Activation of Vascular endothelial growth factor receptor-3 expression by homeobox transcription factor, Prox1

by

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A Thesis submitted to
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In Partial Fulfillment of the Requirements for the Degree of

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Department of Biochemistry and Medical Genetics
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THE UNIVERSITY OF MANITOBA

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

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This is dedicated to

my beloved father **Shri. Hanumantha Reddy** garu, who I miss the most but I have spent very little time, my mother, brother and the rest of the family.

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Contents

Abstract

Prox1 is a homeobox transcription factor that has been shown to be essential for determining lymphatic endothelial cell fate. *Prox1* null mice do not have defects in vasculogenesis and angiogenesis but have defects in the budding and sprouting of the early lymphatic endothelial cells. During normal development, a subpopulation of venous endothelial cells, upon expression of *Prox1*, up-regulates the expression of lymphatic endothelial markers like Vascular endothelial growth factor receptor-3 (VEGFR-3), Podoplanin and LYVE-1 and down-regulates the expression of blood endothelial markers such as CD34, Neuropillin-1 and Laminin. However, the role of Prox1 in the regulation of its down-stream targets involved in lymphangiogenesis has not been determined.

Mutations in the *VEGFR-3* gene have been reported in the congenital lymphedema Milroy's disease. Also, there are many studies showing the correlation between the expression of VEGFR-3 and the degree of lymph node metastasis. Adenoviral mediated expression of Prox1 induces VEGFR-3 expression in blood endothelial cells. In this study, we determined that Prox1 activates *VEGFR-3* expression in a DNA binding independent manner. This Prox1 mediated co-activation of VEGFR-3 expression does not need the nuclear receptor boxes, which are crucial for its role as a co-repressor. We were also able to determine that the minimal 266 bp *VEGFR-3* promoter is necessary and sufficient for Prox1 mediated activation. Thus, this study furthers our understanding of the molecular mechanism of lymphatic endothelial cell fate determination mediated by Prox1.

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Abbreviations

Ad	Adenovirus
AdEGFP	Adenoviral vector expressing Enhanced Green Fluorescent Protein
AdProx1	Adenoviral vector expressing Prox1
AdProx1HDPDΔ	Adenoviral vector expressing Prox1HDPDΔ
Ang-2	Angiopoietin-2
BEC	Blood Endothelial Cells
CYP7A1	Cholesterol 7-α-hydroxylase
FTZF-1	Fushi tarazu Factor-1
HNF-4α	Hepatocyte nuclear factor-4α
LEC	Lymphatic Endothelial Cells
LRH-1	Liver Receptor Homologue-1
LYVE-1	Lymphatic Vessel Endothelial receptor-1
Prox1	Prospero related homeobox transcription factor 1
Prox1HDPDΔ	Prox1 Homeodomain Prosperodomain deleted version
SLP-76	SH-2 domain Leukocyte Protein-76
Syk	Splenic Tyrosine Kinase
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3
VEGF-C	Vascular Endothelial Growth Factor-C

LITERATURE REVIEW

Introduction

Compared to the blood vascular system, the lymphatic system has been historically less well studied primarily due to the lack of lymphatic specific markers (Wigle et al., 2002). Recent studies have shown that several genes have crucial roles in the formation of new lymph vessels (Lymphangiogenesis). *Prox1* is the mammalian ortholog of the fly gene *prospero* and has been shown to be essential for determining lymphatic endothelial cell fate (Wigle et al., 1999; Wigle et al., 2002). *Prox1* null mice have no defects in either vasculogenesis or angiogenesis but have defective budding and sprouting of the lymphatic endothelial cells and thus lymphangiogenesis is arrested (Wigle et al., 1999). During normal embryonic development, the Prox1 positive cells express Platelet/endothelial cell adhesion molecule (PECAM1), a pan-endothelial marker and they gradually down regulate the blood vascular endothelial markers such as CD34, Signal transducer and activator of transcription 6 (STAT6), Neuropilin-1 and Integrin α5 (Hong et al., 2002; Hong et al., 2004b; Petrova et al., 2002).

As well, ectopic expression of Prox1 in blood endothelial cells upregulate lymphatic specific markers such as Vascular endothelial growth factor receptor-3 (VEGFR-3), Podoplanin, Lymphatic vessel endothelial receptor-1 (LYVE-1) and Integrin α9 (Hong et al., 2002; Petrova et al., 2002). VEGFR-3 is initially expressed in all endothelial cells but later becomes restricted to lymphatic endothelial cells (Dumont et al., 1998; Kaipainen et al., 1995). The study of

lymphatic development using lymphatic markers such as VEGFR-3 (or *flt4*), Podoplanin, LYVE-1 and Prox1 have supported Sabin's centrifugal model of lymphangiogenesis in which the lymphatics originate from the pre-existing venous system (Wigle et al., 1999; Wigle et al., 2002; Wigle and Oliver, 1999). Prox1 also plays a crucial role in lens fibre elongation, hepatocyte migration during liver development, maintenance of cholesterol homeostasis and pancreas development (Burke and Oliver, 2002; Cui et al., 2004; Dudas et al., 2006; Dudas et al., 2004; Duncan et al., 2002; Dyer et al., 2003; Qin et al., 2004; Schneider et al., 2006; Song et al., 2006; Sosa-Pineda et al., 2000; Wigle et al., 1999).

Even though *Prox1* has been shown to have a crucial role in lymphangiogenesis, the identity of its direct downstream targets that are required for lymphangiogenesis is largely unknown. Based on our literature review, we have selected VEGFR-3 as being a putative candidate Prox1 target gene (Hong et al., 2002; Petrova et al., 2002). We will study Prox1 regulation of *VEGFR-3* expression at both the mRNA and protein level.

Structure and functions of the Lymphatic system

Lymphatic vessels are a network of thin walled capillaries which are lined by a continuous layer of endothelial cells. The lymphatic system consists of lymphatic vessels, lymph nodes and organs. A primary function of the lymphatic system is to collect lymph, a protein rich fluid which contains cellular debris and immune cells, from the extracellular space and return this solution to the blood vascular system (Karkkainen and Alitalo, 2002). Unlike the blood vascular system, the lymphatic system is a one-way portal system therefore it can only transport lymph from the tissue spaces to the blood system and not *vice versa* (Pepper and Skobe, 2003). Lymphatic vessels are present throughout the body with the central nervous system being the only exception. Other functions of lymphatic system include the absorption of fat and fat soluble vitamins from the digestive system and mediation of the immune response by collecting antigens from the skin and presenting then to the T-cells in the lymph nodes (Marieb, 2004; Saharinen, 2004; Oliver, 2002).

Lymphatic capillaries join to form the pre-collecting tubes, which in turn join to form the lymphatic vessels. These lymphatic vessels contain a number of valves, which enable the one way flow of the lymph from the tissue spaces back into the blood vascular system. The rhythmic contraction and relaxation of the smooth muscle in the walls of the lymphatic vessels helps to pump the lymph. The lymphatic vessels join either of two lymphatic ducts, the thoracic duct or the right lymphatic duct. The former collects the lymph from the lower abdomen, pelvis, lower limbs, and the left half of the head, neck, and chest. The latter

collects lymph from the right side of the body above the diaphragm. Both of these ducts join the venous system near the junction between the internal jugular vein and the subclavian vein on the left and right sides of the body respectively.

The lymphatic vessels feed into lymph nodes, which filter bacteria and other foreign bodies in the lymph before it is drained back into blood system. These lymph nodes are distributed throughout the body and consist of the nodules and the sinuses (spaces) filled with lymph. The nodules are composed of a mesh-like tissue rich in lymphocytes and macrophages that are involved in the immune response of the body to detect and neutralize foreign bodies. These lymph nodes vary in size depending upon the number of lymphocytes present, and whether there is an underlying infectious disease. The larger lymph nodes consist of a germinal layer, where the lymphocytes undergo cell division and actively multiply. The smaller lymph nodes, referred to as the nodules, are present beneath the epithelia of the digestive, respiratory and the urinary tracts, and thus restrict the entry of bacteria and viruses into the body.

The Lymphoid organs are the bone marrow, the spleen and the thymus. The lymphocytes (T-cells and B-cells), which are produced in the bone marrow, undergo maturation in the thymus. The spleen resembles the lymph nodes in structure, shape and function despite that it filters blood instead of lymph. The spleen consists of the lymphoid tissue (white pulp) which store macrophages which are involved in cleansing the blood that enters the spleen.

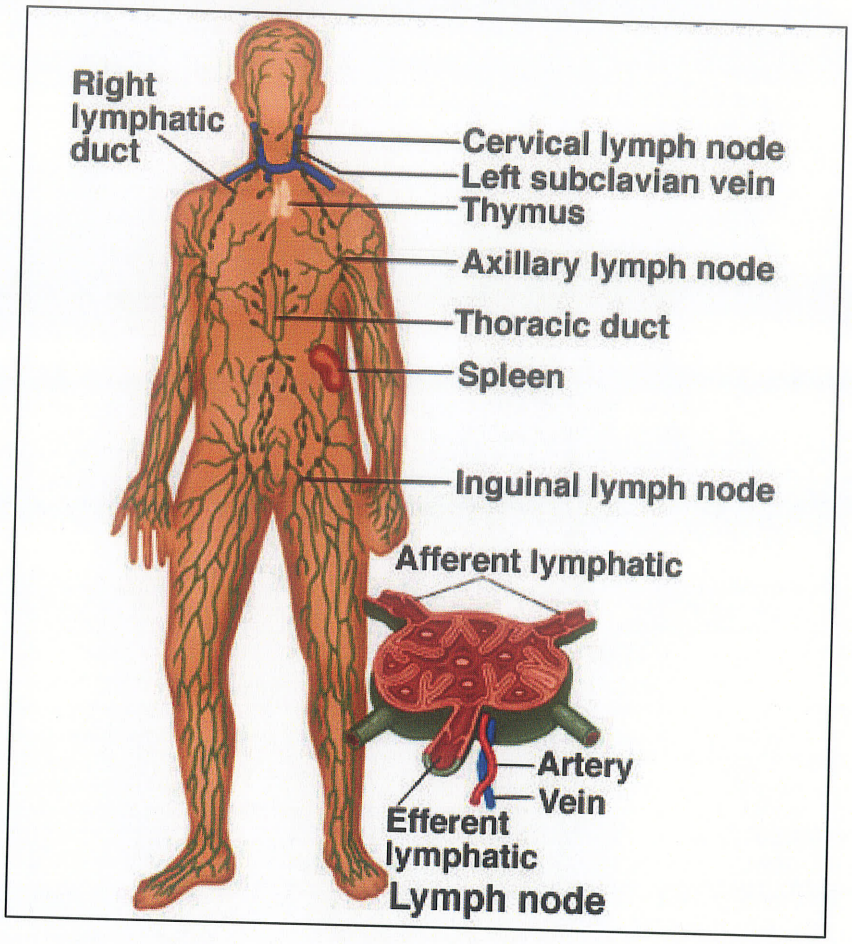


Figure 1: Organization of the Lymphatic organs in the human body
The picture shows the major lymph nodes, lymphatic vessels, thymus and spleen. In the inset we can see the transverse section of a lymph node showing the afferent lymphatic vessels collecting lymph to the lymph node and the efferent vessels, which transport lymph away from the lymph node to the blood system. (Copied with permission from McGraw-Hill Human Sexuality Image Bank (http://www.mhhe.com/socscience/sex/common/ibank/ibank/0138.jpg))

Phylogeny of the Lymphatic system

The lymphatic system is a necessity for animals that have a closed vascular system. It was previously believed that the lymphatic system arose secondary to the formation of the blood vascular system during evolution (Wilting et al., 2004). This hypothesis has been contradicted by the growing evidence that the first vascular system had a more lymphatic function (Wilting et al., 2004). Although the blood vascular system develops earlier in ontogeny than the lymphatic system, the lymphocyte developed earlier than the erythrocytes in phylogeny (Wilting et al., 2004). Currently, it is postulated that the blood vascular system developed secondary to the lymphatic system as a transport system for nutrients and waste removal. The complexity of the lymphatic system increases during evolution. The first appearance of the lymphatic system occurs among the *chondrichthyes* fishes (Wilting et al., 1999). It had been previously thought that the lymphatics originated during the shift from aquatic to terrestrial life since amphibians and reptiles show a well developed, complex lymphatic system. Amphibians have a separate lymphatic heart, which propel lymph through the lymphatic vessels. Amphibians also have sub-dermal lymph sacs below the skin which maintain the humidity of the skin thus helping in respiration (Jeltsch et al., 2003; Wilting et al., 1999). An even more complex lymphatic system appears in birds and mammals. The main difference between the avian and the mammalian lymphatic system is the number of lymph nodes in mammals (Jeltsch et al., 2003; Wilting et al., 1999).

History and Ontogeny of Lymphatic system

The word lymphatic is derived from the Latin word *lymphaticus* which means "distracted and confused" (Yoffey and Courtice, 1970; (Witte et al., 1997). The presence of "White blood" was first mentioned by Hippocrates but it was Gasparo Aselli (1627 AD) who first discovered the presence of "milky veins" in the mesentery of a well-fed dog and termed them *lacteis veins* (Witte et al., 1997). These *lacteis veins*, now called as lacteals, absorb the digested food (chyle) consisting of fat from the small intestine, which gives the cloudy milky appearance. The role of the lymphatic system in draining the extravasated lymph from the tissue spaces to the blood vascular system was demonstrated by Drinker in the early 20th century (Skobe and Detmar, 2000).

