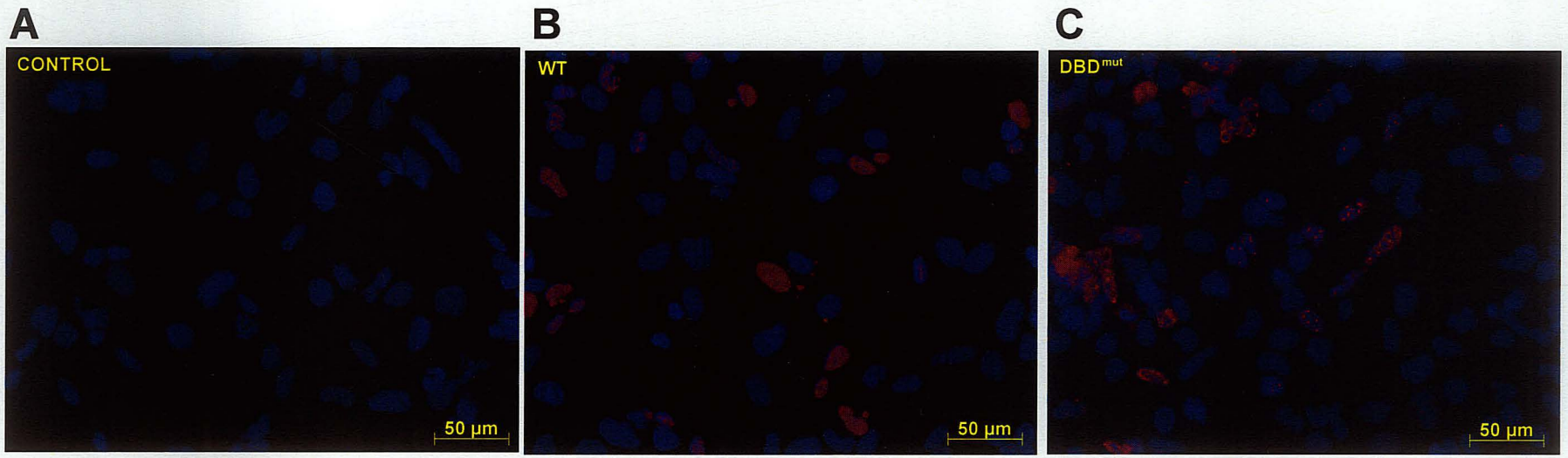


Figure 5. Nuclear localization of Prox1 versions in HEK 293 cells.
Anti-FLAG Immunocytochemistry (red) of cells transfected with pCMV-Tag 4A vector (Control) **(A)** or the respective Prox1 version **(B-H)**. Nuclei were stained with DAPI (blue). The different Prox1 versions localize to the nucleus (pink staining). Prox1PDΔ localizes to both the nucleus and cytoplasm (**F,G** arrows). **B**: Prox1 Wild Type (WT). **C**: Prox1 DNA Binding Domain (DBD^{mut}). **D**: Prox1 Homeodomain deletion (HDΔ), **E**: Prox1 Homeodomain + *Prospero* Domain deletion (HDPDΔ). **F**: Prox1 *Prospero* Domain deletion (PDΔ). **G**: Enlarged view of F. **H**: Prox1 NR1 mutation ($NR1^{mut}$). **I**: Prox1 Q rich deletion (QΔ).

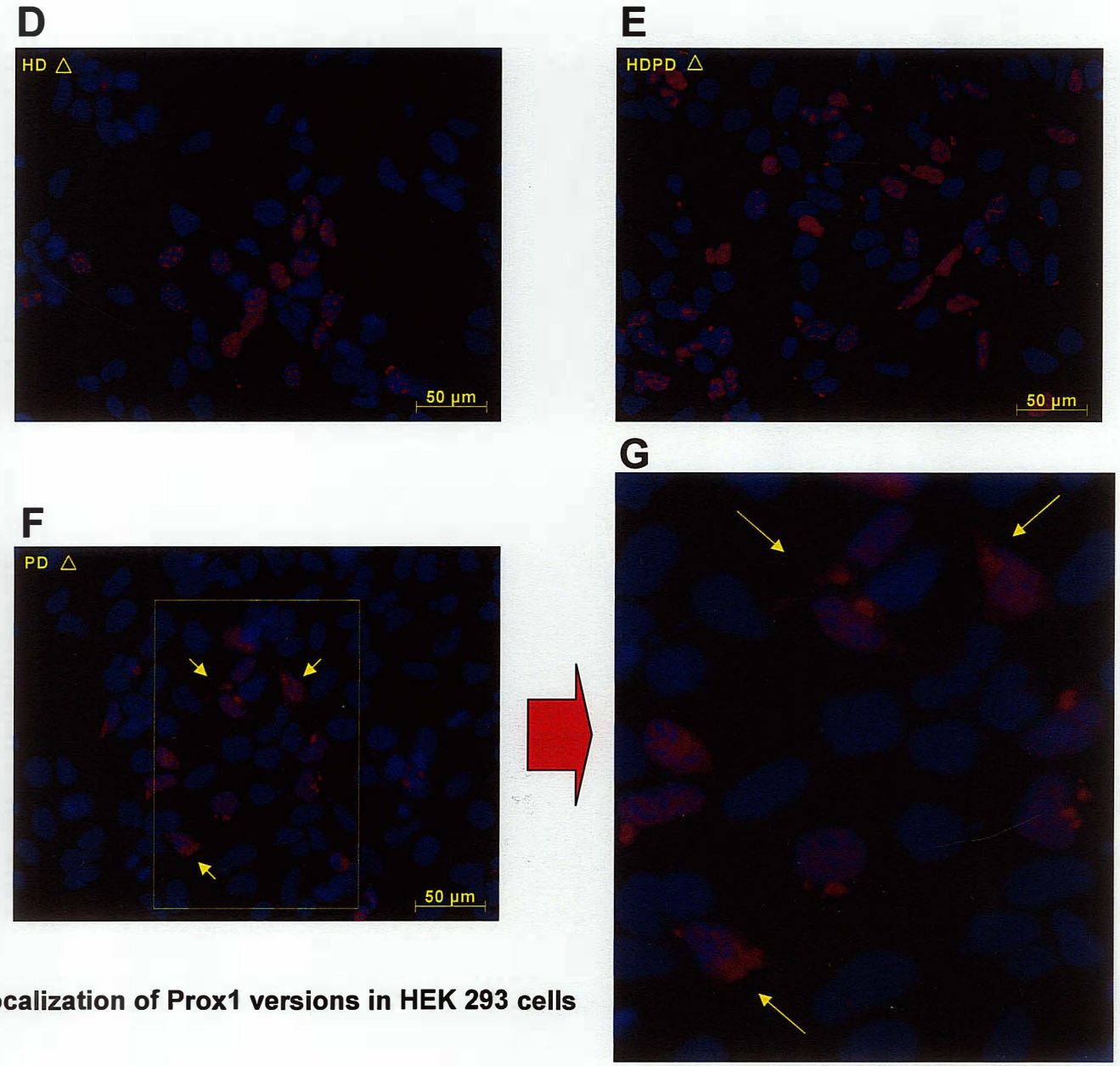


Figure 5. Nuclear localization of Prox1 versions in HEK 293 cells (Cont.)

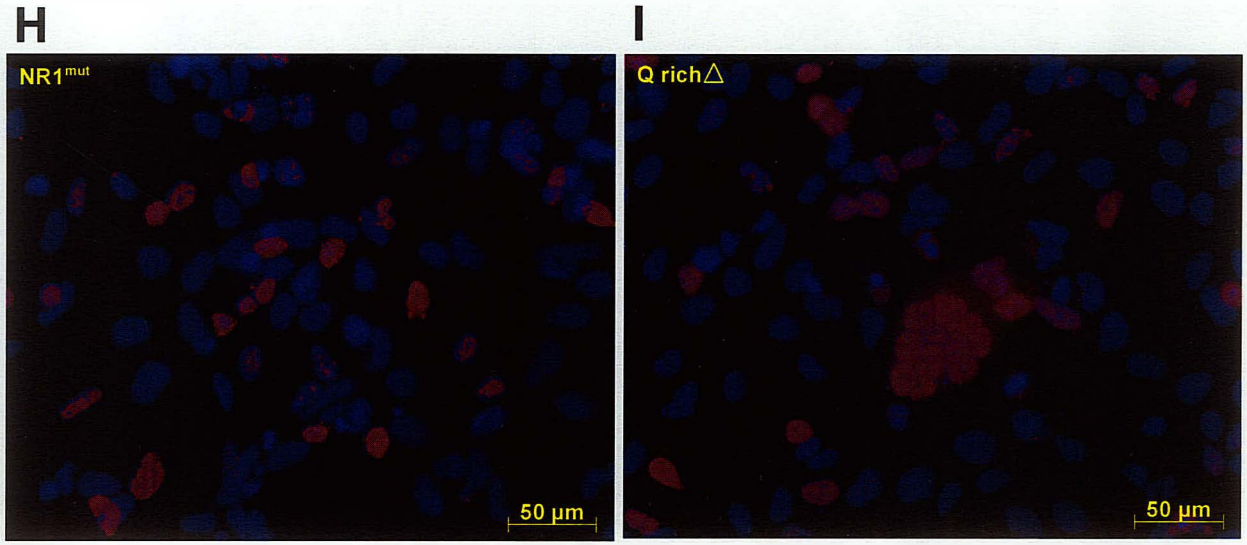


Figure 5. Nuclear localization of Prox1 versions in HEK 293 cells
(Cont.)

were expressed similarly, except for Prox1 PDΔ, which is expressed at lower levels **(Figure 6)**. In addition, the western blots results showed that Prox1 is susceptible to degradation, as lower molecular weight bands were often observed **(Figure 6).**

Immunocytochemistry results from U2OS cells showed that Prox1 WT and Prox1 HDΔ (the only versions tested in this cell type) localized exclusively to the nucleus **(Figure 7)**. Western blot results of transfected U2OS cells revealed that Prox1 WT and Prox1 HDΔ proteins were equivalently expressed **(Figure 8)**.

2. Determining how Prox1 activates the 1Kb *Cyclin E1* promoter

2.1. HEK 293 cells

Previous work has shown that *Cyclin E1* transcription is up-regulated by Prox1 in an apparently DNA-binding dependent manner [2]. In order to determine which domains of Prox1 are responsible for this activation and, in this way, learn about the mechanism by which Prox1 activates this promoter, luciferase assays were performed using HEK 293 cells transfected with the 1 Kb mouse *Cyclin E1* promoter, also referred to as ProCE [116] , and the different Prox1 versions.

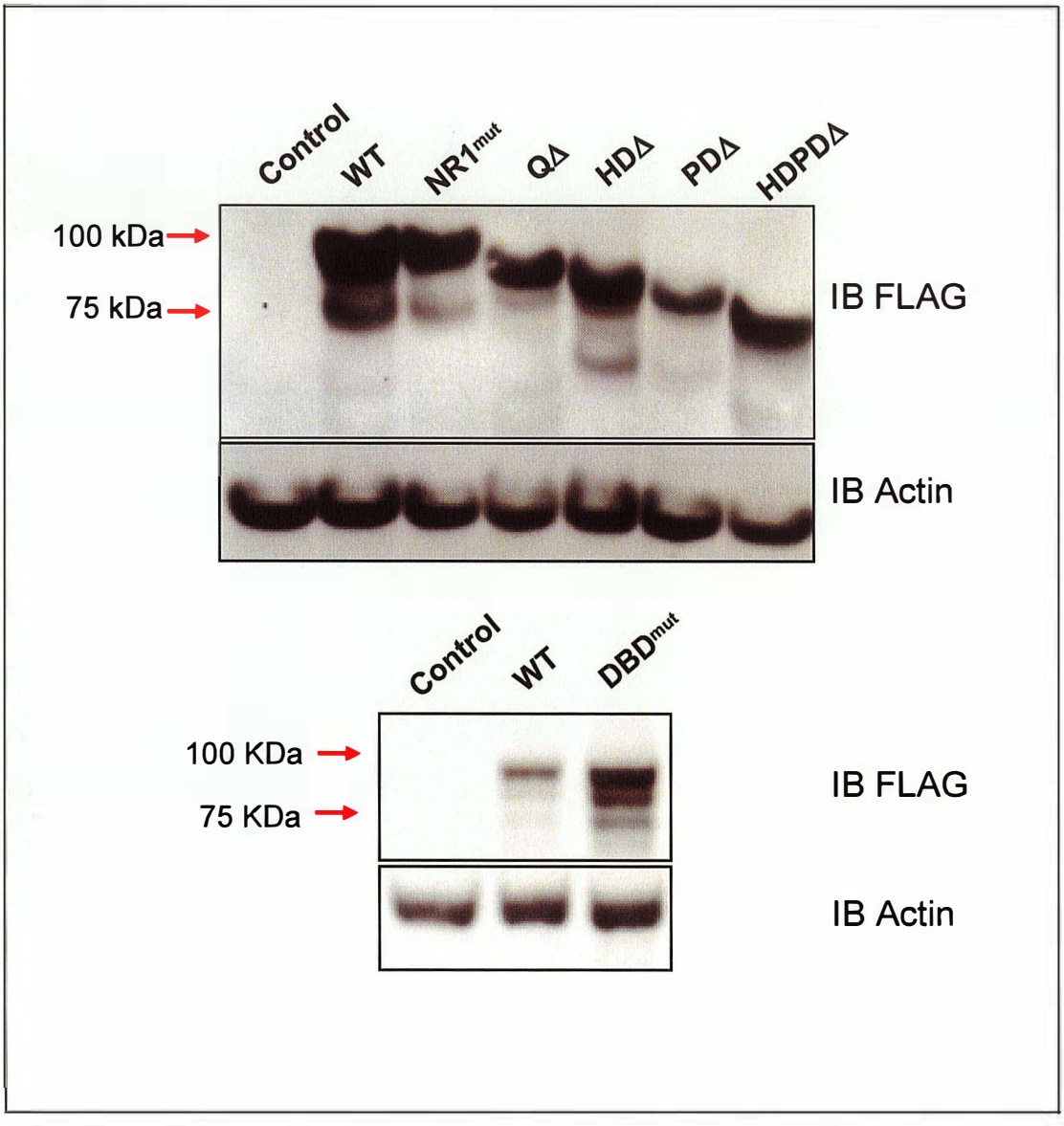


Figure 6. The different *Prox1 mutants are equivalently expressed in HEK 293 cells.*

Anti-FLAG Western blot of total cell lysates derived from cells transfected with pCMV-Tag 4A empty vector (Control) or the respective Prox1 version. The blot was probed with an anti-actin anitibody used as a loading control. Similar amounts of the different Prox1 versions were detected. Lower bands suggest degraded Prox1 proteins. WT=Prox1 Wild type. $NR1^{mut}$=Prox1 NR1 mutation. QΔ=Prox1 Q rich deletion. HDΔ=Prox1 Homeodomain deletion. PDΔ=. Prox1 *Prospero* Domain deletion. HDPDΔ=Prox1 Homeodomain + *Prospero* Domain deletion. DBD^{mut}= Prox1 DNA Binding Domain mutation.

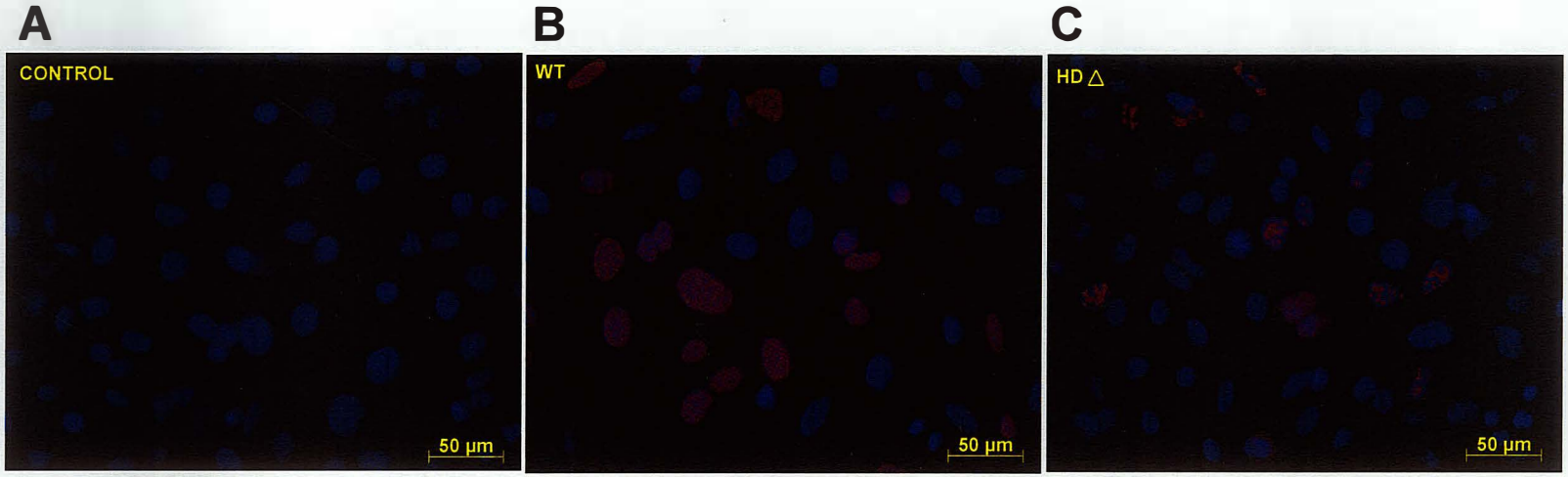


Figure 7. P*rox1 subcellular localization in U2OS cells.*
Anti-FLAG Immunocytochemistry (red) of cells transfected with the pCMV-Tag 4A vector (Control) **(A)** or the respective Prox1 version **(B,C)**. Nuclei were stained with DAPI (blue). The different Prox1 versions localize to the nucleus (pink staining). **B**: Prox1 Wild type (WT). **C**: Prox1 Homeodomain deletion (HDΔ).

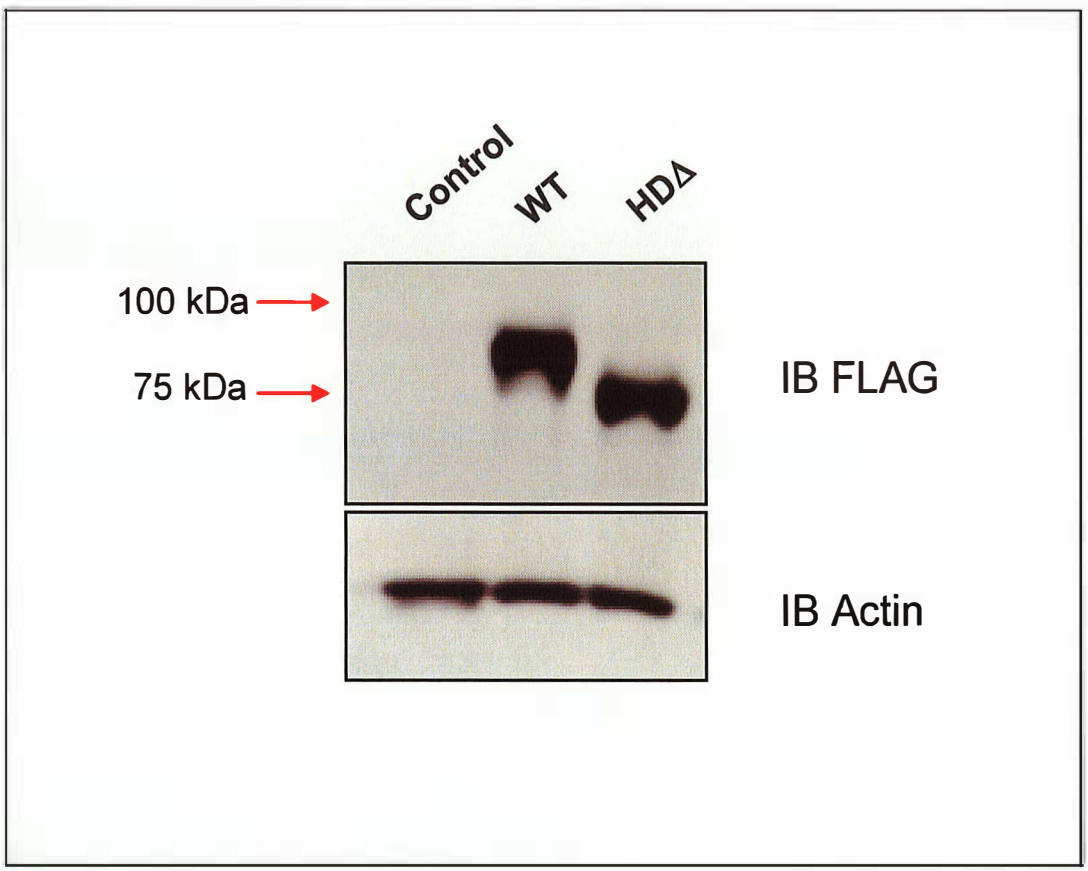


Figure 8. ***Prox1 expression in U2OS cells.***
Western blot of cells transfected with pCMV-Tag 4A vector (Control) or the respective Prox1 version. Actin was used as a loading control. Prox1 Wild-type (WT) and Prox1 Homeodomain deletion (HDΔ) were expressed in equivalent amounts.

2.1.1. Prox1 activation of the *Cyclin E1* promoter is independent of Prox1 directly binding DNA

In order to establish whether Prox1 mediated activation of the *Cyclin E1* promoter was DNA-binding dependent or independent, the abilities of Prox1 WT, Prox1 DBD^{mut} and Prox1 HDΔ versions of Prox1 to activate the *Cyclin E1* promoter were compared. In the Prox1 DBD^{mut}, two amino acids of the Prox1 homeodomain [94], were mutated to a negatively charged glutamate residues **(Figure 4)**. Similar mutations have been previously shown to prevent DNA binding due to electrostatic repulsion [122]. In Prox1 HDΔ, the entire homeodomain (HD) was deleted **(Figure 4)**, which prevents DNA binding and also abolishes any protein-protein interactions mediated by the homeodomain [58, 59, 61, 71].

Results of the luciferase reporter assay showed that Prox1 WT activated the 1 Kb *Cyclin E1* promoter by approximately 4 fold **(Figure 9)**. Interestingly, Prox1 DBD^{mut} and Prox1 HDΔ were also able to activate this promoter to a similar level **(Figure 9).** This result suggests that Prox1 activates the *Cyclin E1* promoter in a DNA-binding independent manner in this cell line. Also, these results indicate that the homeodomain is not necessary for activation of this promoter.

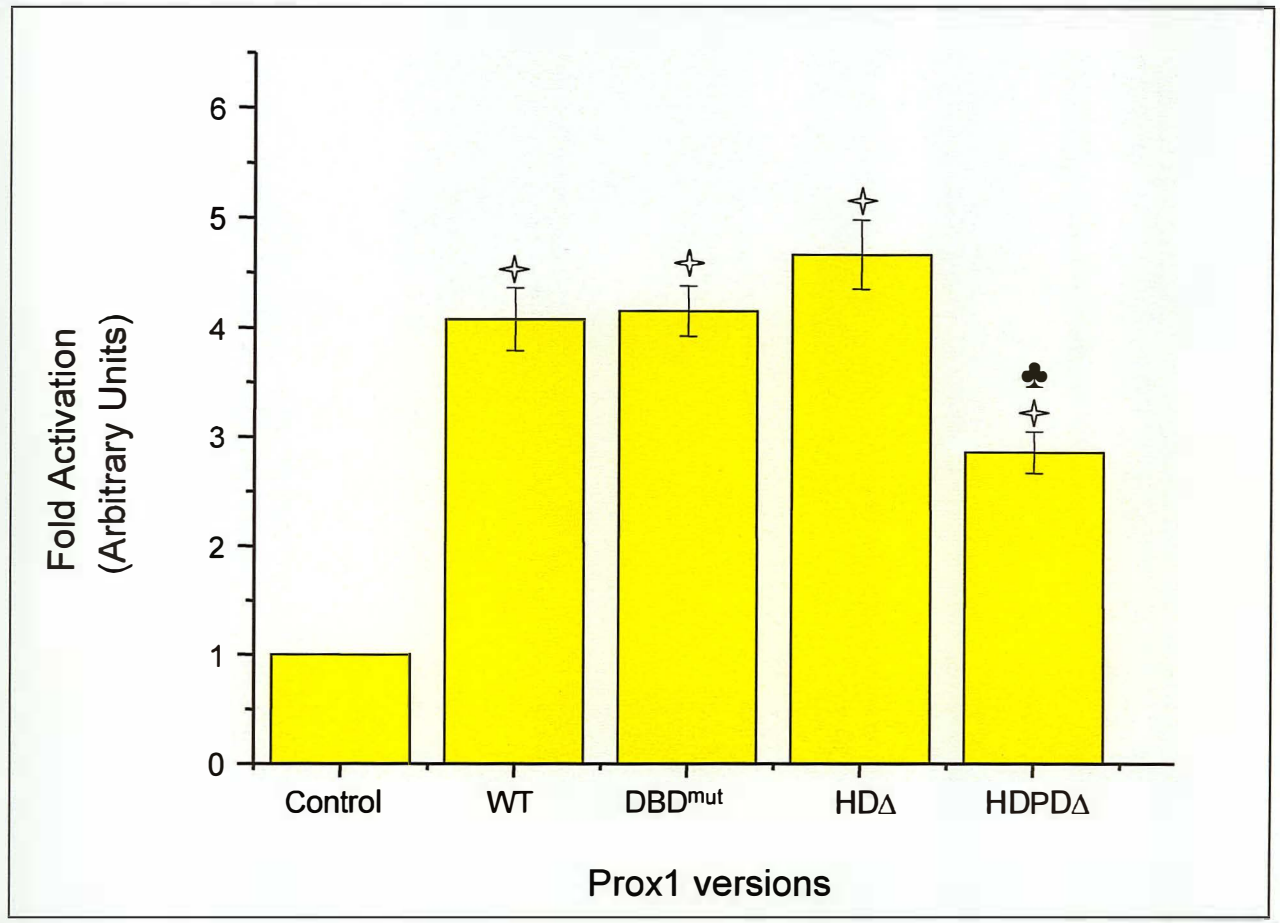


Figure 9. ***Prox1 does not need to bind to DNA to activate the mouse Cyclin E1 promoter in HEK 293A cells.*** Luciferase assay showing Prox1 DNA-independent activation of the mouse *Cyclin E1* promoter. Cells were cotransfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β–galactosidase vector and a luciferase reporter construct carrying the ProCE promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 12, $p < 0.05$. WT=Prox1 Wild-type. DBD^{mut}=Prox1 DNA Binding Domain mutation. HDΔ=Prox1 Homeodomain deletion. HDPDΔ=Prox1 Homeodomain + *Prospero* Domain deletion. (✧): Significantly different from control. (♣): Significantly different from the treatments not marked with the same symbol.