The origin of the lymphatic system has been debated since the proposal of two contradictory models one by Florence Sabin and another by Huntington / McClure at the turn of the 20th century (Sabin, 1902; Huntington and McLure 1908). Sabin, using ink injection experiments, proposed a centrifugal model in which the lymphatics derive from the venous system (Sabin 1902). This model also proposes that the lymphatic endothelial cells bud off from the vein and it is only later that the lymph sacs are formed. Recent studies based on the expression of the lymphatic marker *Prox1*, a homeobox transcription factor, and studies of the Vascular Endothelial Growth Factor-C (*VEGF-C*) knockout mice support Sabin's hypothesis of a venous origin model for lymphatics in mammals (Karkkainen et al., 2004; Wigle and Oliver, 1999). On the other hand, Huntington and McClure proposed the independent mesenchymal origin of the lymphatic

system and that it is only later during development that lymphatic vessels are connected to the venous system (Wilting et al., 2000). Using grafting experiments in the avian chorioallantoic membrane (CAM) Wilting *et al.*, (2000) have showed that the lymphangioblasts formed independently of the venous system during avian lymphangiogenesis. Van der Jagt in 1932 proposed another model which is a fusion model of the centrifugal and centripetal models and according to this model lymphatics can be both of venous and mesenchymal origins (Papoutsi et al., 2001; Wilting et al., 2001).

Molecular mechanism of lymphangiogenesis

Modern studies on the development of the lymphatic system have been accelerated after the recent discovery of the lymphatic specific markers such as *Prox1*, *VEGFR-3*, *VEGF-C*, *Vascular endothelial growth factor-D* (*VEGF-D*), *LYVE-1*, and *Podoplanin*. Lymphangiogenesis starts at around embryonic day (E) 10.5 in mouse, week 6.5-7 in humans and E4.5 in chick (Jeltsch et al., 2003).

Prox1, the earliest known marker that is specific for lymphatic endothelial cells, is expressed at E9.5 in a subpopulation of endothelial cells of the anterior cardinal vein (Wigle and Oliver, 1999). This subpopulation of endothelial cells starts budding and sprouting from the vein upon expression of *Prox1* (Wigle et al., 2002). *Prox1* null mice show defective polarization and budding of lymphatic endothelial progenitors which leads to a complete lack of lymphatic vessels by E12.5 (Wigle and Oliver, 1999). *Prox1* positive endothelial cells have increased expression of lymphatic markers such as VEGFR-3, LYVE-1 and downregulate

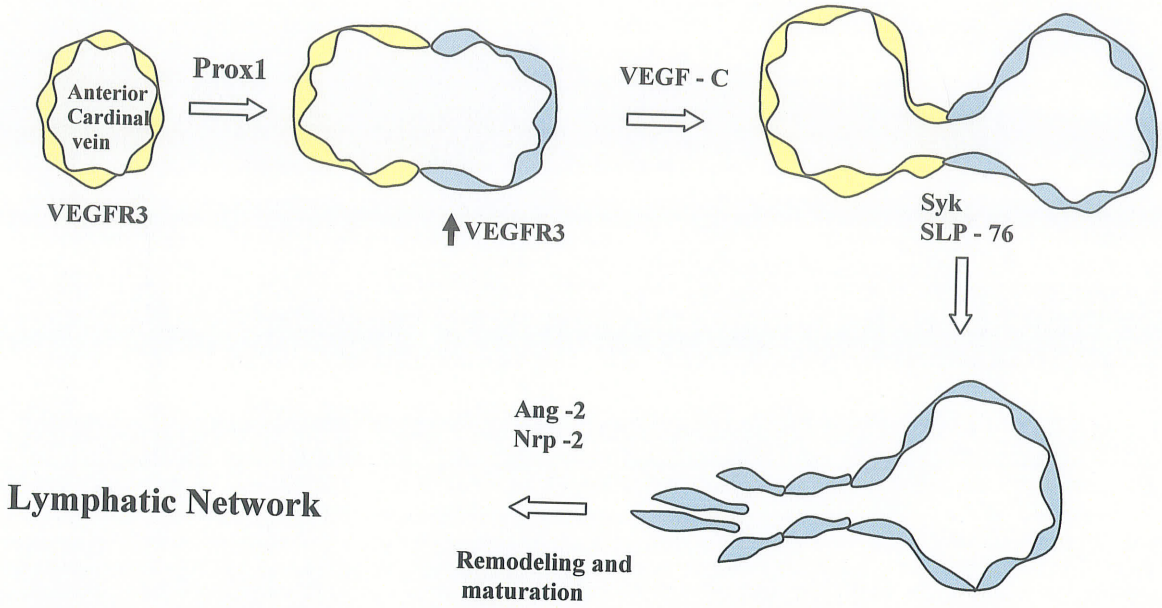
expression of blood endothelial markers such as CollagenIV and Laminin (Hong et al., 2002). This reciprocal pattern of regulation suggests that Prox1 functions as an endothelial cell fate switch.

VEGFR-3 is a cell surface receptor tyrosine kinase, whose ligands include VEGF-C and VEGF-D. VEGF-C is secreted by the mesenchymal cells and forms a chemotactic gradient signal in the regions of lymph sac formation (Karkkainen et al., 2004). The polarized endothelial cells, which express VEGFR-3, migrate towards the VEGF-C signal and form the primary lymph sacs. This migration of polarized lymphatic endothelial cells is aided by Integrin α9β1 which can bind to both VEGF-C and VEGF-D (Vlahakis et al., 2005). *VEGF-C* null mice lack functional lymphatics since the *Prox1* positive cells fail to migrate and remain trapped in the cardinal vein (Karkkainen et al., 2004). Treatment of mice with soluble VEGFR-3 protein, which sequesters VEGF-C away from its target cells, represents a model of human lymphedema (Veikkola et al., 2001). Furthermore, the human primary lymphedema, Milroy's disease, is caused by a dominant negative mutation in VEGFR-3 (Brice et al., 2005; Evans et al., 2003). These findings confirm the critical role of VEGFR-3/VEGF-C signaling pathway during lymphangiogenesis.

These primary lymph sacs are separated from the veins upon the expression of the hematopoietic signaling molecules such as *SLP-76* and *Syk* by the liver (Abtahian et al., 2003). SLP-76 is an adaptor protein whereas Syk, is a tyrosine kinase receptor. In knockout mice for either *SLP-76* or *Syk*, the primary lymphatic vessels remain attached to the veins and thus inhibit further formation

of the lymphatic network (Abtahian et al., 2003). Once the primary lymph sacs are separated from the veins, they undergo maturation and remodeling mediated by Neuropilin-2 and Angiopoietin-2 to form the mature lymphatic vessels (Gale et al. 2002). Tie2, a receptor tyrosine kinase present on the lymphatic endothelial cells, acts as a receptor for Angiopoietin-2, expressed by surrounding mesenchymal cells, and mediates inter-endothelial interactions. The loss of Angiopoietin-2 function leads to the formation of leaky lymphatic vessels (Gale et al. 2002). Neuropilin-2 is a non-tyrosine kinase transmembrane glycoprotein that can interact with various VEGF ligands and is found in lymphatic endothelial cells not in venous endothelial cells. *Neuropilin-2* null mice have mild defects in lymphatic capillaries formation during embryogenesis but the adults do not have any detectable functional deficits (Chen et al., 2000; Yuan et al., 2002).

There are many unanswered questions about the process of lymphangiogenesis such as what signal triggers Prox1 expression in a restricted population of cells of the embryonic vein and what are the direct down stream targets of Prox1 that are required for lymphangiogenesis?



(Modified from Nature immunology, Judah & Arja, 2004)

Figure 2 : Molecular Players in Lymphangiogenesis: A subpopulation of venous endothelial cells at E9.5 express Prox1 which triggers the cell fate switch to become lymphatic endothelial cells and these cells have increased expression of lymphatic markers such as VEGFR-3 and Podoplanin. VEGF-C, a ligand for VEGFR-3, is secreted by surrounding mesenchymal cells at the regions of primary lymph sac formation and acts as a gradient signal for the migration of the lymphatic endothelial cells to form the lymph sac. Once the primary lymph sac is formed, Syk and SLP-76 signaling molecules separate the primary lymph sac from the venous system and this lymph sac undergoes further maturation and remodeling in the presence of Ang-2 and Nrp-2 and form the complete mature lymphatic network.

Abnormalities associated with Lymphatic system dysfunction

Since, the lymphatic system controls fluid and cellular transport, it is essential for the control of infectious diseases, the return of interstitial fluid to the blood circulation and tumour metastasis. Thus, lymphatic dysfunction leads to a spectrum of different disorders in humans.

Lymphedema

Insufficient lymphatic function leads to the accumulation of lymph in tissue spaces and subsequent enlargement and often inflammation of the affected tissue (Browse et al., 1986). This condition is termed as Lymphedema. The Lymphedemas are classified into primary lymphedemas/congenital lymphedemas or the secondary lymphedemas/acquired lymphedemas (An and Rockson, 2004).

Primary Lymphedemas:

Congenital primary lymphedemas result from a primary genetic defect. These congenital lymphedemas, which are more common among females rather than males, are estimated to occur in one out of 6000 people (Dale, 1985). These lymphedemas are mainly autosomal dominant with reduced penetrance and variable expression but they can also be autosomal recessive or X linked (Brice et al., 2002; Dumont et al., 1998; Karkkainen and Alitalo, 2002; Karkkainen et al., 2004). Hereditary lymphedemas are classified based on the age of disease onset. Milroy's disease has a congenital onset, Meige disease occurs at puberty

and lymphedema *tarda* occurs after the age of 35 (Brice et al., 2002; Dumont et al., 1998; Karkkainen and Alitalo, 2002; Karkkainen et al., 2004). Milroy's disease is characterized by lymphedema of the lower limbs and is generally not associated with any major abnormalities except for hydrocoeles and some urethral abnormalities. Familial inheritance studies showed the linkage of Milroy's disease to the chromosome 5q at the location of *VEGFR-3* (Karkkainen et al., 2004). In most cases Milroy's disease develop as a result of a dominant negative mutation in the kinase domain of *VEGFR-3* (Brice et al., 2002; Dumont et al., 1998; Karkkainen and Alitalo, 2002; Karkkainen et al., 2004). Various mutations in *VEGFR-3* kinase domain like G857R, R1041P, R1044P, P1114L and H1035R leading to Milroy's disease are shown to be leading to a failure in autophosphorylation which interrupts the downstream signalling cascade (Karkkainen et al., 2004).

Meige disease appears at puberty and is the most common primary lymphedema. Lymphedema *tarda* is the least common among the primary lymphedemas and appears generally after the puberty (after the age of 35) (Ferrell et al., 1998; Finegold et al., 2001; Karkkainen et al., 2000; Levinson et al., 2003). Lymphedema-distichiasis is an autosomal dominant disorder with variable age of onset. Chromosomal mapping studies have attributed LD to the mutations in *FOXC2,* a member of winged helix family of transcription factors (Ferrell et al., 1998; Finegold et al., 2001; Karkkainen et al., 2000; Levinson et al., 2003). Mutations in *FOXC2* have also been attributed to other lymphedemas without dischitiasis such as in the case of yellow nail syndrome and

lymphedema-ptosis (Finegold et al., 2001).

Lymphedema can occur along with major developmental syndromes like Noonan's syndrome or Turner's syndrome (45, XO karyotype females). Turner's syndrome occurs in of every 2,500 female babies through out the world. The common symptoms among the females with Turner's syndrome are mainly short stature, and infertility but appearance of extra skin near the neck (webbed neck), swelling of the hands and legs due to accumulations of lymph (Lymphedema), bowed arms, and other skeletal defects like dislocated hip are also found. Even though there is no genetic cure, growth hormone treatment for the children to grow taller and estrogen treatment for the adolescents to promote the development of secondary sexual characteristics are available.

Secondary Lymphedema:

Secondary lymphedema is more prevalent than primary lymphedema. In technologically well developed countries, Secondary lymphedema occurs mainly post-surgically or after radiotherapy for cancer due to the destruction of the lymphatic vessels and lymph nodes in the affected region (Karkkainen and Alitalo, 2002). Surveys report that 26% of the breast cancer treated patients develop lymphedema. Even though 23% cancer patients treated with either surgery or radiotherapy developed lymphedema with in the first 2 years this number gradually raised to 45% among the patients treated after 15 or more years (Erickson et al., 2001). In tropical countries, secondary lymphedema often occurs due to the disruption of the lymphatic system by the parasite *Wuchereria*

bancrofti, which is transmitted by the female *Culex* musquitoe (Price, 1975). In Asia, Latin America, the Pacific and Africa, where filariasis is endemic, 100 million people are estimated to be suffering from this disease (El Setouhy et al., 2004). The most effective treatment for lymphedema in the cancer patients includes massage therapy and compression banding. In the case of filariasis patients, diethylcarbamazine and ivermectin drugs were used to prevent further spread of the disease from the infected patients but there is no cure available for the already suffering patients (El Setouhy et al., 2004).

Role of the Lymphatic system in Cancer metastasis:

The role of the lymphatic system in the spread of cancer to other parts of the body had been controversial but recent studies have shown that the lymphatic system acts as a primary route for tumour spread (Padera et al., 2003). Previously, many studies had supported the passive role of lymphatic system in cancer metastasis. However, there were doubts about the existence of functional intra-tumoural lymphatic vessels due to the high interstitial pressures found inside tumours that would collapse the thin walled lymphatic vessels (Jain and Fenton, 2002). Fluorescent dye flow experiments by Kendrick *et al.,* showed that there were no functional intratumoural lymphatic vessels but did show that the existing lymphatic vessels in the periphery of the tumour were enlarged (Kendrick et al., 2003). These enlarged peri-tumoural lymphatic vessels may be sufficient to facilitate the migration of cancer cells into the lymphatic system and thus leading to metastasis to the lymph node and distant tissues (Kendrick et al., 2003). There

are many recent studies that support the active role of the lymphatic system in cancer metastasis. The studies have been enabled by the discovery of the lymphatic endothelial specific markers such as *Prox1, LYVE-1, Podoplanin, VEGFR-3* and its ligands, *VEGF-C* and *VEGF-D*. Skobe *et al.,* in their studies on orthotropically transplanted human breast cancer cells in nude mice have found that the expression of intratumoural lymphatics is induced by the overexpression of VEGF-C (Angeli et al., 2006; Cassella and Skobe, 2002; Pepper and Skobe, 2003; Pepper et al., 2003; Rafii and Skobe, 2003; Roberts et al., 2006; Skobe et al., 2001; Swartz and Skobe, 2001). They have also observed a correlation between the expression of VEGF-C and the degree of lymph node metastasis in their mouse model. This correlation was further supported by similar studies on human patients with head and neck carcinomas, thyroid carcinoma and melanoma (Birck et al., 1999; Salven et al., 1998; Valtola et al., 1999). Other studies have supported the idea that the intra-tumoural lymphatics are not functional in the VEGF-C models (Detmar and Hirakawa, 2002; Oliver and Detmar, 2002; Skobe et al., 2001). This finding also suggests that the dilation of the peripheral lymphatics is sufficient to promote lymph node metastasis. Whether lymphatics play an active or passive role in tumour metastasis is not clear however their importance for cancer metastasis has been established. Targeting of the VEGFR-3/VEGF-C/VEGF-D signal pathway has been considered to be a promising therapeutic target for treating cancer metastasis since this signaling has been shown to be crucial for both angiogenesis and lymphangiogenesis (Detmar and Hirakawa, 2002; Oliver and Detmar, 2002;

Skobe et al., 2001).