2.1.2. The Prox1 *Prospero* Domain has a role in the *Cyclin E1* promoter activation

Given that the role of the *Prospero* Domain (PD) in Prox1 is still undefined, a truncated version of Prox1 lacking both the homeodomain and the *Prospero* Domain, was created (Prox1 HDPDΔ) **(Figure 4)**. Interestingly, this version of Prox1 was able to activate the *Cyclin E1* promoter but not to the same extent as either Prox1 WT or Prox1 HDΔ. The level of activation was significantly reduced by 1 fold **(Figure 9)**, suggesting a role for the *Prospero* Domain in mediating the transcriptional activation of *Cyclin E1* by Prox1.

To confirm this hypothesis, a new truncated version of Prox1 lacking only the *Prospero* Domain, was created (Prox1 PDΔ) **(Figure 4)**. Luciferase assay results showed that Prox1 PDΔ was able to activate this promoter but that the level of activation achieved was lower but not significantly different than that observed with the Prox1 HDPDΔ version **(Figure 10)**. These results suggest that the *Prospero* Domain is required for the full activation by Prox1 of the *Cyclin E1* promoter.

With the intention of identifying the Prox1 domain(s) responsible for the remaining activation of the *Cyclin E1* promoter in the absence of the *Prospero* Domain, more Prox1 mutant versions were created and their ability to activate this promoter was evaluated.

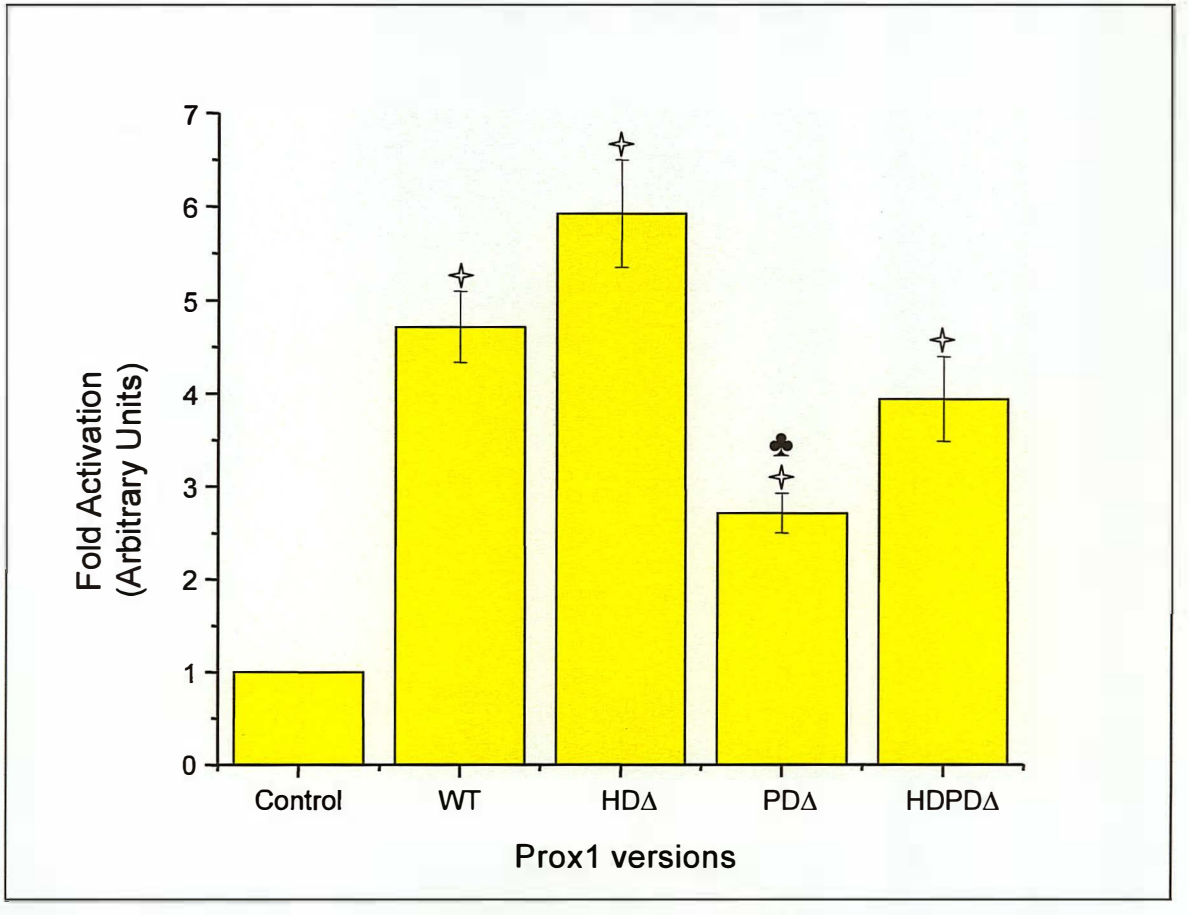


Figure 10. ***Prox1 Prospero Domain has an important role in Cyclin E1 promoter activation.***
HEK 293 cells were cotransfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β-galactosidase vector and a luciferase reporter construct carrying the ProCE promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 15, $p < 0.05$. WT=Prox1 Wild-type. HDΔ=Prox1 Homeodomain deletion. PDΔ=Prox1 *Prospero* Domain deletion. HDPDΔ=Prox1 Homeodomain + *Prospero* Domain deletion. (✧): Significantly different from control. (♣): Significantly different from the treatments not marked with the same symbol.

2.1.3. The Prox1 NR1 box is not required for *Cyclin E1* promoter activation

Prox1 has been shown to interact with LRH-1 and SF-1, two members of the *Fushi tarazu* factor 1 subfamily of orphan nuclear receptors in mammals [73, 75], and with the SF-1 zebra fish ortholog Ff1b [76]. Prox1 functions as a co-repressor for these transcription factors. The LXXLL motif is known to mediate co-factor binding to nuclear receptors and has been termed the nuclear receptor box (NR box). Prox1 has three predicted NR boxes in its amino acid sequence **(Figure 4)**. The NR1 and NR2 boxes are located in its N-terminal region, while NR3 is located in the *Prospero* Domain. The NR1 box has been shown to be the most important LXXLL motif required for Prox1 binding with LRH-1, SF-1 and Ff1b [73, 75, 76]. In addition, a recent study identified a consensus LRH-1 responsive element in the *Cyclin E1* promoter and showed that LRH-1 was able to induce transcription by binding to this element [123].

To investigate whether Prox1 activates the *Cyclin E1* promoter *via* interaction with LRH-1, a version of Prox1 with the NR1 box mutated was created (Prox1 $NR1^{mut}$) **(Figure 4)**. Interestingly, this version of Prox1 was able to fully activate the *Cyclin E1* promoter **(Figure 11)**, suggesting that Prox1 does not activate this promoter through its interaction with LRH-1 and that NR1 is dispensable for Prox1 mediated activation of this promoter. Recently, luciferase assays performed with a version of Prox1 in

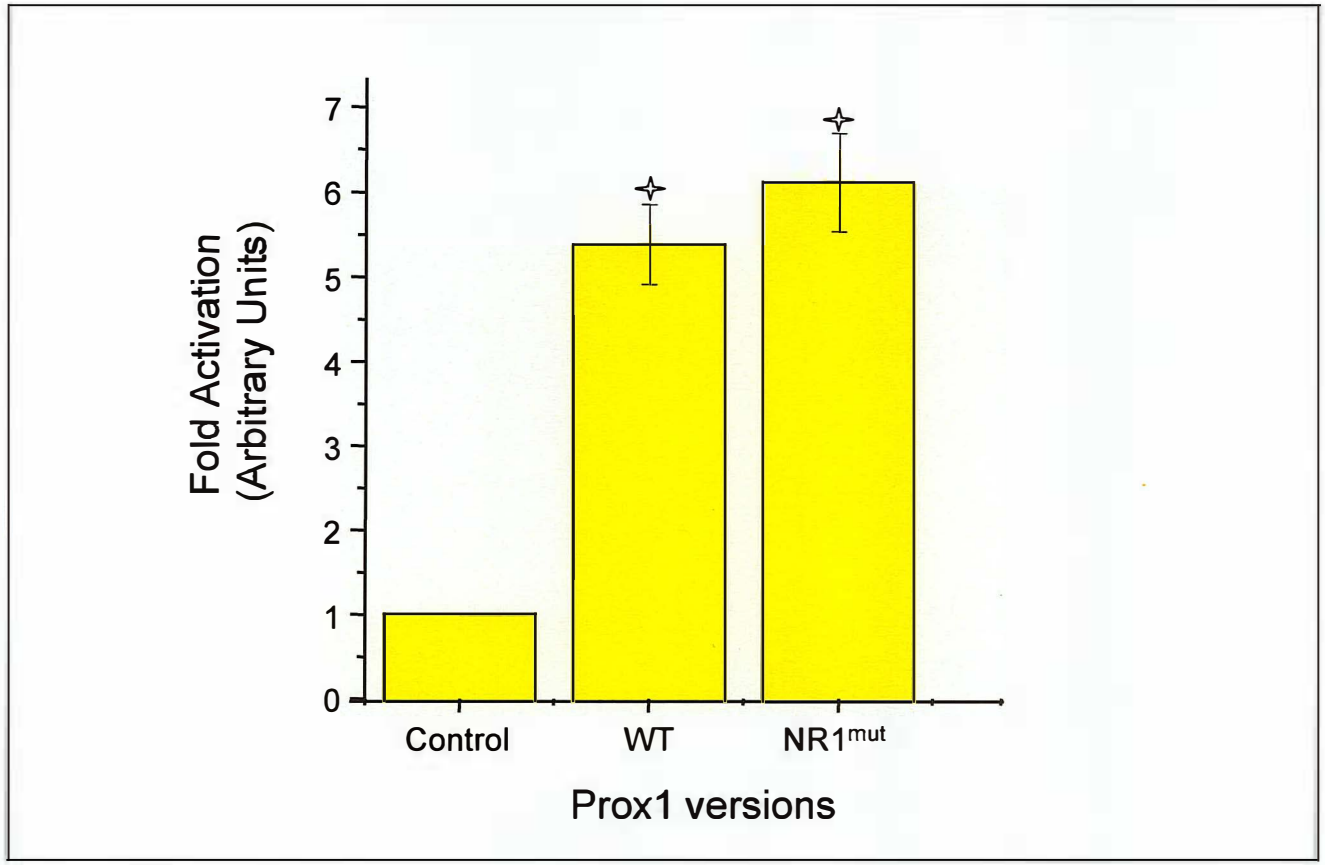


Figure 11. ***Prox1 NR1 is dispensable for Prox1- mediated activation of the Cyclin E1 promoter.***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β-galactosidase vector and a luciferase reporter construct carrying the ProCE promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 9, $p < 0.05$. WT=Prox1 Wild-type. $NR1^{mut}$=Prox1 NR1 mutation. (✧): Significantly different from control.

which NR1 and NR2 were deleted showed that this version was able to activate this promoter to an equivalent level to the one observed when Prox1 WT was used (data not shown) (Baxter and Wigle, unpublished data). This demonstrates that neither NR1 nor NR2 are required for Prox1-mediated activation of the *Cyclin E1* promoter.

2.1.4. The Prox1 Q rich region is not required for the *Cyclin E1* promoter activation

Transcriptional regulatory proteins are modular. Typically they have a DNA binding domain, that targets them to the promoters of the genes they regulate, and a separate activation or repression domain, by which they regulate the assembly or activity of the transcriptional machinery itself [124]. The domain organization of Prox1 has not been fully defined and in fact, the Prox1 activation/repression domains have not yet been identified. Activation domains have typically been classified on the basis of their amino acid composition, depending on whether they are rich in glutamine, proline or acidic amino acids [125, 126]. In order to know whether the Prox1 glutamine rich region (Q rich region) has a role in the activation of the *Cyclin E1* promoter, a version of Prox1 with the Q rich region deleted was created (Prox1 QΔ) **(Figure 4)**. Luciferase results showed that Prox1 QΔ was able to fully activate the *Cyclin E1* promoter **(Figure 12)**, suggesting that the Q rich region in Prox1 is not necessary for this activation. Luciferase assays using only the Q rich region of Prox1 showed

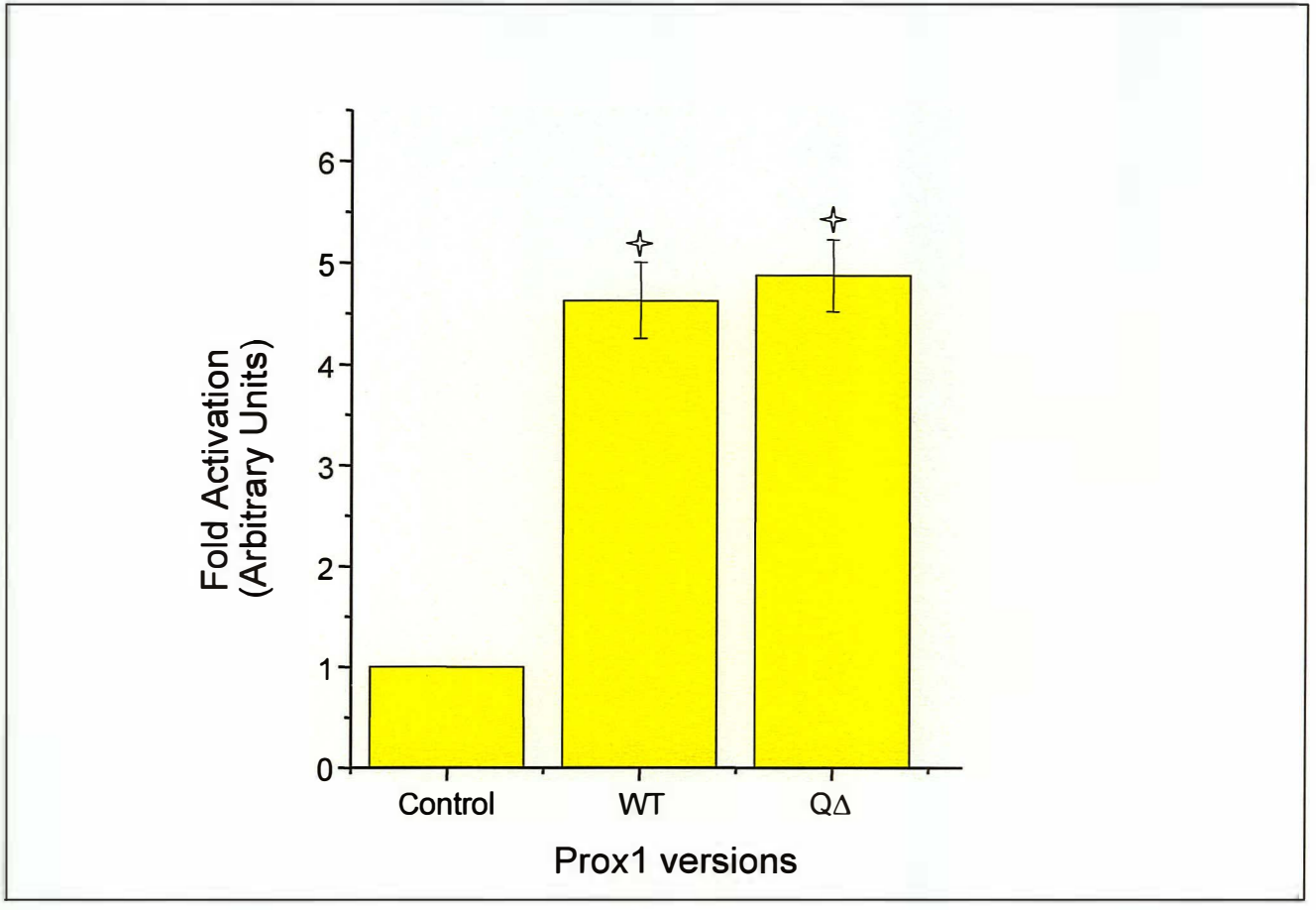


Figure 12. ***Prox1 Q rich region is dispensable for Cyclin E1 promoter activation.***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β-galactosidase vector and a luciferase reporter construct carrying the ProCE promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 12, $p < 0.05$. WT=Prox1 Wild-type. QΔ=Prox1 Q rich deletion. (✧): Significantly different from control.

that this region was unable to activate the *Cyclin E1* promoter at all (data not shown). Western blot results indicated that this version was not expressed, so it was not possible to draw any conclusions regarding the ability of this region to activate *Cyclin E1* transcription by itself (data not shown).

2.2. U2OS cells

Since Prox1-mediated activation of the *Cyclin E1* promoter was shown not to be cell type specific [2], luciferase assays were performed using U2OS cells transfected with the mouse ProCE promoter and, Prox1 WT and Prox1 HDΔ. As well, it was in this cell type that Prox1 activation of *Cyclin E1* had previously been postulated to be DNA-binding dependent [2].

2.2.1. Prox1 activates the *Cyclin E1* promoter in a DNA-binding independent manner

In order to establish that Prox1 DNA-binding independent activation of the *Cyclin E1* promoter was cell type-independent, Prox1 WT and Prox1 HDΔ versions of Prox1 were used.

Luciferase results showed that Prox1 activates the transcription of the *Cyclin E1* promoter 5.5 fold **(Figure 13)**, confirming Prox1 cell type-independent activation of this promoter. Prox1 HDΔ was able to activate this promoter but to a lesser extent than Prox1 WT **(Figure 13)**. Even

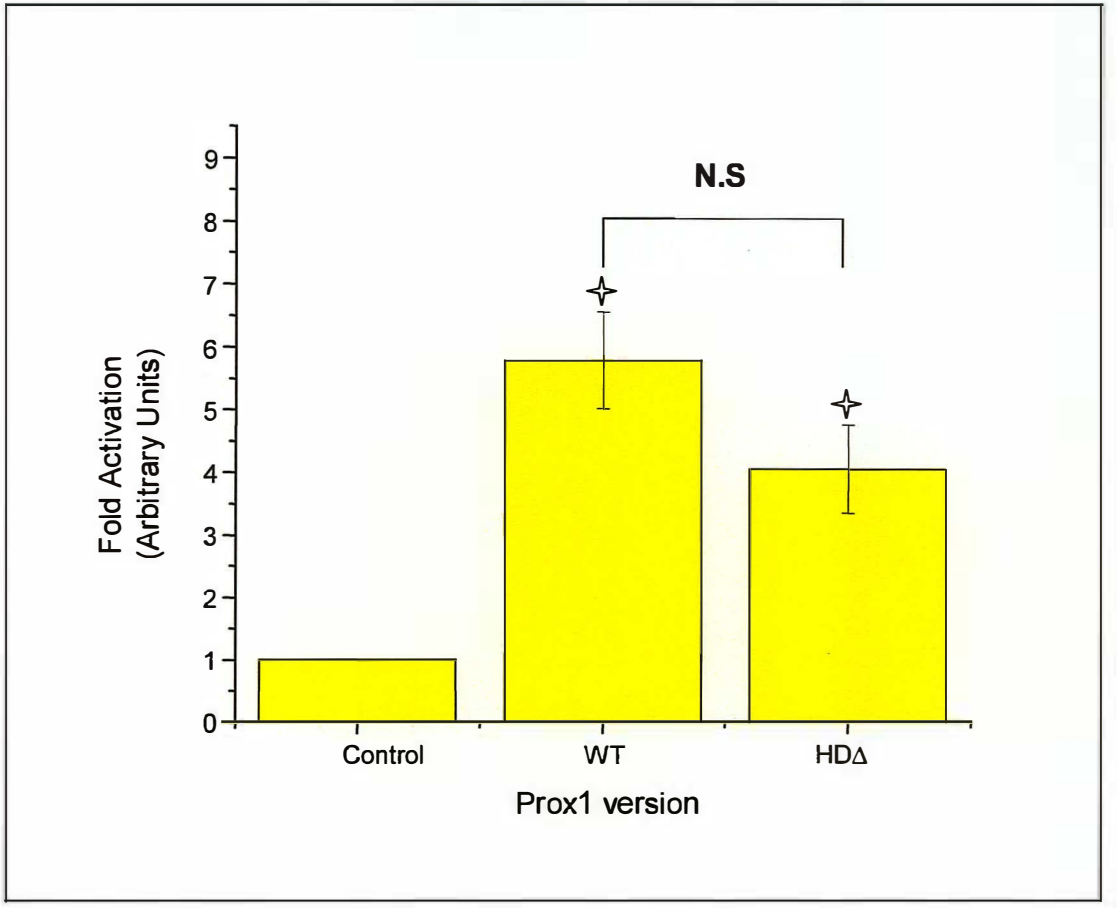


Figure 13. ***Prox1 activates the Cyclin E1 promoter in U2OS cells.***
U2OS cells were transfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β-galactosidase vector and a luciferase reporter construct carrying the ProCE promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. $N \geq 12$, $p < 0.05$. WT=Prox1 Wild-type. HDΔ=Prox1 Homeodomain deletion. (✧): Significantly different from control. N.S=Not significantly different.

though the activation mediated by Prox1 HDΔ was decreased 1.5 fold, this difference was not statistically significant, suggesting that Prox1 mediates the activation of the *Cyclin E1* is DNA-binding independent and this activation is not cell type specific, i.e. limited to HEK 293 cells. This result conflicts with Petrova *et al.* who showed that Prox1 mediated activation of *Cyclin E1* is DNA-binding dependent in U2OS cells [2].

3. Prox1 activates a 4XE2F synthetic reporter construct

The E2F family of proteins are key regulators of *Cyclin E1* transcription [115, 116, 118]. In addition, Prox1 has been shown to activate a 6XE2F artificial promoter [2]. In order to study the manner in which Prox1 activates transcription of E2F responsive promoters, a synthetic reporter construct which contains four consensus E2F binding sites (4XE2F), and the different versions of Prox1 were used.

3.1. Prox1 activates the 4XE2F synthetic reporter construct in a DNA-binding independent manner

To study whether Prox1 mediated activation of the 4XE2F synthetic reporter construct was DNA-dependent or independent, Prox1 WT, Prox1 DBD^{mut} and Prox1 HDΔ versions of Prox1 were used.

Luciferase assay results showed that Prox1 WT stimulates the activity of this promoter approximately 2.5 fold **(Figure 14)** Interestingly, Prox1 DBD^{mut} and Prox1 HDΔ were also able to similarly activate this promoter **(Figure 14)**, suggesting a Prox1 DNA-binding independent activation. Also, this result indicates that the Prox1 homeodomain is not required for Prox1 interaction with other co-regulatory proteins involved in this activation. These results suggest that Prox1 activates the *Cyclin E1* promoter and the E2F artificial promoter in a similar manner and, as well, raises the possibility that Prox1 may activate the *Cyclin E1* promoter through a mechanism that involves E2F proteins and/or E2F/pRb complexes.

3.2. Role of *Prospero* Domain plays a role in the activation of the 4XE2F synthetic reporter construct

To study if the Prospero Domain has a role in Prox1 mediated activation of the 4XE2F synthetic reporter construct, the ability of Prox1 HDPDΔ to activate this artificial promoter was evaluated.

Luciferase assay results showed that Prox1 HDPDΔ was not able to fully activate this promoter **(Figure 14)**. The level of activation reached by this version of Prox1 was significantly lower than that observed for Prox1 WT, suggesting a role for Prox1 Prospero Domain in this activation. This result also supports the idea that Prox1 activates the *Cyclin E1* promoter and the 4XE2F artificial promoter through a similar mechanism.