Kaposi sarcoma

Kaposi sarcomas are malignant dermal tumours that are often found on the skin of individuals infected with human immunodeficiency virus (HIV) (Hong et al., 2004a). Kaposi sarcoma requires infection by herpes simplex virus to form tumours. This infection occurs frequently in AIDS patients since they are immunosuppressed (Hong et al., 2004a). Kaposi tumour cells are generally spindle shaped and can contain spaces filled with red blood cells. The increased growth of lymphatic endothelial cells infected with Kaposi sarcoma associated herpes virus (KSHV) has been observed (Hong et al., 2004a). These tumour endothelial cells express lymphatic endothelial specific markers such as Prox1, VEGFR-3 and Podoplanin. Hong *et al.,* have showed that the infection of blood endothelial cells with KSHV increased the expression of lymphatic markers thus supporting the role of lymphatic endothelial cells in the formation of this tumour (Hong et al., 2004a).

Homeobox genes:

Homeobox genes encode a large family of transcription factors that contain a 60 amino acid helix-turn-helix motif termed the homeodomain. The homeodomain consists of three α helices of which two lie parallel and are crossed by the third helix that is termed as the recognition helix (Banerjee-Basu and Baxevanis, 2001). The recognition helix of the homeobox genes often binds

to a core binding site composed of four bases – TAAT/ATTA in genomic DNA (Banerjee-Basu and Baxevanis, 2001). The temporal and spatial expression of homeobox genes varies among species and between species. The homeobox is homologous between all homeobox genes and is conserved during evolution (Banerjee-Basu and Baxevanis, 2001).

Currently there are more than 200 homeobox genes that have been found. Thirty nine of these genes are grouped into four clusters of HOX transcription factors, which play a vital role in controlling the unique appearance of the body segments during the anterior-posterior body patterning. These HOX transcription factors are expressed spatially in a pattern determined by their place in the HOX gene cluster (Banerjee-Basu and Baxevanis, 2001). The genes at the 3' end of the cluster are expressed in the rostal regions of the organism and those at the 5' end are expressed at the caudal regions of the organism. Most of the homeobox genes are not organized into these clusters (Banerjee-Basu and Baxevanis, 2001). Homeobox transcription factors are not only involved in body patterning but have a wide range of roles during embryogenesis such as controlling cell proliferation, cell differentiation and organogenesis (Banerjee-Basu and Baxevanis, 2001). This diverse set of functions regulated by homeobox genes is attributed to their ability to regulate transcription of many different downstream genes.

Structure and Function of Prox1

Prox1 is the mammalian ortholog of the *Drosophila melanogaster* gene

prospero. It is a homeobox transcription factor of 737 amino acids and has a predicted molecular weight of 84 kDa. The carboxyl terminal region of Prox1 consists of a 60 amino acid homeodomain linked to a 100 amino acid prospero domain, which is unique to the *Pros* family of transcription factors and well conserved through evolution (Hassan et al., 1997; Yousef and Matthews, 2005). The mouse Homeo-Prospero domain (HPD) is sufficient for binding to a specific DNA sequence C A/t C/t N N T/c divergent from the typical homeodomain protein binding site. The mouse HPD is 78% similar to the same region of its *Drosophila* ortholog, *prospero*, suggesting that there are conserved functional similarities across evolution (Hassan et al., 1997; Yousef and Matthews, 2005). This conserved HPD region is required for Prox1 to bind DNA. Unlike most homeobox genes, the *Prox1* homeodomain has the tetrapeptide WFSN instead of WFQN in its recognition helix (Hassan et al., 1997; Yousef and Matthews, 2005). The three dimensional structure of the HPD reveals the divergent organization of the prospero homeodomain compared to that of typical homeobox proteins. This divergence is largely attributed to the integration of the homeodomain with the prospero domain to form a single larger structural unit (Hassan et al., 1997; Yousef and Matthews, 2005). This unique structure might also explain the atypical DNA binding specificity of prospero. The HPD consist of six α helices α1-α6 (Yousef and Matthews, 2005). Like, typical homeodomains, the *Prospero* homeodomain consists of three α helices (α1- α3) in helix-turn-helix fashion (Yousef and Matthews, 2005). The *Prospero* homeodomain differs from typical homeodomains in having more bulky hydrophobic residues on the surface in the

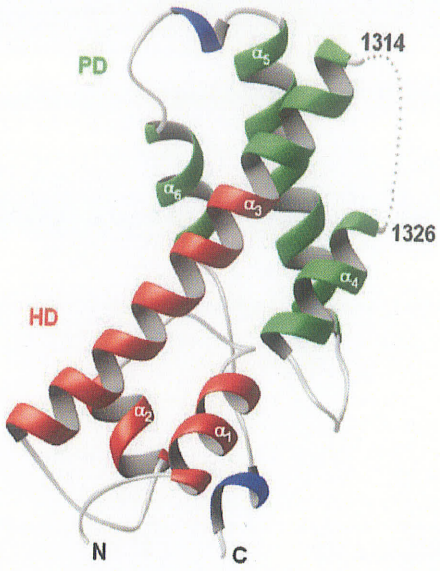
loop region between the α1 and α2 helices and the middle of α3, which help in the formation of strong hydrophobic interactions with the prospero domain (Yousef and Matthews, 2005). The prospero domain consists of four α helices; a part of α3, α4, α5 and α6 arranged in an anti-parallel, right turning, and up-down-up-down topology (Fig 3) (Yousef and Matthews, 2005).

The nuclear export signal present in the α1 helix of the homeodomain is masked by the α6 helix present at the extreme carboxyl terminus of the of the prospero domain once the protein is in the nucleus (Yousef and Matthews, 2005). Removal of the prospero domain leads to the export of prospero into the cytoplasm via the Exportin pathway (Yousef and Matthews, 2005). Protein-DNA binding of prospero is predicted to involve three potential domains. The α3 helix, which forms the DNA recognition helix, binds to the major grove (Yousef and Matthews, 2005). Whereas, the amino terminal arm of the homeodomain and the prospero domain are predicted to make contact with minor grove and the back bone respectively (Yousef and Matthews, 2005).

The amino-terminal domain of Prox1 contains two putative I/LXXLL motifs, termed as NR boxes, that are important for Prox1 binding to the ligand binding domain of nuclear receptors such as Liver receptor homologue-1, Fushi tarazu factor-1 and HNF4α (Fayard et al., 2004; Steffensen et al., 2004). The amino terminal domain of Prox1 also contains a glutamine rich (Q-rich) region whose function is unknown and a nuclear localization signal (NLS). Even though the exact location of the NLS is not yet identified it is predicted to be in the first 224 bp of the N-terminal region (Wigle et al., 1999). We have also found in our

immunocytochemisty experiments that the C-terminal region consisting of just the homeodomain and the prospero domain was not localized to the nucleus (Baxter and Wigle., unpublished results).

A.



(Copied with permission from Structure, Yousef and Matthews., 2005)

B.

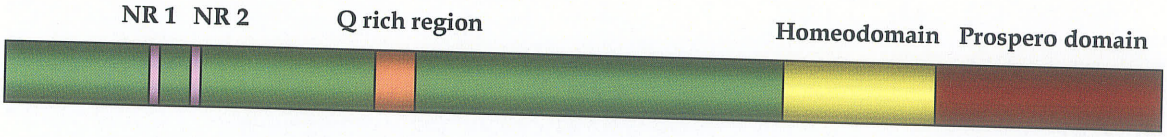


Figure 3: Structure of Prox1: A). Crystallographic studies of the homeodomain and the prospero domain of the prospero family homeobox transcription factors show a single structural unit of Homeo-Prospero domain. Out of the six α helices α1, α2 and a part of α3 forms the homeodomain, the other part of α3, α4, α5, and α6 form the Prospero domain. The DNA recognition helix is formed by the α3 and the nuclear export signal is present in α1 helix. B). The Linear structure of Prox1 showing the major functional domains.

Molecular mechanisms of Prox1 regulation

Given the vital role of Prox1 in the development of lymphatic system, liver, pancreas and eye, several targets have been investigated. Microarray analysis reports revealed a myriad of genes regulated by Prox1 during lymphangiogenesis (Petrova et al., 2002 and Hong et al., 2002) and pancreatic development (Wang et al., 2005). Some of these could be putative downstream target genes regulated directly by Prox1. These targets include BEC markers (CD34, CD31 and STAT6) that are down regulated and LEC markers (VEGFR-3, Podoplanin and LYVE-1) which are upregulated upon the ectopic expression of Prox1. Prox1 also regulates a variety of cell cycle regulatory genes depending on the cell type either directly or indirectly. In the developing lymphatic vasculature and eye, Prox1 promotes cell cycle exit and promotes cell differentiation (Wigle et al., 1999). Conversely, Prox1 prevents cell cycle exit and there by allowing further rounds of cell cycle in the progenitor cells of pancreas by preventing the accumulation of the cell cycle inhibitor Cdkn1b/p27 (Wang et al., 2005). Several other Prox1 targets like *βB1-Crystallin*, *γ-Crystallin* and *SOX2* during eye development and *CYP7A1* gene expression during liver development have been investigated (Cui et al., 2004; Lengler and Graw, 2001; Lengler et al., 2001).

Generally, transcription factors activate or repress by directly binding to the promoter and such transcription factors are called activators or repressors. Transcription factors can also regulate the activation or repression of its downstream targets by indirectly binding to the promoter through other members of the transcription machinery and these transcription factors are called co-

activators or co-repressors (Fig 4). Interestingly, except as a repressor, Prox1 has been shown to act as an activator, co-activator and a co-repressor (Cui et al., 2004; Qin et al., 2004).

Prox1 - the activator:

Currently, Prox1 has been reported to act as an activator of a set of target genes. One such target is the fibroblast growth factor receptor-3 (FGFR-3), which has been shown to play divergent roles like endothelial cell proliferation, migration and differentiation during lymphangiogenesis independent of the VEGF-C/VEGFR-3 signalling pathway. Shin *et al.*, have shown that Prox1 activates the *FGFR-3* promoter by directly binding to the promoter via its DNA binding domain (Shin et al., 2005). The proximal 220 bp *FGFR-3* promoter, which has several putative Prox1 binding sites (C(a/t)(c/t)NNC(t/c) and (T)AAGACG), is necessary for the activation by Prox1. Another direct target, which has been reported to be activated by Prox1 at the transcriptional level by binding to the promoter, is *βB1-Crystallin* gene. Like *FGFR-3* promoter, the *βB1-Crystallin* gene promoter also possesses similar Prox1 putative sites in the proximal promoter region (Cui et al., 2004). *SOX2* and *γ-Crystallins* are the other genes which have similar Prox1 putative sites and are also proposed to be activated by Prox1 by direct interaction with the respective promoters (Lengler et al., 2001).

Prox1 – the Co-activator

In unpublished data from our lab, Bocangel and Wigle for the first time, have shown the DNA binding independent activating role of Prox1 in the *Cyclin E1* gene expression. This transcriptional activation of *Cyclin E1* does not require the homeodomain of Prox1 which is crucial for DNA binding but requires the prospero domain and the N-terminal region of Prox1 for full activation. Thus, this finding points to the involvement of the prospero domain and the N-terminal region in binding to the various other co-factors to activate the *Cyclin E1* transcription. The Prox1 mediated activation of *Cyclin E1* transcription is predicted to be through two E2F sites, one located upstream and the other on the downstream of the transcription start site as Prox1 was found to activate the 6XE2F or 4XE2F artificial promoters which contain 6 or 4 copies of the E2F binding site respectively (Petrova et al., 2002). Prox1 is hypothesized to be activating the transcription of *Cyclin E1* by removing the repression mediated by HDAC3 via pRb/E2F interaction (Steffensen et al., 2004).

Prox1 – the Co-repressor

Prox1 interacts with members of orphan nuclear receptor family NR5A. These nuclear receptors having the AF2 domain are reported to be binding to the proteins with the LXXLL motifs. Prox1 has two such motifs LRKLL (NR1 box) ISQLL (NR2 box) and was shown to bind human Liver receptor homologue-1 (LRH-1) of NR5A2 subfamily and Hepatocyte nuclear factor-4α (HNF-4α) of NR2A1 subfamily (Qin et al., 2004; Song et al., 2006). Both LRH-1 and HNF-4α

are involved in the regulatory network of *cholesterol 7-α-hydroxylase* (*CYP7A1*) gene expression. The Prox1 NR1 box, LRKLL motif, plays an essential role in interacting with AF2 domain of LRH-1 and HNF-4α individually. Prox1 binds to these factors and represses *CYP7A1* expression (Qin et al., 2004; Song et al., 2006). A similar interaction of Prox1 with zebra fish orphan nuclear receptor of Ff1b (NR5A4) during the inter-renal development of the zebra fish was also demonstrated (Steffensen et al., 2004).