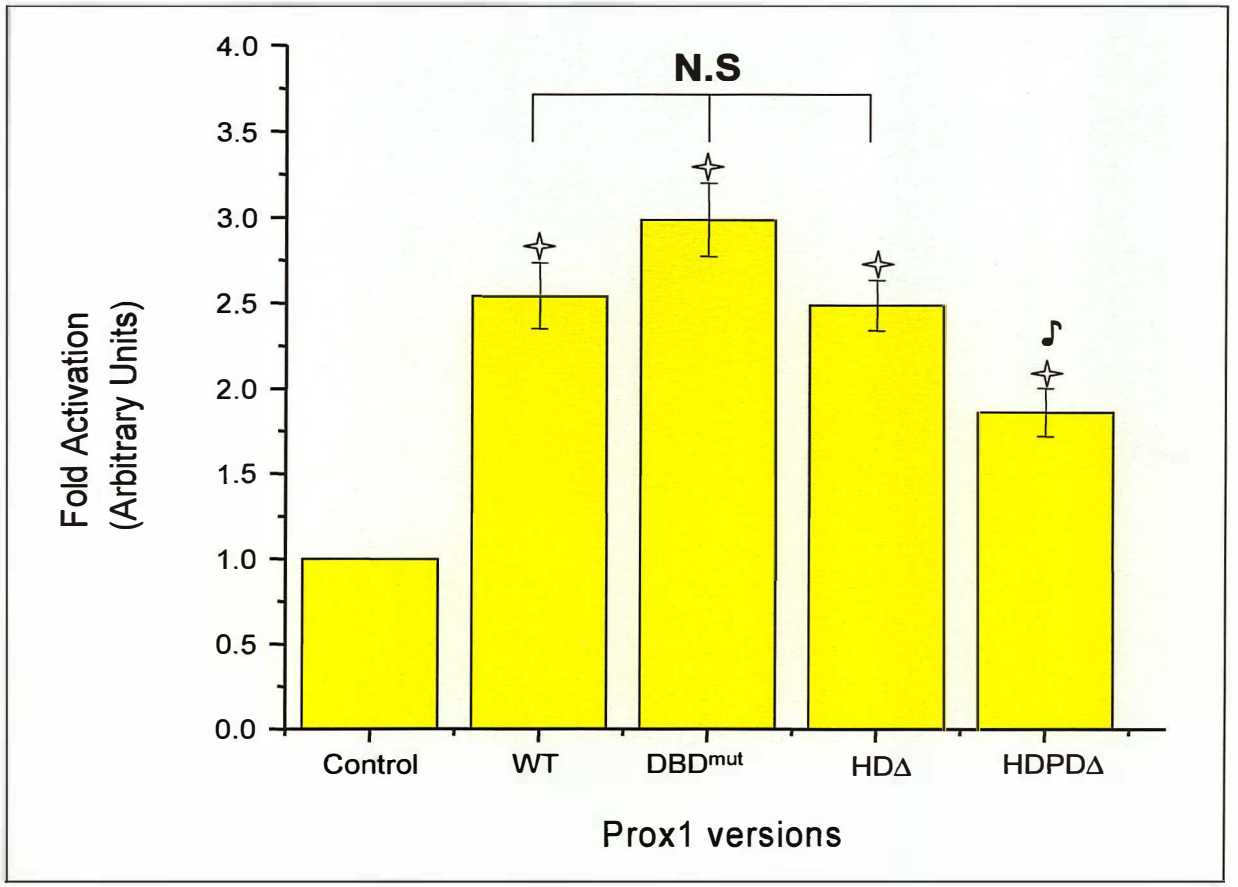


Figure 14. ***Prox1 activates the 4XE2F synthetic reporter construct***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with the respective Prox1 version, the β-galactosidase vector and the 4XE2F synthetic reporter construct. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 11, $p < 0.05$. WT=Prox1 Wild type. DBD^{mut} = Prox1 DNA Binding Domain mutation. HDΔ=Prox1 Homeodomain deletion. HDPDΔ=Prox1 Homeodomain + *Prospero* Domain deletion. (✧): Significantly different from control. (♪) indicates that HDPDΔ is significantly different from WT and DBD^{mut}, but not from HDΔ. N.S=Not significantly different.

4. Prox1 activates a proximal *Cyclin E1* promoter

With the aim of identifying the region of the *Cyclin E1* promoter involved in Prox1 mediated activation, deleted versions of this promoter were used for luciferase assay experiments in HEK 293 cells.

4.1. Prox1 activates a 557 bp version of the *Cyclin E1* promoter

In order to narrow the region of the *Cyclin E1* promoter involved in the activation mediated by Prox1, and to confirm that Prox1 does not exert this activation through an interaction with LRH-1, a 557 bp *Cyclin E1* promoter version that does not include the LRH-1 responsive element was used **(Figure 15)**.

Luciferase assay results showed that Prox1 activates this version of the *Cyclin E1* promoter 4 fold **(Figure 16, A)**. This level of activation is similar to that obtained when we used the intact 1Kb *Cyclin E1* promoter (ProCE) **(Figure 9)**. This result confirms that Prox1 does not activate the *Cyclin E1* promoter through an interaction with LRH-1, and indicates that the region upstream of the 557 bp promoter is not required for Prox1 mediated activation.

4.2. *Prox1 activates a 206 bp version of the Cyclin* E1 promoter

Given that the two major regulatory modules of the *Cyclin E1* promoter are located proximal to the start site for transcription [115, 116],

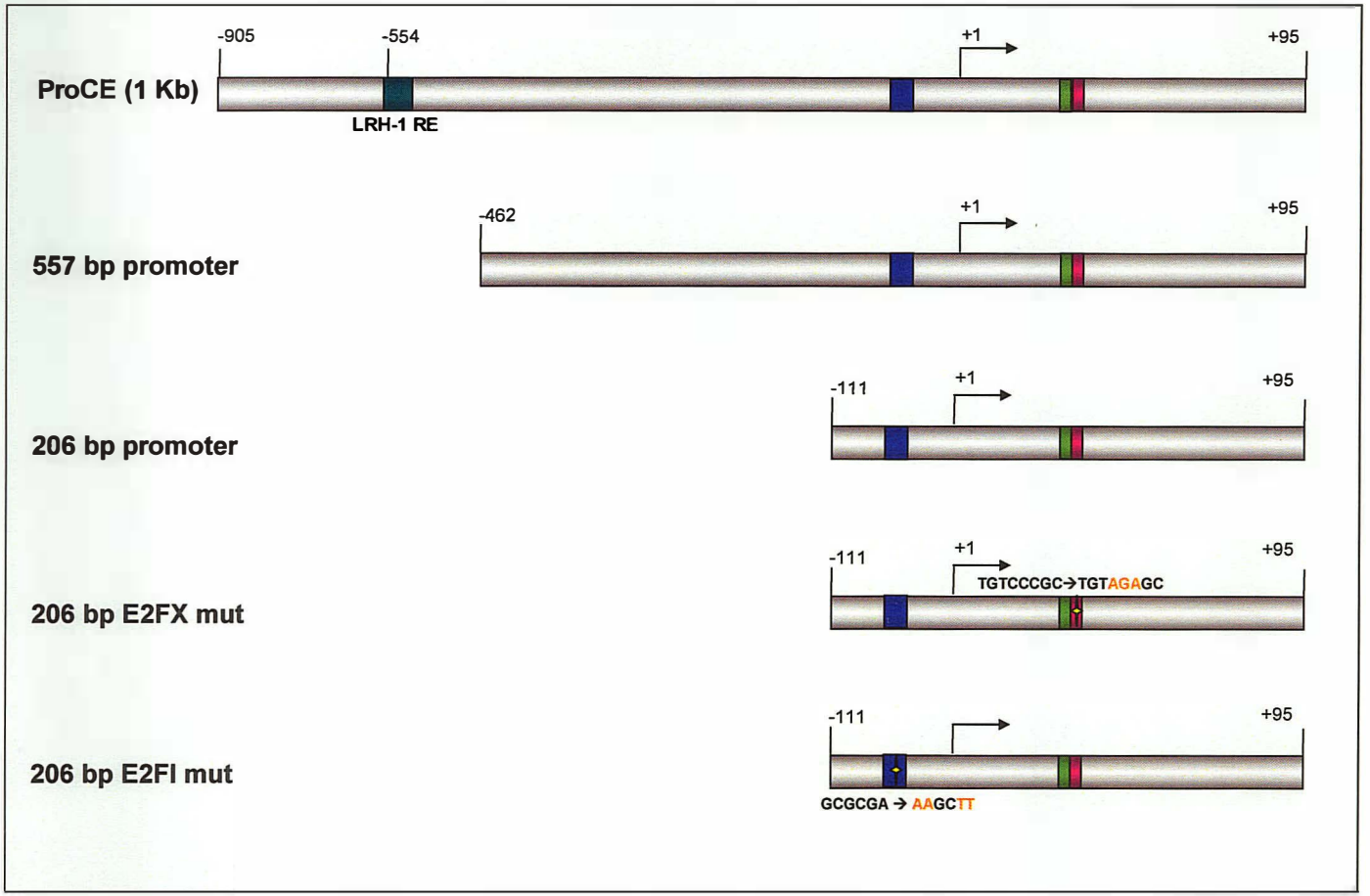


Figure 15. ***Different Cyclin E1 promoter constructs.***
Start of transcription of the mouse *Cyclin E1* gene is indicated as +1. The E2F-Sp1 element is depicted as a purple box. CERM is depicted by the green/pink boxes. In CERM, the green box symbolizes the AT-rich element and the pink box represents the E2FX site. Mutated nucleotides (†) are indicated in orange.

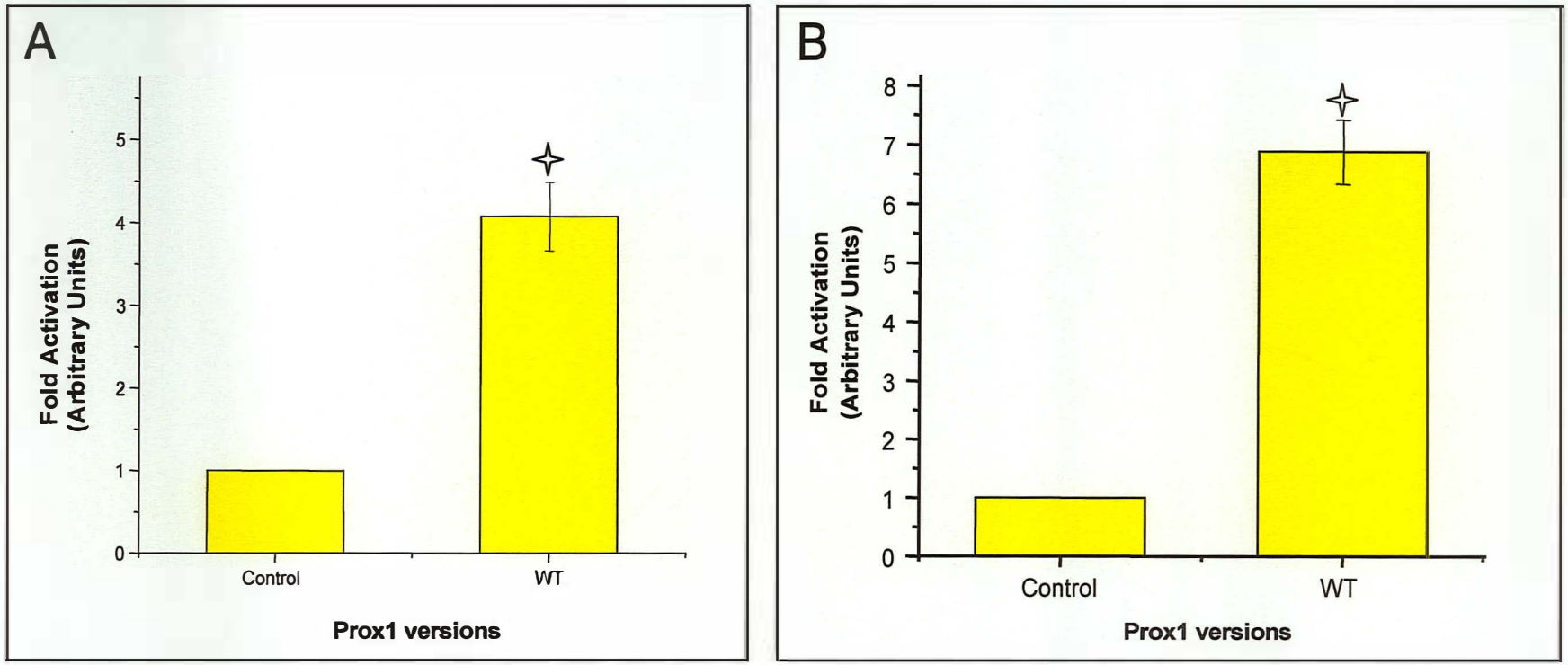


Figure 16. ***Prox1 activates shorter versions of the Cyclin E1 promoter.***
Luciferase Assay showing Prox1 activation of a 557 bp (**A**) and a 206 bp (**B**) version of the Cyclin E1 promoter. HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with Prox1 WT (WT), the β-galactosidase vector and a luciferase reporter construct carrying a 557 bp or a 206 bp version of the Cyclin E1 promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N= 9, $p < 0.05$ (A). N= 11, $p< 0.05$ (B). (✧): Significantly different from control.

a 206 bp version of this promoter was created, which encompasses these two modules (E2F-Sp1 and CERM) **(Figure 15).** The ability of Prox1 to activate this promoter was then evaluated.

Luciferase assays results showed that even this small region of the *Cyclin E1* promoter was responsive to Prox1 expression **(Figure 16, B)**. Interestingly, Prox1 mediated activation of this truncated promoter was 3 fold higher than that observed when the 1 Kb or 557 bp promoters were used **(Figure 9)**. This difference could be a result of the removal of a repression module located between positions -462 and -111 of the promoter. Interestingly, a comparison among the basal levels of transcription of the 1Kb, 557 bp and 206 bp *Cyclin E1* constructs, showed that the 557 bp promoter is significantly less active than the other two promoters. Furthermore, there is not a significant difference between the basal transcription levels of the 1 Kb and the 206 bp promoter constructs **(Figure 17)**. Prox1 is likely to activate the *Cyclin E1* and the artificial 4XE2F promoters in a similar manner and, since the 206 bp version of the *Cyclin E1* promoter includes two E2F responsive regulatory modules, we predicted that Prox1 activates the *Cyclin E1* promoter *via* one or both of these two modules.