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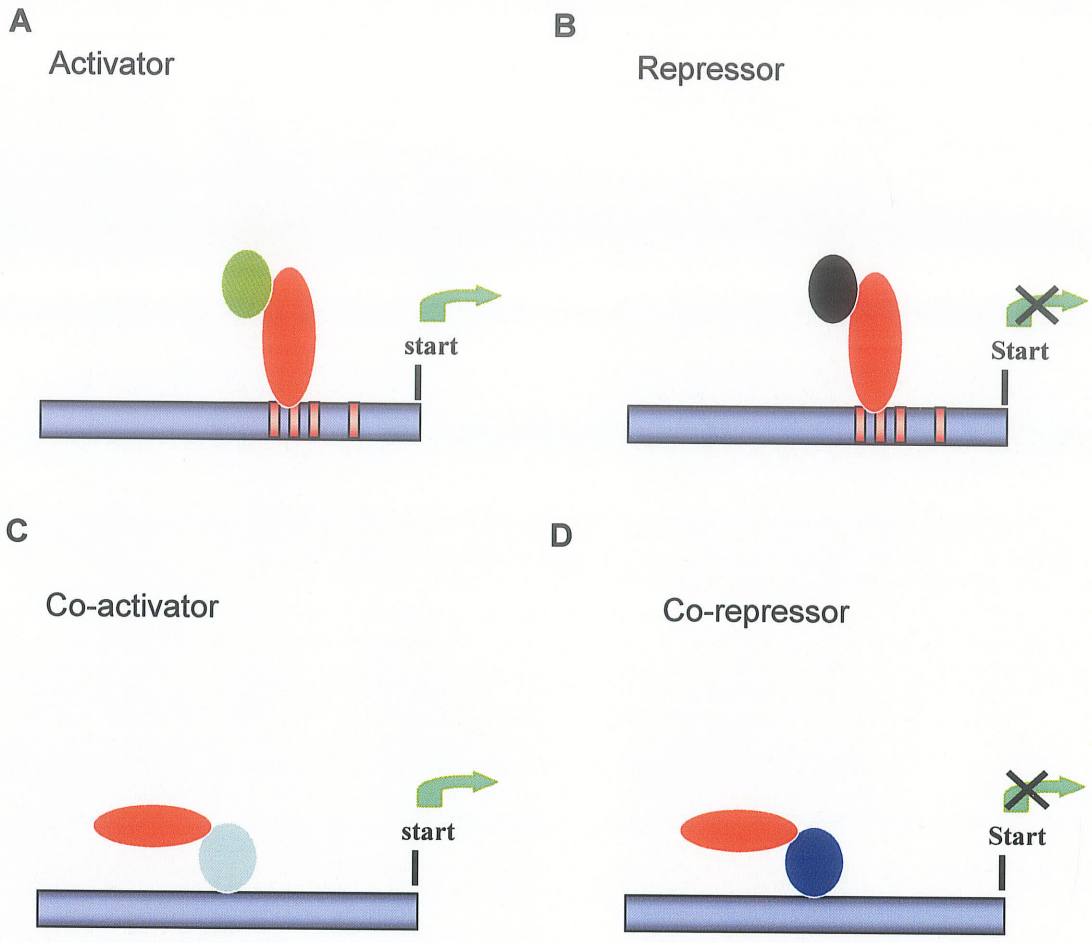

Fig 4: General molecualr mechanisms of transcriptional factors: A transcription factor can regulate the expression of its target gene by either directly binding to its DNA (Regulator) or by recruiting to the promoter with the help of other transcriptional co-factors (Co-regulator). The transcription factors that upregulate the gene expression by directly binding to the DNA are called activators (A) and those that repress are called repressors (B). Similarly, the transcription factors that activate upon recruitment on to the promoter with the help of other co-factors are called co-activators (C) and those repress are called co-repressors (D). Prox1 has been shown to activate, co-activate and co-repress its downstream targets.

Vascular Endothelial Growth Factor Receptor-3

Endothelial receptor tyrosine kinases are divided into two major families, the vascular endothelial growth factor receptor family (VEGFR family) and the Angiopoietin family of receptors (Tie1 and Tie2). The VEGFR family includes VEGFR-1, VEGFR-2 and VEGFR-3. Members of VEGFR family contain an external ligand binding domain and an internal tyrosine kinase domain (Iljin et al., 2001). The external ligand binding domain of the VEGFR family is composed of seven immunoglobulin homology repeats. Unlike VEGFR-1 and VEGFR-2, VEGFR-3 contains of two disulphide bonds in its fifth immunoglobulin homology domain which helps in keeping the ligand binding domain intact with the rest of the receptor.

The ligands of VEGFR's include VEGF, VEGF-B, VEGF-C and VEGF–D, which preferentially bind to their corresponding receptor (Alam et al., 2004; Iljin et al., 2001). VEGF and VEGF-B binds specifically to VEGFR-2 whereas VEGF-C and VEGF-D preferentially bind to VEGFR-3 (Iljin et al., 2001). VEGFs are initially produced as pre-pro-proteins and undergo proteolytic cleavage to become activated. The fully processed molecules can show varied specificity when compared to the incompletely processed forms (Veikkola et al., 2001). Even though VEGF-C secreted as a 60 kDa protein binds specifically binds to VEGFR-3 and partially to VEGFR-2 but when completely proteolysed and processed to a 20 kDa fragment, binds both VEGFR-3 and VEGFR-2 effectively and promote the dimerization. The binding of the ligands (VEGF-C or VEGF-D) to the receptors (VEGFR-3 or VEGFR-2) results in either the formation of

homodimers (VEGFR-3/VEGFR-3 or VEGFR-2/VEGFR-2) or heterodimers (VEGFR-3/VEGFR-2). This dimerization of the receptors triggers the transphosphorylation of the specific tyrosine residues in the intracellular domain. The intracellular domain consists of a juxtamembrane region, two kinase domains and a carboxyl terminal domain. The two kinase domains are separated by a kinase insert domain. All regions in the intracellular domain contain various tyrosine residues which get phosphorylated upon activation of VEGFR-3 by its ligands similar to other members of tyrosine kinase family of receptors. There are 16 tyrosine residues in the intracellular domain of VEGFR-3. Among the 16 residues, three are present in the juxtamembrane region, two in the first kinase domain, five in second kinase domain and six in the carboxyl terminal domain. In VEGFR-1 and VEGFR-2 there are no tyrosine residues in the kinase insert but VEGFR-2 has three tyrosines. The VEGFR family members show conserved tyrosine residues serving the similar functions in the various domains. Tyr 812 in VEGFR-3 juxtamembrane domain is conserved in VEGFR-1 (tyr-794) and VEGFR-2 (tyr-801). Similarly to Tyr-1054 and 1059 in kinase domain of VEGFR-2, which play a positive regulatory signal upon the activation of the receptor, VEGFR-3 also consists of Tyr-1063 and 1068. These phosphorylated tyrosine residues trigger the downstream of signaling cascade by forming the docking sites for the adaptor proteins like Shc and Grb2 which in turn trigger the Ras mediated mitogen activated protein kinase (MAPK) pathway and promote cell growth and proliferation (Fournier et al., 1999; Salameh et al., 2005). In the case of VEGFR-3, tyr-1337 present in the carboxyl terminal domain is shown to act as

a docking site for Shc and Grb2 adaptor proteins (Fournier et al., 1999; Salameh et al., 2005). VEGFR-3 is also thought to trigger PI3kinase/Akt pathway and promote cell survival. The phosphorylated tyrosine residues in the carboxyl terminal tail vary depending on the type of ligand bound and dimerization (homo or heterodimerization) and thus leading to the differentiated regulation of these signaling cascades. It has been shown that Tyr-1337 is active only in the VEGFR-3 homodimer but not in the VEGFR-3/VEGFR-2 heterodimer (Dixelius et al., 2003). This might lead to the disruption in the Shc and Grb2 signaling (Dixelius et al., 2003) and these points to the versatile mechanisms composed of a variety of ligands and receptors involved in lymphangiogenesis (Dixelius et al., 2003).

Early during embryogenesis, both *VEGFR-2* and *VEGFR-3* are initially expressed in blood endothelial cells (Dumont et al., 1998). In later development VEGFR-3 expression becomes restricted to lymphatic endothelial cells. VEGFR-3 is expressed at (E) 8.5 before the actual start of the lymphangiogenesis (E9.5 – E10.5). *VEGFR-3* null mice die early during embryogenesis because of a failure in the development of blood vasculature and thus it is believed to have an important role during embryonic angiogenesis (Dumont et al., 1998). However, the study of the role of VEGFR-3 in lymphangiogenesis is complicated since the mice die before lymphangiogenesis begins (Dumont et al., 1998). Mutations in the tyrosine kinase domain of VEGFR-3 leads to lymphedema and most patients with Milroy's disease carry mutations in the VEGFR-3 gene leading to its dysfunction (Brice et al., 2005; Evans et al., 2003). Various mutations leading

primary congenital lymphedema are present in the kinase domain of the VEGFR-3 and thus leading to the dysfunction of the signaling cascade. The reported mutations that show familial inheritance are G857R, R1041P, R1044P, and P1114L (Fig 5).

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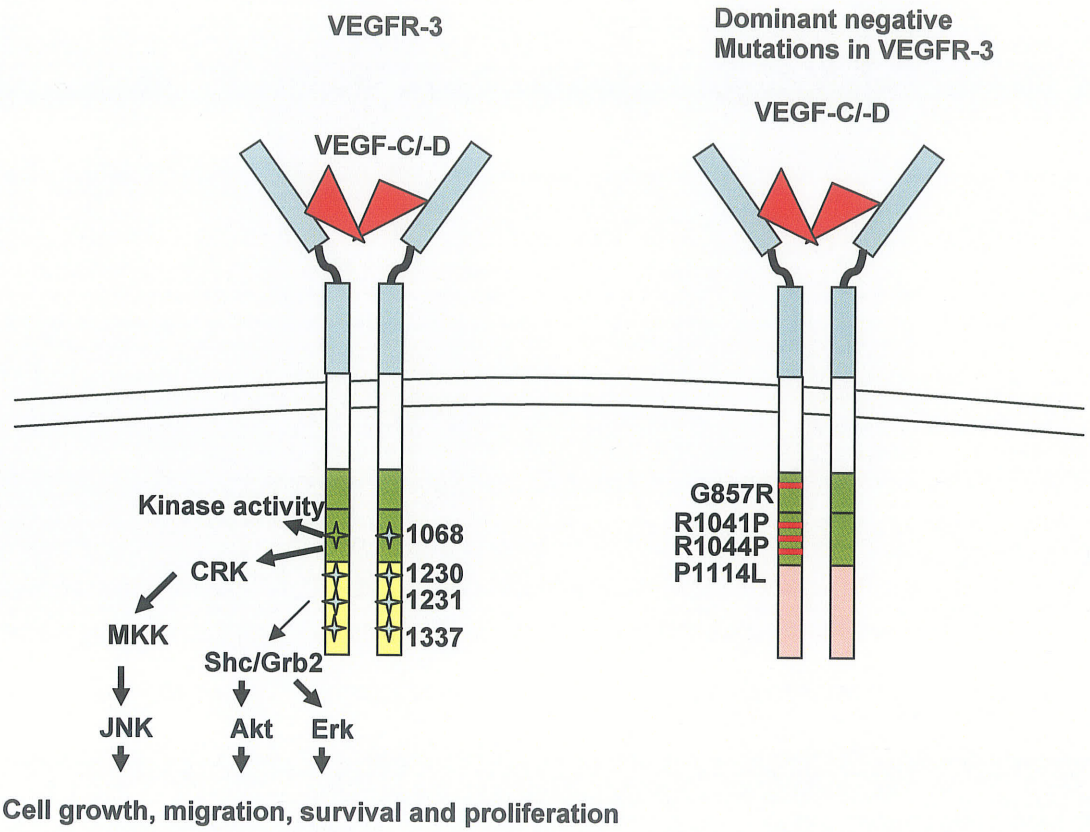

Fig 5: Role of VEGFR-3 signaling: Binding of VEGF-C/-D to the extracellular domain triggers the dimerization and transphosphorylation of the tyrosine residues in the kinase domain (green) and thus activating the carboxyl domain of the receptor (yellow). The phospho tyrosine residues in the activated carboxyl domain help in the recruitment SH2 containing adaptor proteins (Shc and Grb2) and trigger the downstream signaling cascade. Dominant negative mutations in kinase domains have been reported in congenital primary lymphedema. These mutations in kinase domain result in the inhibition of transphosphorylation and thus interrupting the downstream signaling cascade.

Rationale

An understanding of the molecular mechanism of lymphangiogenesis is pivotal as the lymphatic system has been shown to be a major route of cancer metastasis (Karkkainen and Alitalo, 2002). As well, dysfunction of the lymphatic system leads to lymphedema a common side effect of cancer therapy. Studies of breast cancer patients treated either with radiation or surgery have revealed that at least 26% of such patients developed lymphedema of the arms within the first 2 years of treatment and about 45% developed after a decade of treatment. Lymphedemas due to the genetic defect i.e., the primary lymphedemas are less common but have been reported in considerable number of cases. In tropical countries, secondary lymphedemas due to the transmission of nematode, *Wucheraria*, by infection of the mosquitoes is more prevalent. Thus, these studies on the development of lymphatic system will help in developing better treatment methods for lymphedema and cancer metastasis.

The homeobox gene, *Prox1,* has been shown to be crucial in determining lymphatic endothelial cell fate, differentiation and maturation (Wigle et al., 2002). *Prox1* null mice have severe edema and subsequently die at about embryonic day E14.5-E15.5 (Wigle and Oliver, 1999). Microarray studies of the Human umbilical vein endothelial cells (HUVECs) infected with adenovirus expressing *Prox1*, have shown an increase in lymphatic endothelial markers (VEGFR-3 and Podoplanin) and decrease in the blood endothelial specific markers (CD34 and STAT6) (Hong et al., 2002; Petrova et al., 2002). Microarray analysis cannot differentiate between which genes are direct targets and which are indirect

targets. Until now, Prox1 has been shown to directly regulate *Fibroblast growth factor receptor-3* (*FGFR-3*) and chicken *βB1-Crystallin* gene expression by DNA binding dependent mechanism (Cui et al., 2004; Shin et al., 2006). Prox1 has also been shown to act as a co-repressor in the regulation of *Cholesterol 7-α-hydroxylase* (CYP7A1) gene expression by binding to the hepatocyte nuclear factor (HNF)4α transcription factor via its N-terminal LXXLL motif (Song et al., 2006). Our data have shown that Prox1 acts as a co-activator in the *Cyclin E1* gene expression (Baxter and Wigle ; Unpublished data).

During embryogenesis, *VEGFR-3* is initially expressed in blood endothelial cells but shows increased expression in the lymphatic endothelial cells after the expression of *Prox1* is initiated (Dumont et al., 1998). Many studies have shown the importance of VEGF-C/VEGFR-3 signaling mechanism in the migration of Prox1 positive lymphatic endothelial during the formation of primary lymph sacs (Dumont et al., 1998; Karkkainen et al., 2004). VEGFR-3 is also thought to be crucial for angiogenesis since, *VEGFR-3* null mice died at E8.5 i.e before the actual start of lymphangiogenesis (Dumont et al., 1998). Dominant negative mutations in VEGFR-3 tyrosine kinase domain leading to congenital lymphedema have been reported in many cases (Brice et al., 2005). Prox1 has been shown to significantly upregulate *VEGFR-3* expression not only at the mRNA level using microarrays but also at the protein level (Hong et al., 2002; Petrova et al., 2002). Like the *FGFR-3* promoter, which has been shown to be upregulated by Prox1 by directly binding to DNA, the *VEGFR-3* promoter is a TATA less and CAAT less promoter (Iljin et al., 2001; Shin et al., 2005). Both the *FGFR-3* and *VEGFR-3*

promoters show a striking similarity with various transcription factor binding sites. A manual survey of the 1.8 kb VEGFR-3 5' upstream promoter region shows seven putative Prox1 binding putative sites of which three are in between -847 to -758 bases upstream and four that are near the proximal promoter region (-218 to -33 bases upstream). Unlike the *VEGFR-3* null mice, *FGFR-3* null mice do not have severe lymphatic or angiogenic abnormalities (Shin et al., 2005). Since, both Prox1 and VEGFR-3 are crucial for the development of lymphatic system this study would give an insight into the molecular mechanism of lymphangiogenesis. Thus, we predict that *VEGFR-3* is candidate target gene of Prox1.

Hypothesis

We hypothesize that Prox1 activates *VEGFR-3* transcription via a DNA binding dependent mechanism.

Objectives

The objectives of this study are

1) To determine whether Prox1 mediated activation of *VEGFR-3* expression is via a DNA binding dependent or DNA binding independent mechanism.
2) To determine the region/regions of Prox1 crucial for the activation of the *VEGFR-3* promoter.
3) To determine the 5' upstream promoter region of *VEGFR-3* necessary for activation by Prox1.

By achieving these objectives we will establish how Prox1 activates *VEGFR-3* expression.

Materials and methods

Cells and cell culture

Human embryonic kidney cells (HEK 293 cells) were obtained from the American type culture collection (ATCC) and cultured in HyQ's Dulbecco's modified eagles's medium (DMEM) (Hyclone) with 5% fetal bovine serum (FBS) (Hyclone), 4 mM L-Glutamine and 1% penicillin-streptomycin (Invitrogen) and are used between 7-30 passages for adenoviral production and Lipofectamine transfections. Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics were cultured in HyQ's HAM's/F-12 medium (Hyclone) with 20% FBS, 1 mM L-glutamine and 1% penicillin-streptomycin.

PCR and construction of plasmids

A 1.8 kb mouse *VEGFR-3* promoter sequence was obtained by using a FailSafe PCR kit (EPICENTRE, Madison, Wisconsin). In this PCR reaction, a Bacterial artificial chromosome (BAC) encoding the mouse *VEGFR-3* gene (Invitrogen, ID# RP23-58E13) was used as a template and Px11 and Px12 (please refer to Table 1) as primers. The *VEGFR-3* insert was first cloned into the pBluescript plasmid by digesting both the *VEGFR-3* promoter and pBluescript with *EcoRI* and *XhoI* and its sequence was verified. The insert was then cut with *EcoRI* , blunted (*klenow* fill in reaction) and *XhoI* digested and then cloned into the pGL3 luciferase reporter vector (Promega Corporation) digested with *SmaI* and *XhoI* for luciferase assay. Similarly, 926 bp, 696 bp, 266 bp, 130 bp, and 90

bp versions of *VEGFR-3* promoter were cloned into pGL3 using primers Px51, Px52, Px 53, Px58 and Px59 respectively as 5' primers and Px12 as 3' primer (refer to Table 1). A *VEGFR-3* promoter version with the proximal Δ432 bp (-432 to -1.8 Kb) was constructed by digesting the 1.8 kb pGL3 *VEGFR-3* promoter with *PstI /XhoI*, then blunted with mung bean nuclease and ligated. Δ260 bp (-266 to -1.8 Kb), *VEGFR-3* promoter version was constructed by cutting the pBluescript 1.8 kb *VEGFR-3* promoter with *BspMI*, blunted (*Klenow fill in* reaction) and digested with *KpnI* and cloned into pGL3 digested with *KpnI* and *SmaI*. A full length 2.9 Kb and 220 bp FGFR-3 promoters which are cloned into the pGL2 basic (Promega) luciferase expression vector were a kind gift from Dr. David Ornitz (Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, Missouri 63110, USA).

A 2.4kb mouse *Prox1* cDNA (Invitrogen) was amplified by PCR using primers Mx10 and Mx49 (Table 2) encoding *BamHI* and *XhoI* restriction sites respectively. The resulting PCR product was cloned into pCR-Blunt vector (Invitrogen). The sequence was verified and the insert excised with *SacII/XhoI* was cloned into pCMV-4A tag vector (Stratagene) that encodes a carboxy terminal FLAG epitope.

Prox1 homeodomain-prospero domain deleted version (*Prox1HDPDΔ*) was obtained by PCR using pCMV-4A-Prox1 as template and primers Px21 and Px8 (Table 2) encoding *BamHI* and *XhoI* restriction sites. The insert was digested with *SacII/XhoI* and cloned into pCMV-4A.

Table 1

Primer Name	5' / 3' primer	Sequence	VEGFR-3 Promoter version	Restriction Enzyme tagged
Px11	5'	GGGAATTCTATGGAGGTAACTCTAGCTG	1.8 kb	EcoRI
Px12	3'	GGCTCGAGTCTAGGCTGGGAGTGAGAGCG	Used with all 5' Primers in this table	XhoI
Px51	5'	GGGAATTCAGCCTCAGTGGCAGTTAGGGA	921 bp	EcoR1
Px52	5'	GGGAATTCGGATCGTGGGAACAGTGGG	696 bp	EcoRI
Px53	5'	GGGAATTCACCCGCAGTGGCCCTGGAG	266 bp	EcoRI
Px58	5'	GGGAATTCGGGGCGGGCTGAGGCTACGGTGA	130 bp	EcoRI
Px59	5'	GGGAATTCGGAGCTCCGCCCCGGGCCGCC	90 bp	EcoRI

Table 2

Primer Name	5' / 3' primer	Sequence	Prox1 version	Restriction Enzyme tagged
Mx10	5'	GCGGATCCTAATACGACTCACTATAGGGC	Full length	BamHI
Mx49	3'	CCCTCGAGCTCGTGAAGGAGTTCTTGTAG	Full length	XhoI
Px21	5'	GCGGATCCGAGAGAGTGGCTGCGCCCCGCTGCTG	Prox1HDPDΔ	BamHI
Px8	3'	CCCTCGAGCTGCATTGCGCTTCCTGAATAAGG	Prox1HDPDΔ	XhoI

Construction of adenoviral vectors:

An Adenoviral vector expressing EGFP was kindly provided by Dr.Grant Pierce (Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, Winnipeg, MB). Adenoviral vectors encoding Prox1 and Prox1HDPDΔ were constructed as described below.

pCMV-4A-Prox1 vector was digested with *SacII* and *KpnI* to isolate Prox1 along with FLAG tag. The Prox1FLAG was first cut with *SacII*, blunted with *Klenow* and then digested with *KpnI* and was cloned into pShuttle-CMV vector cut with *BglII*, blunted with *Klenow* and digested with *KpnI*. Prox1HDPDΔ was similarly cloned into pShuttle-CMV.

pShuttle-CMV-Prox1/pShuttle-CMV-Prox1HDPDΔ vector was first linearized with *PmeI* and along with adenoviral vector, pAdEasy-1, was transformed into the *E.Coli* strain BJ5183, which has enhanced homologous recombination (Fig 6). Several small colonies were selected for kanamycin resistance and were screened with *PacI* and *PacI/XhoI* double digestions. The positive colonies were amplified and the recombinant vector was isolated and linearized with *PacI*. The linearized vector was transfected into QBI 293 cells (Qbiogene), a subclone of HEK 293 cells, using Lipofectamine 2000. Once cytopathic effect (CPE) was reached the cells were collected, lysed by three rounds of freeze thaw cycles and centrifuged. The supernatant was collected and serial dilutions were made from 10^{-1} to 10^{-8} with 100µl of the supernatant and 900 µl of DMEM 5% FBS. The QBI 293 cells plated (0.4 x 10^6 cells per plate) in six 6 cm plates a day before were infected with the serial dilutions from 10^{-3} to 10^{-8} into

respective plates and were incubated at 37° C for 2 hrs. After 2 hrs, the media is removed from the plates and the cells were covered with 5 ml of DMEM 5% Sea plaque agarose (CAMBREX). 3 ml of DMEM 5% sea plaque agarose is added every 4-5 days. The individual viral plaques formed in about 17-20 days were isolated and eluted in 5% DMEM for 24 hrs at 37° C. The eluted viruses were initially amplified by infecting 100,000 QBI 293 cells plated in each well of 24 well plate with 100 µl of eluted virus and were incubated until CPE is reached. After the cells reached CPE, they were subjected to three freeze/thaw cycles and were centrifuged. The supernatant was collected and stored as viral stock of first amplification (VSFA). The VSFA was screened for the expression of recombinant Prox1 by western blot analysis with the anti-FLAG antibody (Fig 7A). The highest expressing plaques were then amplified by infecting $5X10^6$ HEK 293 cells plated in a 75 cm^2 flask with 500 µl of VSFA and were incubated for 72 hrs at 37° C. After 72 hrs, the cells were collected and subjected to freeze/thaw cycles and centrifuged at 5000 RPM for 10 min. The supernatant was collected and was stored as upscale of virus production passage 2 (UVPP2). UVPP2 were further amplified in three 175 cm^2 flasks plated with $10X10^6$ HEK 293 cells in each flask and were stored as the Final amplification virus. The number of viral particles is calculated using the Tissue culture infectious dose method (TCID50) (Qbiogene) (Fig 7B).

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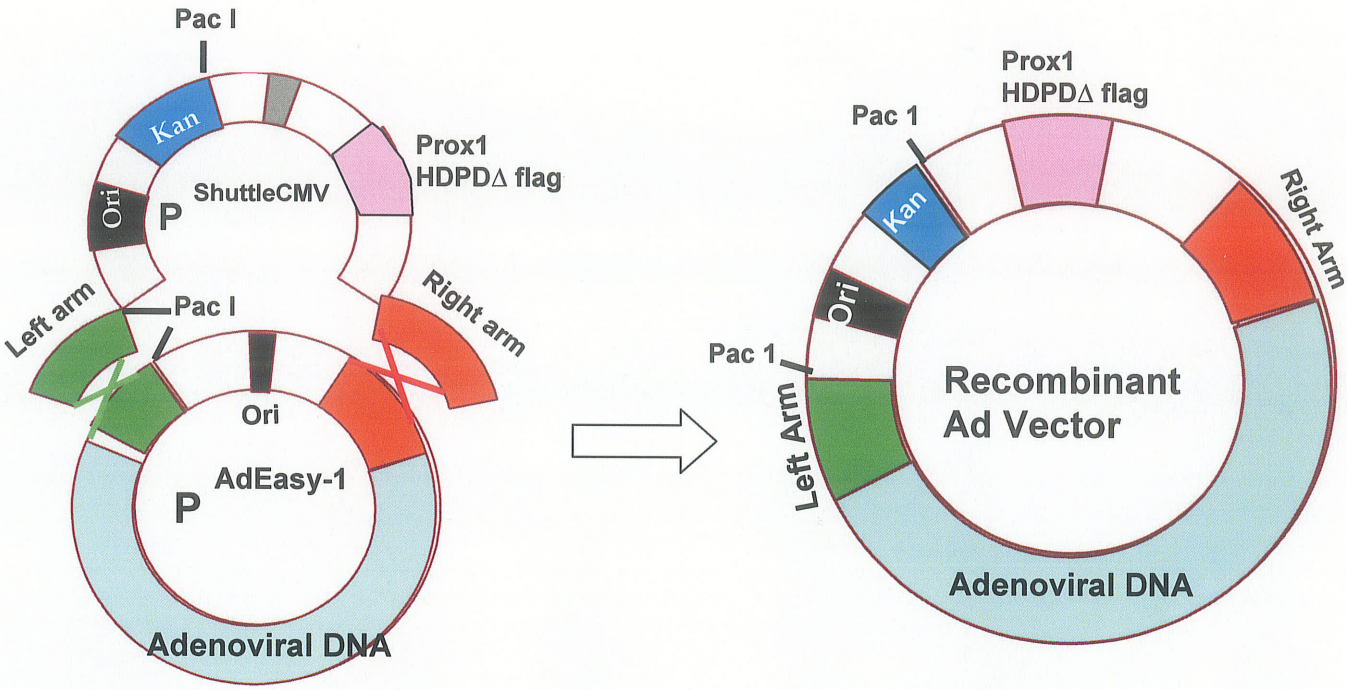


Fig 6: Homologous recombination of pAdEasy-1 and pShuttleCMV-Prox1HDPDΔ: pShuttleCMV and adenoviral vector pAdEasy-1 consists of two homology domains (Left and right arms) and Ori sites. pAd-Easy consists of one PacI site where as pShuttleCMV consists of two such PacI restriction sites. pShuttleCMV vector with Prox1HDPDΔ carrying FLAG tag at its C-terminus was linearized with *PmeI* restriction enzyme. When linearized pShuttleCMV-Prox1HDPDΔ along with pAdEasy-1 was co-transformed into BJ5183 E.coli cells and recombination occurs between the homology domains present in both the vectors. The recombination can also occur between the Ori sites present in both the vectors. Either of the cases results in the recombinant vector carrying the adenoviral genes along with *prox1HDPDΔ* gene. The recombinant vector consists of two *PacI* sites also. This recombinant vector was linearized with *PacI* and transfected into QBI 293 cells in which the adenovirus carrying Prox1HDPDΔ were packaged.