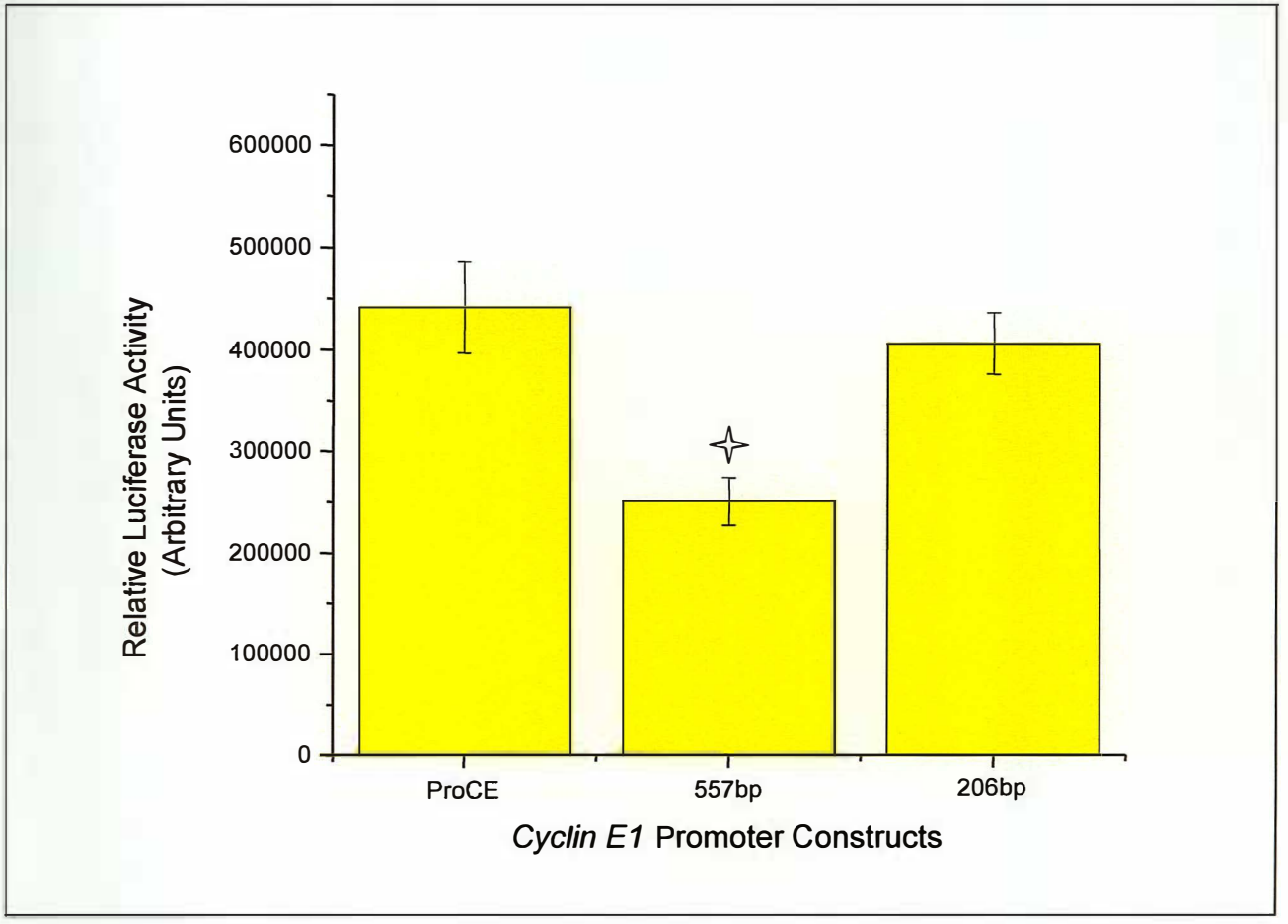


Figure 17. ***Basal transcription of proximal Cyclin E1 promoter versions.***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector, the β-galactosidase vector and a luciferase construct carrying the respective version of the *Cyclin E1* promoter. Luciferase values were normalized to β–galactosidase activity. Error bars indicate standard error. N>9, $p < 0.05$. ProCE=1Kb *Cyclin E1* promoter. 557bp=557 bp *Cyclin E1* promoter. 206 bp=206 bp *Cyclin E1* promoter. (✧): Significantly different from the treatments not marked with the same symbol.

4.3. Prox1 does not activate the *Cyclin E1* promoter exclusively through CERM/CERC

CERM functions as a transcriptional repressor element in which E2Fs, pocket proteins and HDACs are involved (CERC) [101, 116]. Since Prox1 has been shown to directly bind and re-distribute HDAC3 [75], it is possible that Prox1 activates the transcription of *Cyclin E1* by interacting with the HDAC activity present in CERC, destabilizing the complex and releasing repression. In order to test this hypothesis, we created a new version of the 206 bp promoter, in which the E2FX element present in CERM was mutated [116] **(Figure 15)**. The E2FX site present in CERM has been shown to be essential for the formation of the CERM/CERC complex [116]. If Prox1 mediates the activation of transcription of *Cyclin E1* by interacting with CERC and releasing CERM form this complex, then Prox1 should not activate *Cyclin E1* transcription when CERC cannot bind.

Luciferase results showed that Prox1 stimulates the transcription of the *Cyclin E1* promoter even when the E2FX site is mutated (thus when CERC is not bound to CERM). The level of activation stimulated by Prox1 in the E2FX mutated promoter was similar to the level of activation observed with the wild-type 206 bp promoter **(Figure 18, A)**. This suggests that Prox1 mediated activation of the *Cyclin E1* promoter does not exclusively involve CERM/CERC. Interestingly, the basal

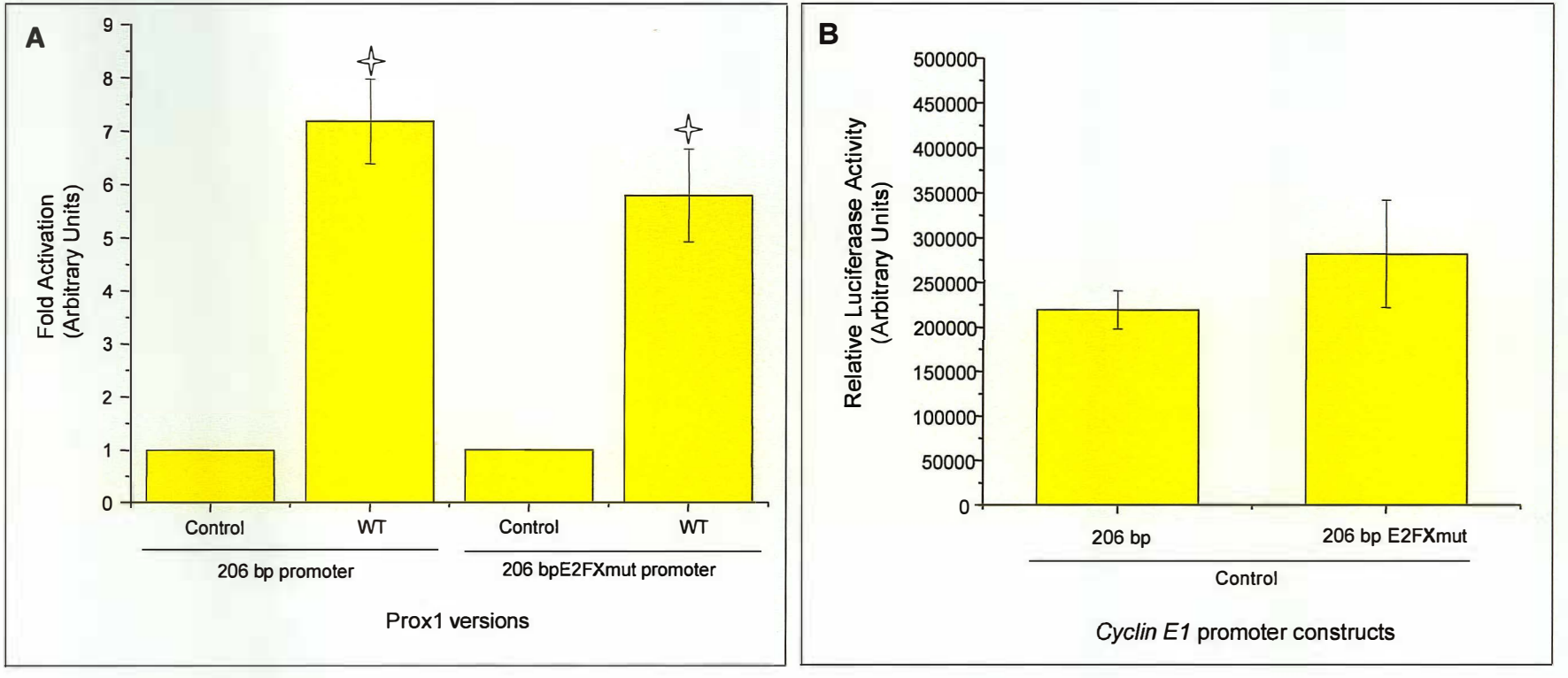


Figure 18. ***Prox1 does not activate Cyclin E1 promoter exclusively through CERM/CERC.***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with Prox1 WT (WT), the β-galactosidase vector and a luciferase reporter construct carrying the 206 bp version of the *Cyclin E1* promoter or the 206 bp with the E2FX site mutated version of it. Error bars indicate standard error. N=9, $p < 0.05$. **A**: Prox1 induced activation relative to each control. (✧): Significantly different from control **B**: Basal transcription of the 206 bp and 206 bp E2FXmut *Cyclin E1* promoter constructs. (✧): Significantly different from the treatments not marked with the same symbol.

transcription level (control) observed in the non-mutated and mutated 206 bp promoters was similar **(Figure 18, B)**.

4.4. Prox1 does not activate the *Cyclin E1* promoter exclusively through the E2F-Sp1 module

The current model for *Cyclin E1* regulation states that the constitutively occupied E2F-Sp1 module acts as a repressor module when pRb and HDAC1 are complexed with E2F1-3 and Sp1 in G_0/early G_1 [118, 119]. This module has been proposed to have a role in the activation of transcription of *Cyclin E1* when pRb and HDAC1 are released from the complex, and E2F1-3 and Sp1 remain bound in mid G_1 [115, 118]. Morrison *et al.* showed that pRb and HDAC1 bind to a nucleosome positioned at the transcription start site of the *Cyclin E1* promoter during its repression [119]. Moreover, they reported that pRb represses this promoter by modulating the level of histone acetylation of this nucleosome [119]. As Prox1 has been shown to interact with HDAC3, it is possible that it interacts with HDAC1 to prevent deacetylation of this nucleosome and, in this way, activate transcription of *Cyclin E1*. Another option is that Prox1 activates *Cyclin E1* transcription by displacing pRb, to interact with E2F1-3 and/or Sp1 and in this way, facilitates activation of transcription.

In order to determine if Prox1 activates the *Cyclin E1* promoter through the E2F-Sp1 module, we created a new version of the 206 bp

promoter, in which the E2FI element present in this module was mutated [115] **(Figure 15)**. The E2FI site has been shown to be essential for activation of *Cyclin E1* in late G_1 [115]. Our luciferase results showed that Prox1 activates the transcription of the *Cyclin E1* promoter even when the E2FI site is mutated. The level of activation induced by Prox1 of the E2FI mutated promoter was similar to that observed with the wild-type 206 bp promoter **(Figure 19,A)**. This finding suggests that the Prox1 mediated activation of the *Cyclin E1* promoter does not exclusively occur through the E2F-Sp1 module. The basal level of transcription observed in the mutated 206 bp promoter was significantly lower than that observed when the wild-type 206 bp promoter was used **(Figure 19, B)**.