Transfections, β-galactosidase and luciferase assays:

Cells were transfected at around 80% confluency. The media of the cells was changed to OptiMEM before transfections. For each microgram of DNA, 2 µl of Lipofectamine 2000 reagent (Invitrogen) was used. The control cells were transfected with pCMV-4A, pcDNA-3-lacZ (a kind gift from Dr. Nasrin Mesaeli, Institute of Cardiovascular Sciences, St.Boniface General Hospital Research Centre, Winnipeg, MB) and pGL3-VEGFR3 promoter whereas, the tests were transfected with pCMV-4A encoding various Prox1 versions/pcDNA3-lacZ/pGL-3-VEGFR3-promoter and were incubated for 4 hrs at 37° C. Triplicates were prepared for each transfection. The media was changed back to the normal growth media after 4 hrs and incubated for a further 48 hrs at 37° C. After 48 hrs the cells were washed with PBS, collected with 100 µl of NP40 lysis buffer with DTT (100 mM Tris pH 7.8, 0.5% NP40 made to 2 ml with ddH2O and 1µl of 1M DTT). 20 µl of the cell lysate collected was added to each luciferase tube in duplicates and to each well in duplicates of the 96 well plate. The luciferase tubes with 20 µl of NP40 lysis buffer with DTT and the wells with 20 µl of water were used as blanks. The luciferase tubes with samples and blanks were used for luciferase assay and the wells with samples and blanks were used for β-galactosidase assay. A freshly prepared luciferase assay buffer (20mM Tricine, 1.07mM $MgCO_3$, 2.67mM $MgSO_4$, 0.1mMEDTA, 33.3mM DTT, 270uM coenzyme A, 470uM luciferin, 530uM ATP) was prepared and the luciferase activity was measured using Lumat LB 9507 Luminometer.

To each well with sample or blank 30 µl of ONPG and 70 µl of dd H2O

were added and incubated for 1 hr at 37°C. After 1 hr the β-galactosidase activity was measured at 418 nm using Dynex MRXTC Revelation.

Western blotting

HEK 293 cells or HUVECs infected with adenovirus were incubated for 24/48 hr (HEK 293 cells) or 48/72 hrs (HUVECs). The cells were harvested and lysed with New RIPA buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1mM, 1% Triton, 1% Na Deoxycholate, SDS 0.1%, pH=7.4) with protease inhibitor (Complete Mini) and centrifuged. The supernatant was collected and Standard protein assay (Bio-Rad Dc Protein Assay) was performed following the manufacturers protocol. Protein samples were heated and loaded onto a 8% poly-acrylamide gel along with the standards (Bio-Rad Dual Color standards). Proteins were then transferred to Nitrocellulose membrane at 100V for 2 hrs or 35v overnight at 4° C. The membrane was blocked with 5% skin milk protein in TBS (6.05 g of Tris, 8.06 g of NaCl, 0.2 g of KCl in 1 Lit of ddH2O at pH 8.0) either overnight at 4° C or for 2 hrs at room temperature. The membrane was washed with TBS and treated with primary monoclonal antibody (anti-FLAG antibody (SIGMA) 1:10,000 dilution in 5% skin milk protein, anti-VEGFR-3 antibody (Chemicon) 1:2,000 dilution and anti-α-Actin antibody (SIGMA) 1:1000) overnight at 4° C. The membrane was washed with TBST (TBS with 0.45% Tween) and then treated with secondary antibody (Goat anti-mouse antibody 1:5000 dilution or Goat anti-rabbit antibody 1:5000 dilution) for an hour. The proteins were detected using Amersham Biosciences ECL plus, Bio-Rad Fluor-

S™ MultiImager machine and Quantity one software (BioRad).

Immunocytochemistry

Cells plated on cover slips (collagen coated cover slips for HUVECs or polylysine coated cover slips for HEK 293) were either transfected using Lipofectamine 2000 or infected with adeniovirus expressing the desired protein for 48-72 hrs depending on the experiment. The cells were washed with 1X PBS once and were fixed onto the coverslips with 4% paraformaldehyde (4% PFA) for 30 min. After 30 min the cells were washed twice with PBS and thrice with PBT (1X PBS with 0.3% Triton X–100). The cells were then blocked for 1 hr with 100 µl of the blocking reagent (5% goat serum in PBT) and treated with primary antibody (Anti-FLAG antibody 1:1000 dilution) overnight. The next day, cells were washed thrice with PBT and treated with fluorescent secondary antibody (Texas-red conjugated goat anti-mouse 1:200 dilution from JacksonImmunoResearch Laboratories) for 1 hr. The cells were washed thrice with PBT, then mounted on to a slide with a mounting media containing DAPI (Molecular Probes) and images were taken with Zeiss Axioskop 2 microscope equipped with a Zeiss Axiocan digital camera.

Results

1. Construction of Adenoviral vectors

The recombinant vector carrying the adenoviral genes as well as the Prox1HDPDΔ was constructed as mentioned in the materials and methods section earlier. The recombinant vector was linearized with PacI and transfected into QBI 293 cells for adenoviral packaging. Among such packaged adenovirus, some show higher protein (Prox1 HDPDΔ) expression while others show lower expression. Several individual viral plaques developed from single virus were isolated, eluted and amplified on a small scale. To check for the expression of Prox1 HDPDΔ by the individual plaqes a western blot was conducted with the cell lysates from the QBI 293 cells infected with adenoviral plaques separately. As in Fig 7A plaque 1 showed higher expression compared to the rest of the plaques. So, we further amplified plaque 1 on a large scale and the viral count was determined using $TCID_{50}$ method. Similarly, AdEGFP (a kind gift from Dr.Pierce) and AdProx1 (which was constructed earlier in our lab) were amplified on a large scale and viral count was determined. The viral count for AdEGFP, AdProx1 and AdProx1HDPDΔ were estimated to be 1.5×10^8 pfu/ml, 1×10^8 pfu/ml and 6.3×10^7 pfu/ml respectively.

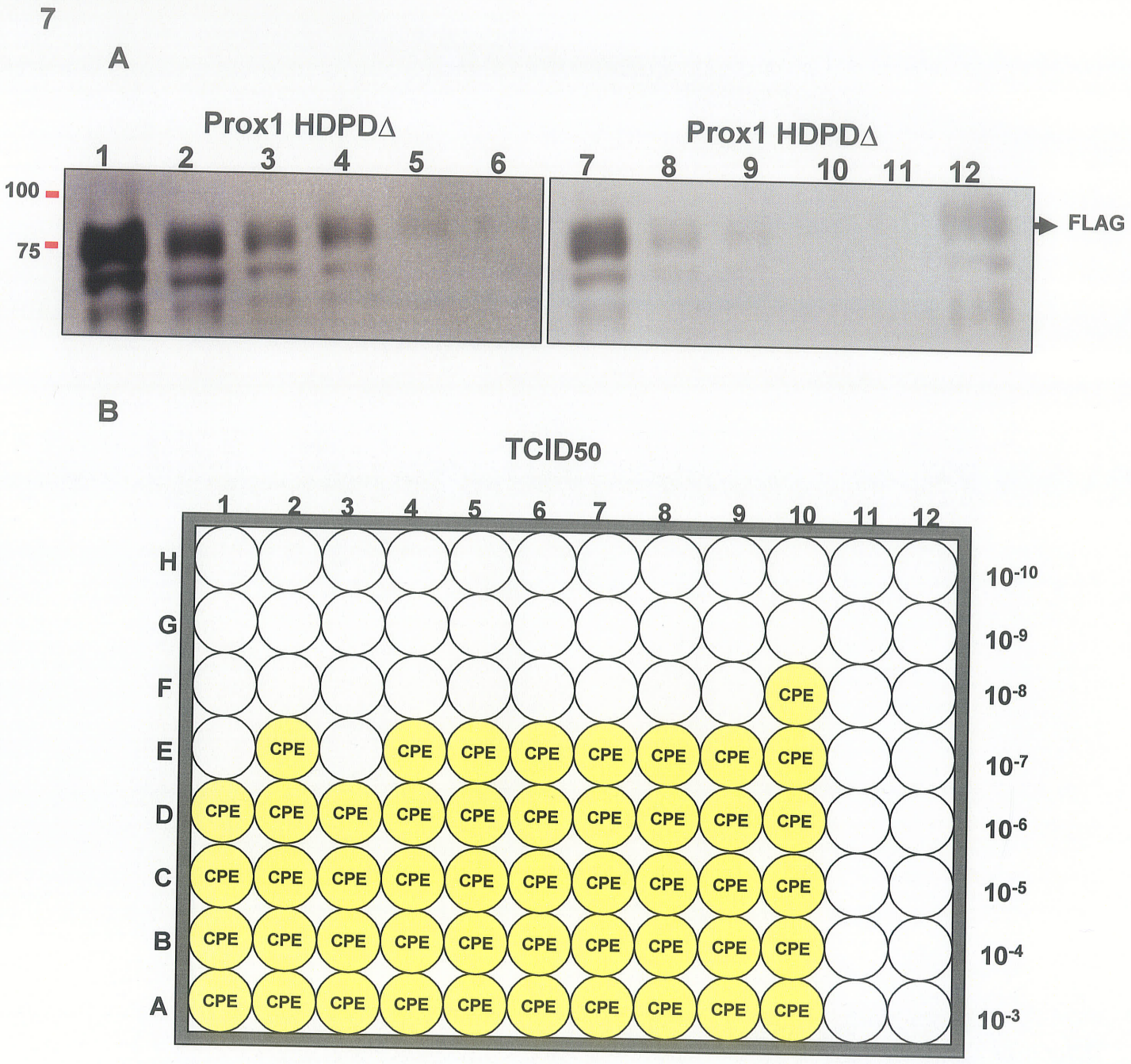


Fig 7: AdProx1HDPDΔ plaque selection and viral count determination: Individual plaques selected were eluted and stored as Viral stock of first amplification (VSFA) seperately. The cell lysates of the HEK 293 cells infected with different plaqes seperately were screened for the expression of Prox1HDPDΔ by western blot (A). Plaque 1 and 7 expressed higher levels of Prox1HDPDΔ and so were amplified further. After final amplification of the adenovirus the number of virus were counted using Tissue culture infectious dose 50 method in a 96 well plate as per the protocol (Qbiogene). Each row (A-H) was infected with different serial dilution of the virus (10^{-3} to 10^{-10} dilution). After 10 days, the wells in which CPE was reached were counted and the number of virus were calculated using the formula $T=10^{1+d(s-0.5)}$ (B).

2. Expression of Prox1 and Prox1HDPDΔ by adenoviral constructs.

Adenoviral vectors expressing EGFP, Prox1, and Prox1HDPDΔ were packaged in QBI 293 cells. Prox1 and Prox1HDPDΔ were tagged with FLAG epitope at their carboxyl-terminus. Expression of recombinant proteins in HUVECs 72 hrs post infection with 50 MOI of adenovirus expressing Prox1 and Prox1HDPDΔ respectively was studied using anti-FLAG antibody in western blotting. HUVECs that were not infected or EGFP infected were used as controls. Prox1 and Prox1HDPDΔ were detected at 90 kDa and 75 kDa respectively (Fig 8A). Prox1HDPDΔ consistently had higher levels of expression as compared to full length Prox1 (Fig 8A). A similar observation was observed in HEK 293 cells 24 hrs post infection as well (Fig 8B). As HEK 293 cells reach CPE within 48 to 72 hrs post infection, samples were collected for western blot only at 24 hrs post infection.

Efficiency of infection

To check if the increased relative expression of Prox1HDPDΔ compared to the Prox1 is due to the increased efficiency of infection i.e. number of cells infected, assessment of infection levels using immunocytochemical technique was performed with HUVECs 72 hrs post infected with varying (0, 5, 25, 50 and 100 MOI) concentrations of adenovirus expressing EGFP, Prox1 and Prox1HDPDΔ. Both Prox1 and Prox1HDPDΔ were localized in the nucleus where as EGFP was expressed throughout the cell (Fig 9). A significant increase in the number of cells infected was observed with the increase in MOI of adenovirus used. 5 MOI of adenovirus infected 20% of cells, 25 MOI infected

50% of cells, 50 MOI infected 75% of cells and a 100 MOI infected 78% of cells (Table 3). There is no difference in the number of cells infected between a 50 MOI and a 100 MOI of adenovirus infection and thus we used 50 MOI in all of the subsequent experiments unless otherwise specified (Table 3). Moreover, there was no difference between the number of cells infected by AdEGFP, AdProx1 and AdProx1HDPDΔ at any particular MOI. This finding implies that the increased expression of Prox1HDPDΔ compared to Prox1 is not due an increased rate of infection.

Time dependent expression

We then sought to determine if there is any difference in the time dependent expression of Prox1 and Prox1HDPDΔ. HUVECs uninfected and infected for 24 hrs, 48 hrs and 72 hrs with AdEGFP, AdProx1 and AdProx1HDPDΔ were studied separately. At 24 hrs post infection Prox1 was expressed more compared to Prox1HDPDΔ (Fig 10A), at 48 hrs post infection both Prox1 and Prox1HDPDΔ had equivalent levels of expression (Fig 10B) and at 72 hrs post infection Prox1HDPDΔ had a higher level of expression as compared to Prox1 (Fig 10C).

8

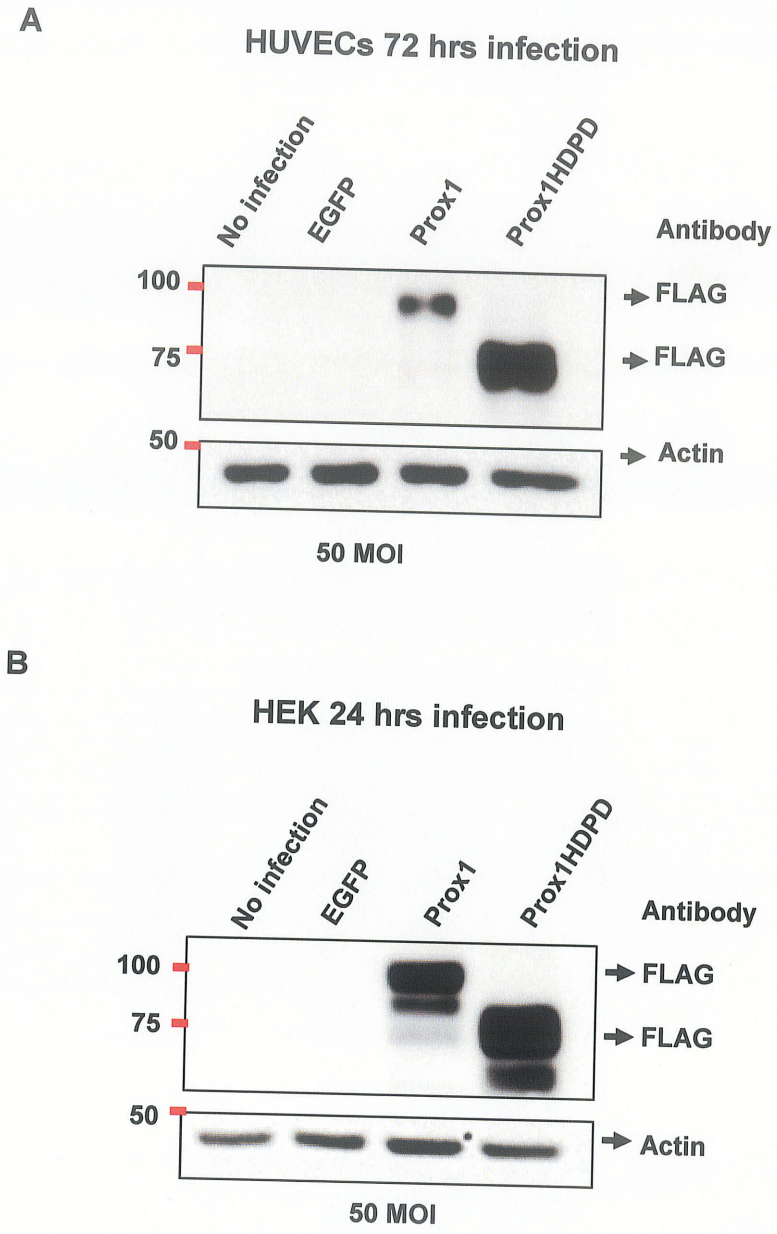
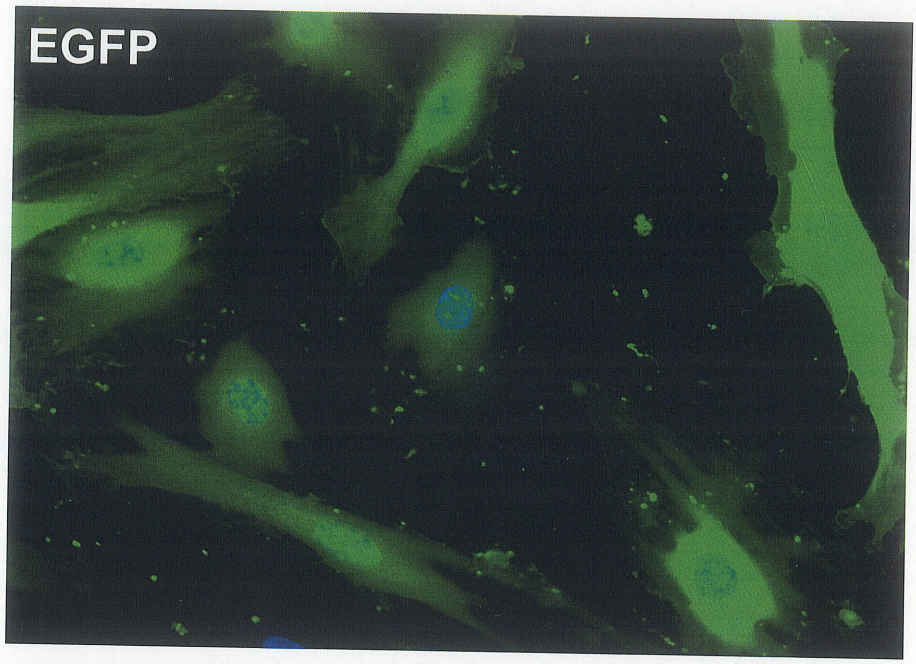


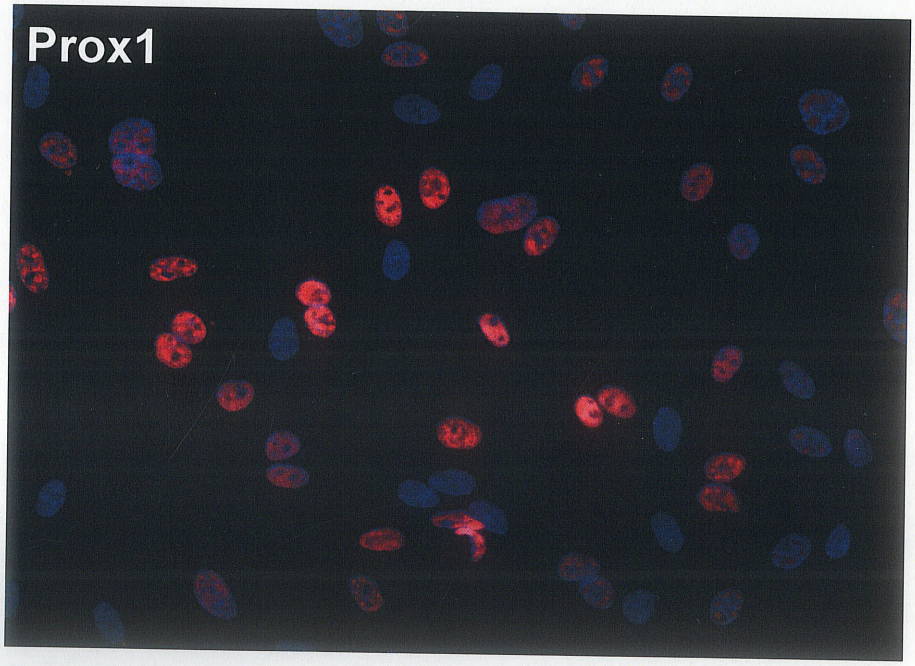
Fig 8: Adenoviral expression of EGFP, Prox1 and Prox1HDPD: HUVECs infected with 50 MOI of adenovirus expressing EGFP, Prox1 and Prox1HDPDΔ for 72 hrs were checked for the protein expression using western blot (A). Actin is used as a loading control. The expression of the Prox1 and Prox1HDPDΔ were also checked in the HEK 293 cells by infecting them with a 50 MOI of the respective adenovirus for 24 hrs (B).

9

A



B



C

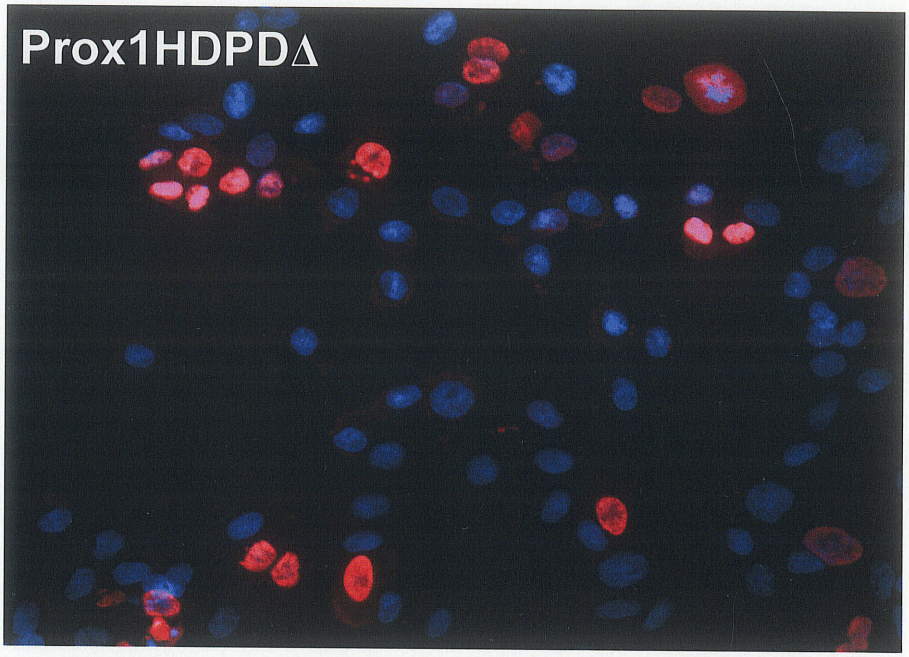


Fig 9: Cellular localization of adenovirally expressed EGFP, Prox1 and Prox1HDPDΔ in HUVECs: The cellular localization of EGFP, Prox1 and Prox1HDPDΔ was determined using immunocytochemistry of the HUVECs infected for 72 hrs. The expression of EGFP (A) is observed through out the cell but both Prox1 (B) and Prox1HDPDΔ (C) were expressed in the nucleus.

Table 3

Adenovirus	Total number of Living cells	Number of Positive cells	Percentage of Positive cells	MOI
EGFP	81	2	2	0
Prox1	56	1	1	0
Prox1HDPDΔ	72	5	6	0
EGFP	53	12	22.6	5
Prox1	58	14	24.1	5
Prox1HDPDΔ	54	11	20.4	5
EGFP	124	60	48.4	25
Prox1	68	39	57.4	25
Prox1HDPDΔ	65	32	49.2	25
EGFP	42	32	76.2	50
Prox1	26	20	76.9	50
Prox1HDPDΔ	38	28	73.7	50
EGFP	44	35	79.5	100
Prox1	68	53	77.9	100
Prox1HDPDΔ	59	38	64.4	100

Table 3: Efficiency of Transduction: The efficiency of infection was tested using immunocytochemistry of HUVECs infected with various MOI of adenovirus expressing EGFP, Prox1 and Prox1HDPDΔ. With the increase in the MOI there was an increase in the percentage of cells infected but there was no difference in the number of cells infected between the 50 MOI and 100 MOI. Interestingly there was no significant difference in the infection between AdEGFP, AdProx1 and AdProx1HDPDΔ at any particular MOI.