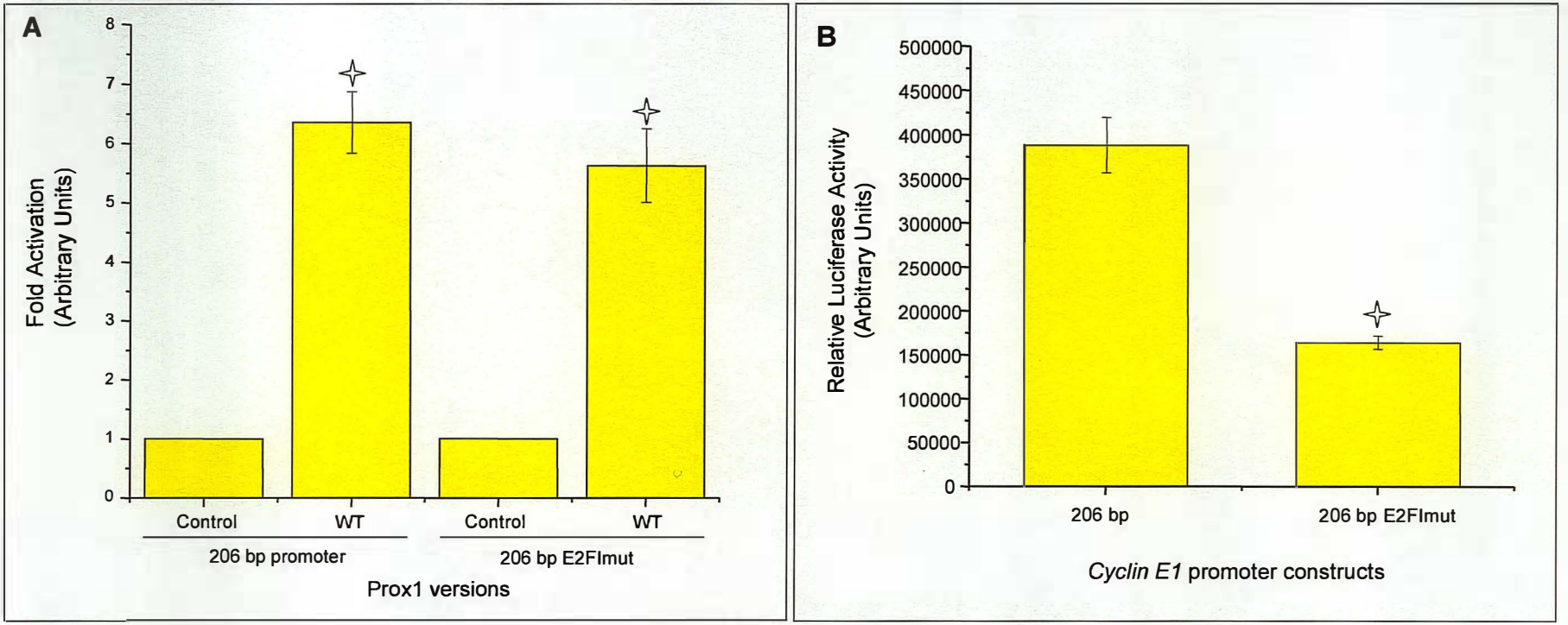


Figure 19. ***Prox1 does not activate Cyclin E1 promoter exclusively through the E2F-Sp1 module***
HEK 293 cells were transfected with pCMV-Tag 4A empty vector (Control) or with Prox1 WT (WT), the β-galactosidase vector and a luciferase reporter construct carrying the 206 bp version of the *Cyclin E1* promoter or the 206 bp with the E2FX site mutated. Error bars indicate standard error. N=6, $p < 0.05$.. **A**: Prox1 induced activation relative to each control. (✧): Significantly different from control **B**: Basal transcription of the 206 bp and 206 bp E2FImut *Cyclin E1* promoter constructs. (✧)Significantly different from the treatments not marked with the same symbol.

VII. DISCUSION

As shown by immunocytochemistry, all of the different Prox1 proteins used in this study localize to the nucleus. Prox1 PDΔ, however, localizes to both the nucleus and the cytoplasm. Interestingly, the *Prospero* Domain has been shown to have a role in regulating the subcellular localization of prospero in *Drosophila* [95, 96]. Mutations that truncated the *Prospero* Domain resulted in the accumulation of prospero in the cytoplasm and in a corresponding decrease in nuclear prospero levels [95, 96]. Prospero contains an Exportin-dependent nuclear export signal (NES) in the α_1 helix of the homeodomain, which is normally masked by the *Prospero* Domain [95]. This hypothesis has been supported by structural studies of prospero, which suggest that the extreme C-terminus of the *Prospero* Domain has a steric effect on the NES region thereby masking it [94]. The key residues of the NES present in prospero homeodomain are conserved among the homeodomains of all Prox1 orthologs [96]. It is possible that the *Prospero* Domain plays a role in regulating Prox1 nuclear subcellular localization by functioning as an NES mask. This role for the *Prospero* Domain would explain why, among all of the Prox1 versions used, only the one lacking the Prospero Domain (Prox1 PDΔ) but containing the homeodomain, was localized to the cytoplasm in a considerable amount of cells. Prox1 HDPDΔ localizes only to the nucleus even though the *Prospero* Domain is absent, perhaps because it also lacks the homeodomain and, as a consequence of this, the NES is not present. This "nuclear restoration" has also been observed

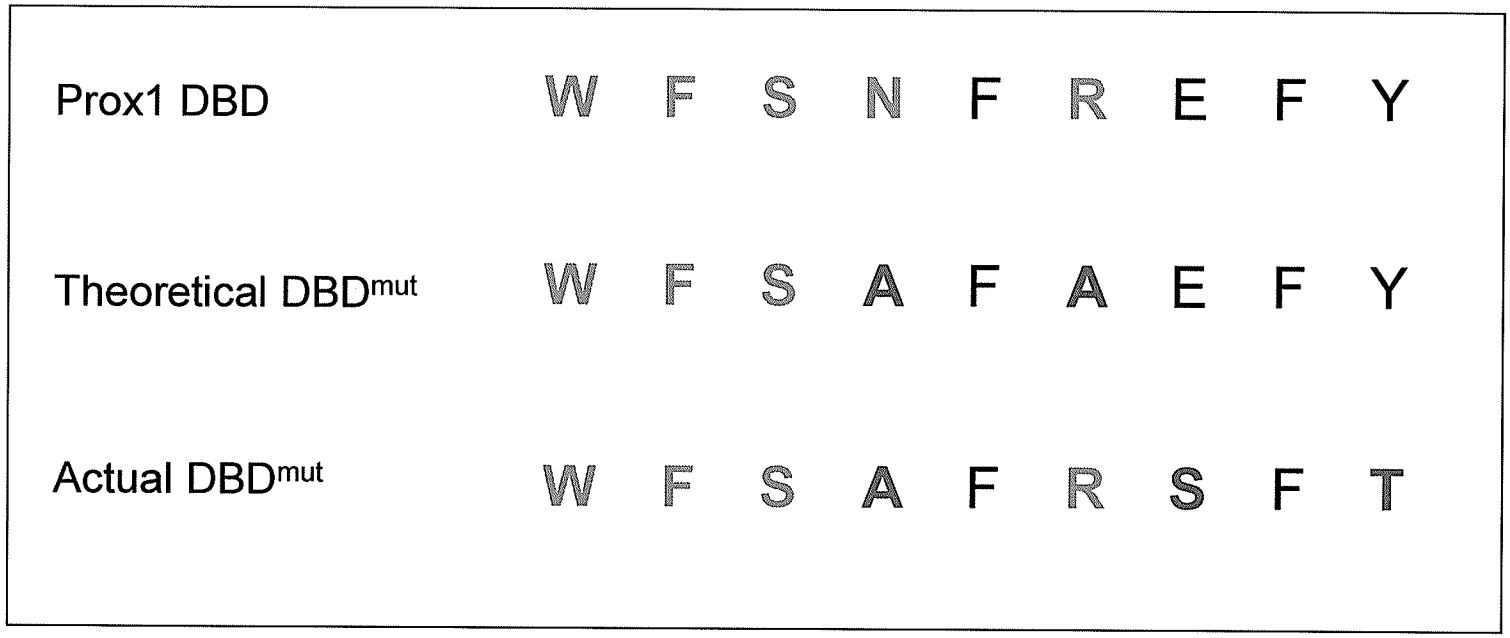
in prospero, when both the homeodomain and the *Prospero* Domain are deleted [95]. The Prox1 nuclear localization signal (NLS) has not been identified yet, but is likely localized to the N-terminal region of Prox1 since Prox1 inactivation in the mouse was achieved by an in-frame insertion of the β-galactosidase gene at amino acid 224 of the protein, and as shown by immunohistochemistry, β-galactosidase localizes to the nucleus [42]. As well, sequence analysis programs predict an NLS-like sequence at amino acid 15 of the protein.

Western blot results showed that the Prox1 PDΔ protein was expressed the least. Prospero contains a proteasome-dependent nuclear exclusion signal in a region including the α_2 and α_3 helices of the homeodomain [96]. Similar to the NES present in prospero, this proteasome-dependent nuclear exclusion signal has been proposed to be masked by the *Prospero* Domain when the prospero is in the nucleus [96]. Interestingly, the key residues of this nuclear exclusion signal are also conserved among all prospero/Prox1 family members [96]. The reduced amount of protein observed for Prox1 PDΔ could be a consequence of Prox1 proteasome-dependent degradation as a result of an unmasked proteasome-dependent nuclear exclusion signal. It would be predicted that Prox1 HDΔ and Prox1 HDPDΔ versions had shown the highest level of protein expression, since they lack the homeodomain and thus the proteasome-dependent nuclear exclusion signal. However, as this increase in the amount of protein was not consistently observed throughout this study, this subject requires further investigation.

Prospero subcellular distribution is regulated during *Drosophila* embryonic nervous system development. Typically, a neuroblast will divide asymmetrically to give rise to a ganglion mother cell (GMC), which will again divide to form the mature neurons and glia [95]. In neuroblasts, prospero is cytoplasmic, whereas in ganglion mother cells (GMC) it is nuclear and establishes GMC-specific transcription [51, 52, 127]. On the other hand, prospero is completely degraded in neurons [51, 52,]. In vertebrates, changes in subcellular distribution of Prox1 have not been conclusively established. One study has shown that during lens development Prox1 changes its subcellular localization [128]. Prox1 was reported to localize mainly in the cytoplasm of lens epithelial cells and to localize exclusively in the nucleus of newly differentiating lens fiber cells [128]. In contrast, Wigle *et al.* showed that, in the lens, Prox1 was nuclear in both the dividing epithelium and lens fibers [84]. Prox1 is also expressed in lymphatics, liver and pancreas, where it plays important roles during development [1, 55, 81, 86, 87]. However, it is unknown whether Prox1 undergoes subcellular distribution changes in these tissues during development. It is likely that Prox1 and prospero regulate their subcellular localization by the same mechanism involving the nuclear export and nuclear degradation exclusion signals present in their homeodomain and the *Prospero* Domain in order to have them masked or more accessible.

Our luciferase reporter assay results revealed that Prox1 DBD^{mut} and Prox1 HDΔ, which are the versions of Prox1 with the DNA binding domain mutated or the entire homeodomain deleted respectively, were able to activate transcription

of the *Cyclin E1* promoter to comparable levels with that observed with Prox1 WT. This result suggests that Prox1 activates transcription of this promoter in a DNA-binding independent manner, and that the Prox1 homeodomain is not required for the protein-protein interactions involved in this activation. Prox1 DNA-binding independent activation of the *Cyclin E1* promoter was observed in both HEK 293 and U2OS cells. This finding suggests that Prox1 mediated activation of the *Cyclin E1* promoter may be cell type independent and that Prox1 likely mediates this activation by a similar mechanism in these two cell types. Surprisingly, Petrova *et al.* reported that a Prox1 mutant version containing **two** amino acids substitutions in its DNA binding domain was not able to activate the 1 Kb *Cyclin E1* promoter [2]. This result suggested that Prox1 activates this promoter in a DNA-binding dependent manner. However, the primers that they published to create their DNA binding domain mutations do not actually mutate the residues they have indicated **(Figure 20)**. Apart from mutating only **one** residue in the DBD of Prox1, these primers mutate two residues located outside of this domain. These additional mutations could have affected the stability of the protein and thereby altered its ability to activate the *Cyclin E1* promoter. It is also possible that the additional mutations have an effect on the three-dimensional structure of the protein, modifying other domains important for the activation of this promoter. Since the authors did not present western blot or immunocytochemistry results using this Prox1 DBD^{mut} version, their results regarding Prox1 DNA-binding dependent activation of *Cyclin E1* can not be considered as.



From [2]

Figure 20. Theoretical and actual versions of Prox1 DBD^{mut} used by Petrova et al.
Scheme showing Prox1 DNA binding domain (DBD, showed in blue) and Prox1 DBD mutants (DBD^{mut}, mutations shown in red). Theoretical DBD^{mut} refers to the mutations that the authors state they introduced to Prox1 DBD [1]. Actual DBD^{mut} refers to the mutations encoded by the primers the authors declare they used.

Prox1 has been reported to regulate promoters in DNA-binding dependent and independent manners. Prox1 has been shown to activate transcription of genes by binding to specific DNA elements in their promoters [90, 91]. These DNA elements (CACTTCC and CACGCCTCT) share homology with the consensus site C(a/t)(c/t)NNC(t/c) originally described for prospero [72]. On the other hand, Prox1 has been reported to repress transcription of genes in a DNA-binding independent manner, by acting as a co-repressor [73, 75, 76]. To our knowledge, ours is the first study in which Prox1 has been shown to **activate** transcription in a DNA-binding independent manner (Co-activator).

An important novel finding of this study is the requirement of the *Prospero* Domain for Prox1 mediated activation of the *Cyclin E1* promoter. As shown by luciferase assays, the absence of the *Prospero* Domain in Prox1 (Prox1 PDΔ) significantly reduces the ability of Prox1 to activate the transcription of *Cyclin E1*. A possible explanation for the decrease in activation observed is that in the absence of the *Prospero* Domain, the NES present in the homeodomain is exposed and as a consequence the protein is being exported to the cytoplasm and less protein remains in the nucleus for transcriptional activation. As western blot results from whole cell lysates showed a reduced amount of protein for Prox1 PDΔ, it is likely that this version of Prox1 is also being degraded, probably as a result of an unmasked proteasome-dependent nuclear exclusion signal. Although the absence of the *Prospero* Domain results in reduced levels of Prox1 remaining in the nucleus, and this would decrease the level of activation of the

Cyclin E1 promoter, we found that the *Prospero* Domain is also required for the process of activation itself. In the absence of the homeodomain but when the *Prospero* Domain is present (Prox1 HDΔ), Prox1 fully activates the *Cyclin E1* promoter. However, when both domains are absent (Prox1 HDPDΔ), the ability of Prox1 to activate transcription of *Cyclin E1* decreases significantly. In Prox1 HDΔ and Prox1 HDPDΔ, the homeodomain is absent and hence, the NES and the proteasome-dependent nuclear exclusion signal are missing. Therefore, the *Prospero* Domain is not required for the regulation of the subcellular localization or degradation of Prox1 in these versions. The only difference between Prox1HDΔ and Prox1 HDPDΔ is the absence of the *Prospero* Domain in the latter. These results strongly suggest that Prox1 requires the *Prospero* Domain for the transcriptional activation of *Cyclin E1*.

As explained above, Prox1 HDPDΔ shows significantly decreased activation of the *Cyclin E1* promoter as compared to Prox1 WT. Since this activation is significantly higher than that observed for the control, other Prox1 domain(s) are likely also involved. In order to identify the domain(s) of Prox1 responsible for this remaining activation, different Prox1 constructs were used. As shown by luciferase assays, neither of the NR boxes present in the N-terminal region of Prox1 (NR1 and NR2) are responsible for this remaining activation, nor is the Q-rich region. The domain organization of Prox1 has not been fully defined. We have shown that among all the described regions of Prox1, the *Prospero* Domain is one of the domains required for *Cyclin E1* activation of transcription. The other domain(s) that contribute to this activation remain to be identified. Since the

Prospero Domain of prospero contacts DNA [94], and the *Prospero* Domain of Prox1 is required for the maximal activation of the *Cyclin E1* promoter, it is possible that this domain plays a role in the activation of transcription of this gene by contacting DNA and, in this way, inducing a conformational change in Prox1 which allows for its optimal transcriptional activity. In the absence of the *Prospero* Domain, this conformational change would not occur and as a consequence the ability of Prox1 to activate the *Cyclin E1* promoter would be reduced.

Luciferase assays using shorter versions of the *Cyclin E1* promoter, confirmed that Prox1 does not activate *Cyclin E1* transcription by interacting with the nuclear receptor LRH-1. In addition, these experiments showed that a region of the *Cyclin E1* promoter region located between nucleotides -111 and +95 is sufficient to respond to Prox1. Interestingly, Prox1 mediated activation of the -111/+95 version of the *Cyclin E1* promoter was 3-fold higher than when either the 1 Kb or 557 bp promoter versions were used. This increase in activation could be explained as a consequence of the elimination of an unidentified repression module(s) located 5' of position -111. Moreover, the basal level of transcription of the 206 bp promoter construct was significantly higher than that observed when the 557 bp was used, supporting our explanation. As the basal transcription levels observed with the 1 Kb and 206 bp promoter constructs are similar and significantly higher than that observed with the 557 bp construct, it is likely that an unidentified "strong" activation module(s) is located 5' to position -462.

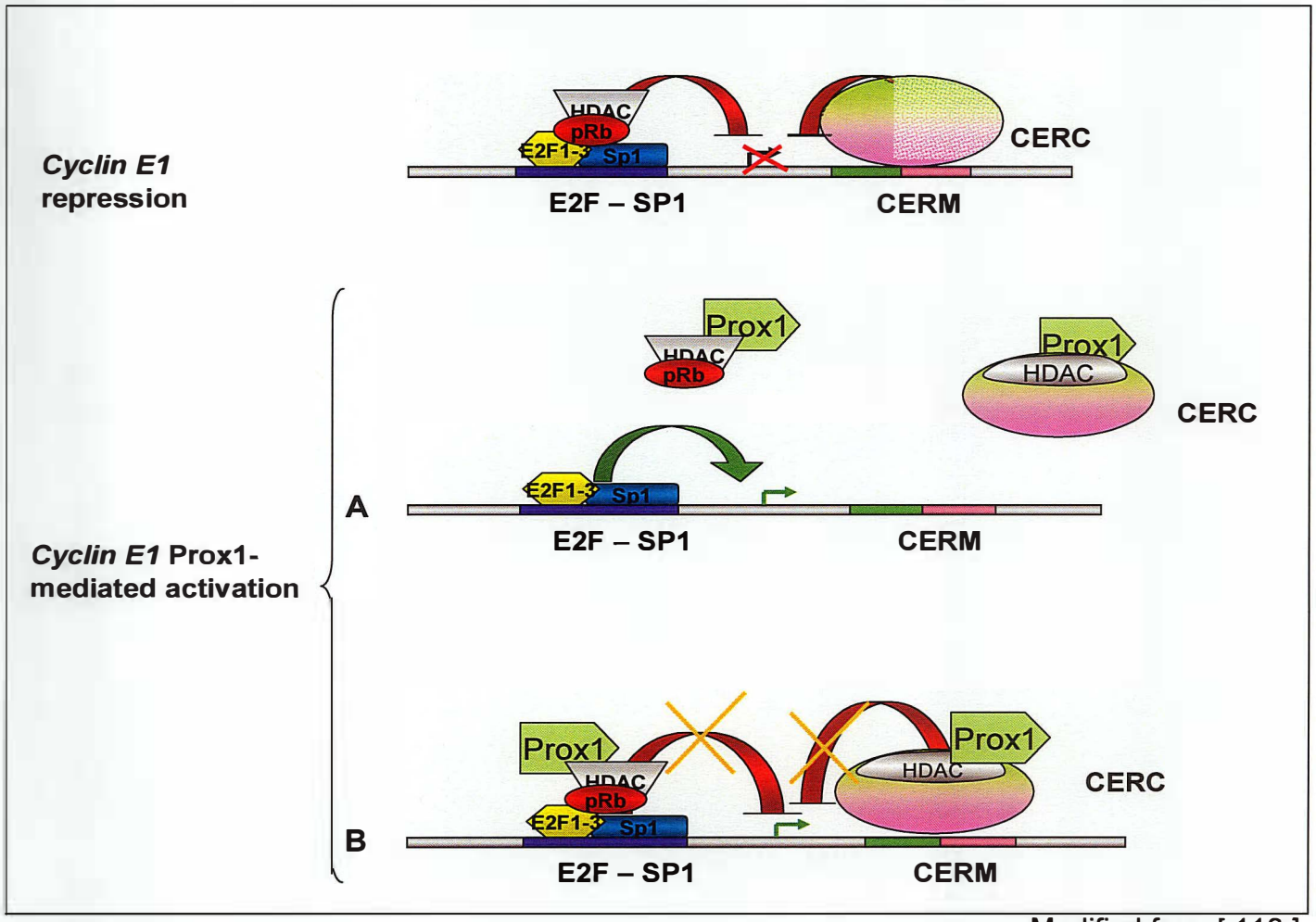
We have also shown that Prox1 activates the 4XE2F artificial promoter in a DNA-binding independent manner and that it requires the *Prospero* Domain for

full activation. Since the -111/+95 region of the *Cyclin E1* promoter is essentially composed of two E2F regulatory modules (E2F-Sp1 and CERM) [115, 116], it is likely that Prox1 mediates the activation of the *Cyclin E1* promoter through one of these two E2F regulatory modules. As our luciferase assay results showed, Prox1 activates the *Cyclin E1* promoter even when the E2FX site in CERM is mutated. This finding indicates that Prox1 does not activate *Cyclin E1* transcription exclusively through CERM. As CERC is not able to bind CERM when the E2FX site is mutated [116], de-repression of the *Cyclin E1* basal level of transcription would have been expected. However, the basal level of transcription for the mutated and wild-type versions of the -111/+95 promoter was similar, probably as a consequence of repression occurring through the E2F-Sp1 module. Another possible explanation is that CERM/CERC mediated repression affects the timing but not the level of induction of *Cyclin E1* transcription [116]. It should be noted that the luciferase experiments used in this study were performed using un-synchronized cells and the detection of luciferase expression was performed at only one time point (48 hours after transfection). The premature activation of *Cyclin E1* transcription in the absence of CERC binding may have not been detected by our studies. We have performed preliminary luciferase assays using synchronized cells and different time points for harvesting. These experiments showed that Prox1 is activate *Cyclin E1* transcription at any stage of the cell-cycle. However, we were unable to detect endogenous *Cyclin E1* activation at late G_1 when the cells were transfected with pCMV-4A empty vector (data not shown). As our luciferase assays showed,

Prox1 was able to activate the *Cyclin E1* promoter even when the E2FI site present in the E2F-Sp1 module was mutated. This result indicates that Prox1 does not activate transcription exclusively through the E2F-Sp1 site. As expected, the basal level of transcription of *Cyclin E1* was significantly decreased when the E2FI mutated 206 bp promoter was used, confirming the requirement of the E2FI site for maximal endogenous activation of this promoter [115]. It is possible that Prox1 activates the mutated promoter by releasing the repression on the CERM module, and in this way increases the basal transcription level observed when the E2FI site is mutated. Our results suggest that Prox1 regulates the *Cyclin E1* promoter through both the CERM and the Sp1-E2F modules **(Figure 21).**

In conclusion, we have provided evidence that Prox1 activates the *Cyclin E1* promoter in a DNA-binding independent and cell type independent manner. We have also shown that this activation requires the presence of the *Prospero* Domain and that the *Cyclin E1* promoter region located between positions -111 and +95 is sufficient to respond to this activation. Also, we found that the *Prospero* Domain plays a role in regulating Prox1 subcellular localization.

Although this study has not established whether *Cyclin E1* is a direct Prox1 target, it has provided important information that Prox1 does not need DNA binding to regulate *Cyclin E1* transcription. It remains to be determined if Prox1 regulates the *Cyclin E1* promoter through protein-protein interactions or if it does



Modified from [118]

Figure 21: A model for Prox1-mediated activation of Cyclin E1 transcription

During *Cyclin E1* repression, CERC is bound to CERM and pRb together with HDAC1 are bound to the E2F1-3-Sp1 complex on the E2F-Sp1 module. **A**: Prox1 activates transcription of *Cyclin E1* by interacting with the HDAC activities present in both modules and therefore removing the repressor complexes. **B**: Prox1 activates transcription by associating with the HDAC activities present in both modules and prevents them from interacting with their targets (nucleosome) , thereby preventing repression.

on the promoter of an upstream gene and therefore indirectly regulates transcription of *Cyclin E1*.

VIII. CONCLUSIONS

1. The *Prospero* Domain of Prox1 has a role in determining Prox1 subcellular localization.

2. Prox1 activates the *Cyclin E1* promoter in a DNA-binding independent manner.

3. The Prox1 homeodomain is not required for the activation of the *Cyclin E1* promoter.

4. The *Prospero* Domain is required for maximal Prox1-mediated activation of the *Cyclin E1* promoter.

5. Prox1 NR1 box and Q rich regions are not required for the activation of the *Cyclin E1* promoter.

6. The *Cyclin E1* promoter region involved in the Prox1-mediated activation is located between nucleotides -111 and +95.

7. Prox1 does not activate transcription of *Cyclin E1* exclusively through either the CERM/CERC or the E2F-Sp1 module.

IX. FUTURE DIRECTIONS

We have shown that the *Prospero* Domain is required for maximal Prox1-mediated activation of *Cyclin E1* transcription. In the absence of the *Prospero* Domain, Prox1 can still significantly activate this promoter, but to a lesser extent. This result suggests that other Prox1 domain(s) also play a role in the activation of transcription of *Cyclin E1*. Luciferase experiments using new Prox1 mutant versions will identify the other Prox1 domain(s) involved in the activation of *Cyclin E1* transcription. Our results have shown that the *Cyclin E1* promoter region responsive to Prox1 levels is located between nucleotides -111 and +95. Also, our results indicate that Prox1 does not activate transcription of *Cyclin E1* through exclusively the CERM/CERC or the E2F-Sp1 modules. Luciferase assays using a *Cyclin E1* promoter construct with mutations in both modules will be important to understand the mechanism by which Prox1 activates transcription of this gene. Immunoprecipitations will determine whether Prox1 interacts with HDAC1/HDAC3 (or other HDACs), E2F proteins as well as pRb. Luciferase assays using synchronized cells and cell lysates obtained at different time points will establish whether Prox1 activates the *Cyclin E1* promoter by affecting the timing or level of induction. Also, in order to establish whether *Cyclin E1* is a direct Prox1 target, chromatin immunoprecipitations (ChIP) will be undertaken. ChIP will determine if Prox1 activates transcription of *Cyclin E1* by binding and preventing the

proteins involved in repression, such as HDACs, from interacting and modifying their target proteins, or by binding to these repression-involved proteins and sequestring them away from the *Cyclin E1* promoter **(Figure 21).**

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