10

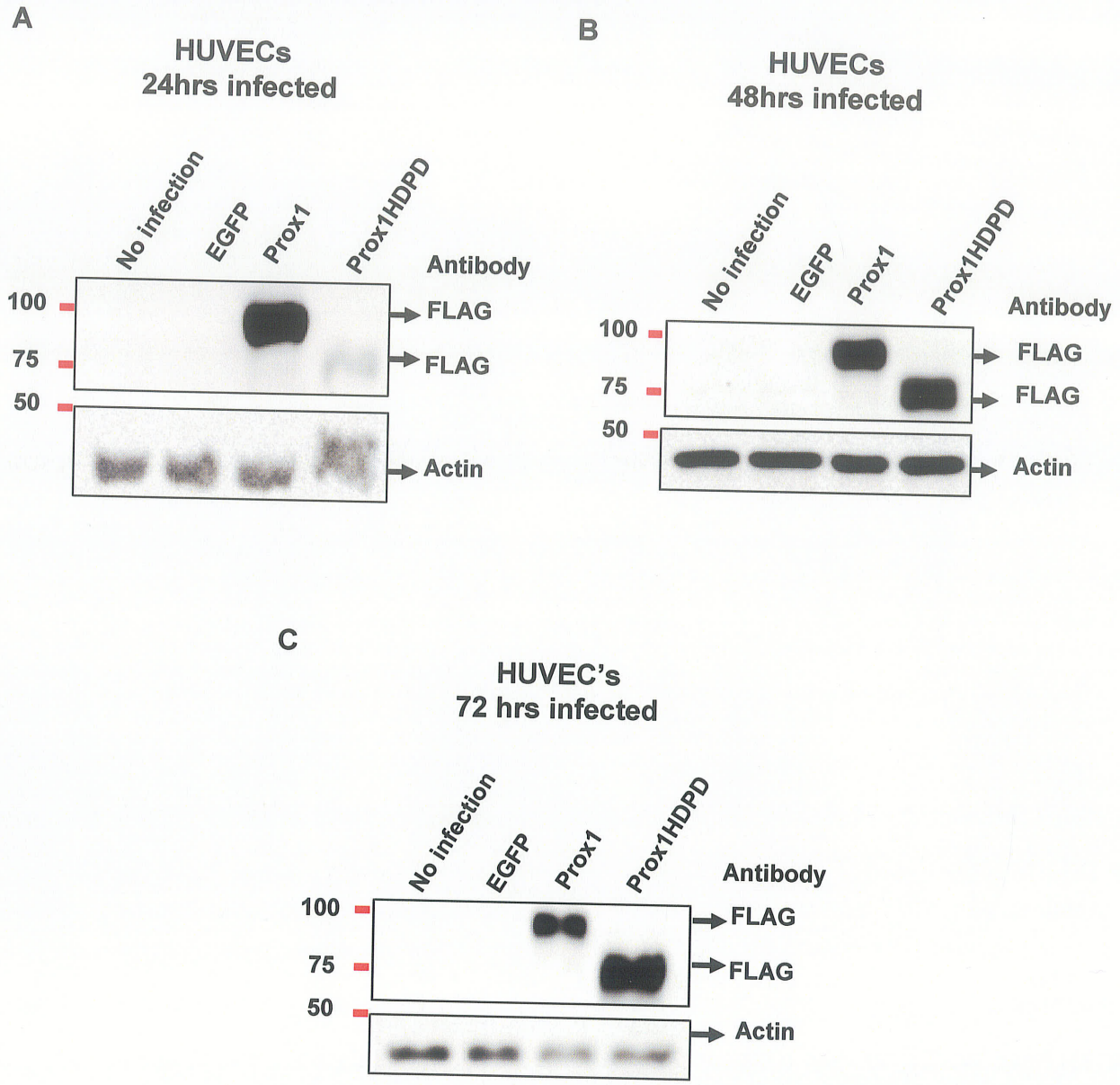


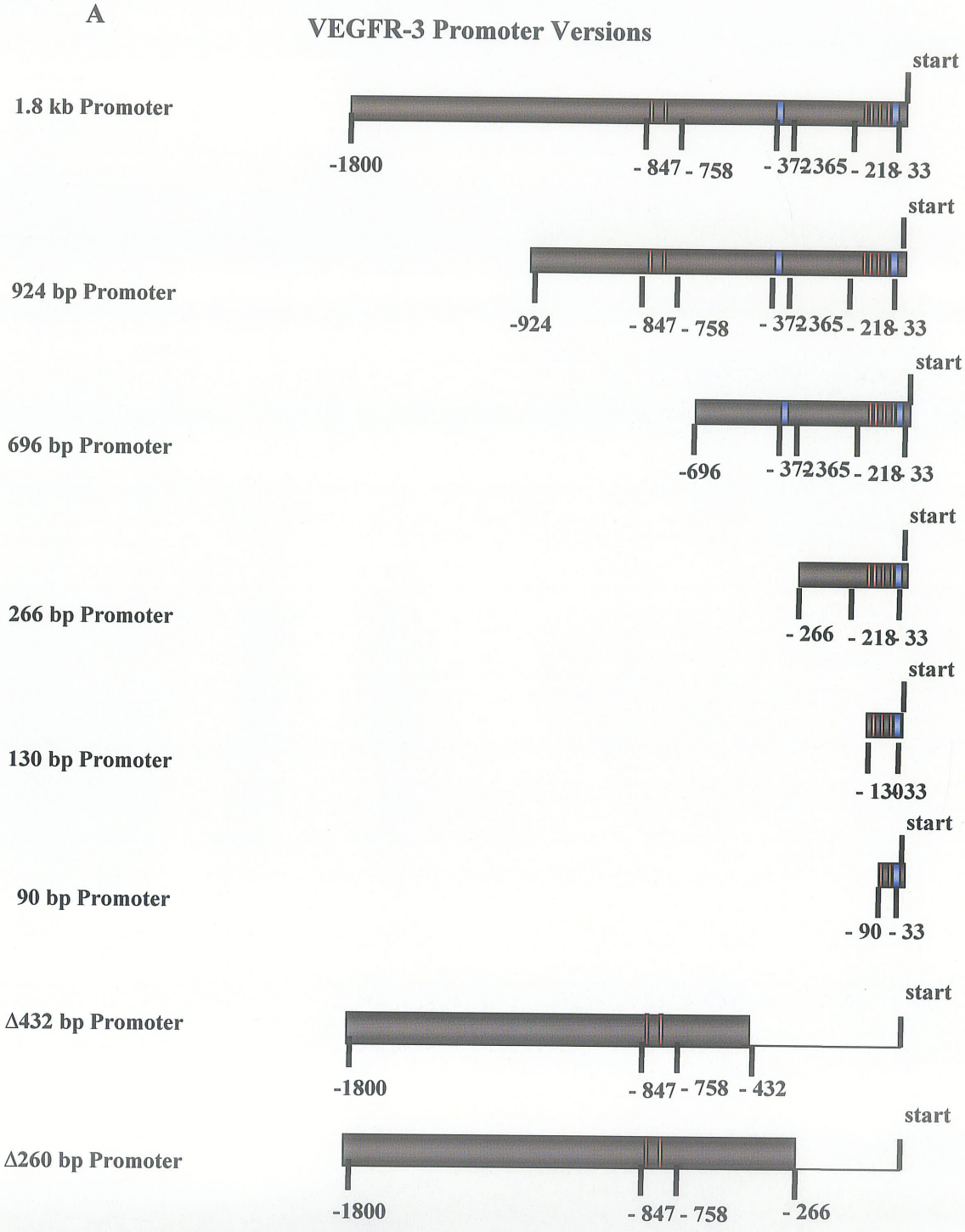
Fig 10: Time dependent expression of EGFP, Prox1 and Prox1HDPDΔ by adenoviral constructs : The expression of Prox1 and Prox1HDPDΔ in the HUVECs infected with a 50 MOI of the respective adenoviral vectors for different time intervals of 24 hrs (A) 48 hrs (B) and 72 hrs (C) were checked using western blot. At 24 hrs Prox1 was immediately expressed but we could observe very low expression of Prox1HDPDΔ but at 48 hrs, both Prox1 and Prox1HDPDΔ expression levels are similar. Contrasting to the observation at 24 hrs, Prox1HDPDΔ expression was consistently more than that of Prox1 at 72 hrs post infection.

3. Identification of the minimal *VEGFR-3* promoter region crucial for activation by Prox1.

To determine whether Prox1 activates transcription of the *VEGFR-3*, we assayed Prox1 mediated activation using a luciferase assay. First we cloned 1.8 Kb segment of the mouse *VEGFR-3* promoter into the pGL3-Basic vector upstream of the luciferase gene. Prox1 significantly activates the 1.8 Kb promoter relative to control (Fig 12A). To determine the minimal *VEGFR-3* promoter region sufficient for activation, luciferase assays were repeated with 926 bp, 696 bp and 266 bp *VEGFR-3* promoter versions. Similar to the 1.8 Kb *VEGFR-3* promoter version, all (926 bp, 696 bp and 266 bp) of these promoter versions were significantly activated by Prox1 as compared to the control (Fig 12B, 12C and 12D). Prox1 was unable to activate Δ432 bp but showed a little increase in activation with Δ260 bp deleted version of *VEGFR-3* promoter (Fig 12G and Fig 12H). This finding shows that the initial 432 bp of the *VEGFR-3* promoter were required for activation by Prox1. Further luciferase assays with 130 bp and 90 bp *VEGFR-3* promoter versions also showed Prox1 mediated increase in transcription as compared to control (Fig 12E and 12F).

11

A


VEGFR-3 Promoter Versions
1.8 kb Promoter
start
-1800
- 847 - 758
- 372365 - 218 33
924 bp Promoter
start
-924
- 847 - 758
- 372365 - 218 33
696 bp Promoter
start
-696
- 372365 - 218 33
266 bp Promoter
start
- 266
- 218 33
130 bp Promoter
start
- 13033
90 bp Promoter
start
- 90 - 33
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start
-1800
- 847 - 758
- 266

B

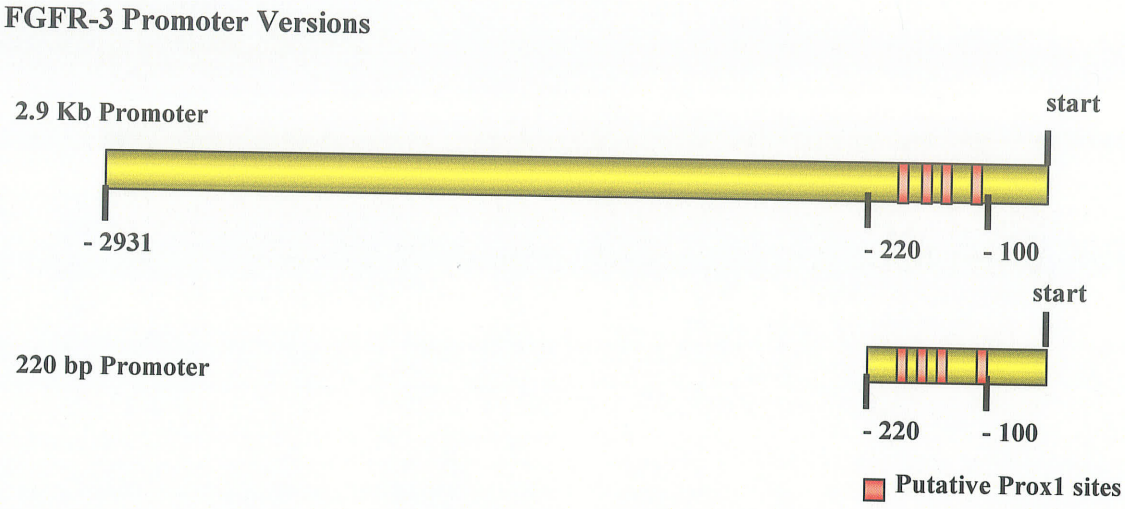
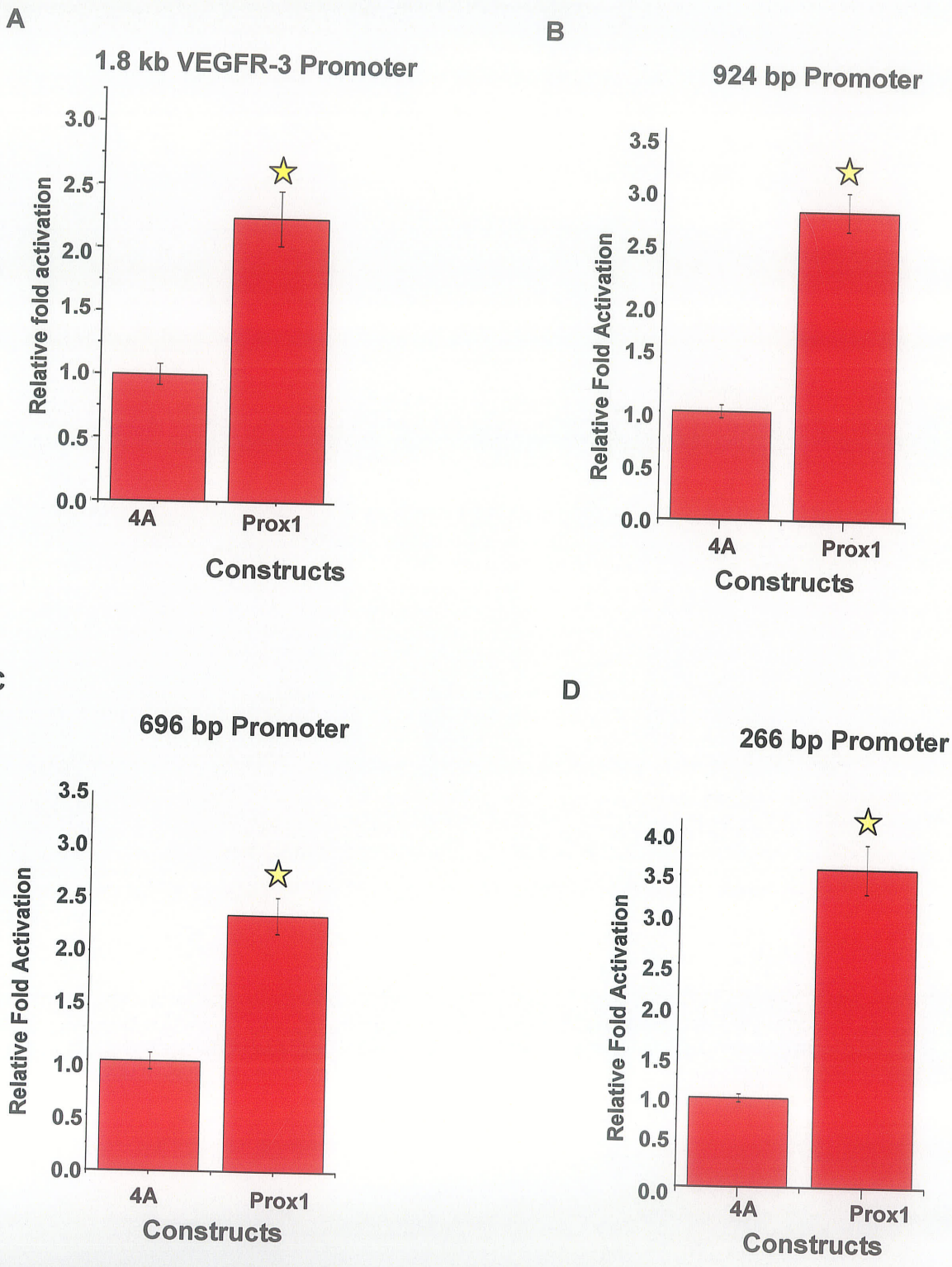


Fig 11: Various VEGFR-3 and FGFR-3 promoter luciferase reporter constructs: **(A)** A mouse full length 1.8 kb promoter was screened manually for putative Prox1 sites. We found six such putative sites, four at the proximal promoter region similar to FGFR-3 promoter and two at the distal region. Using MatInspector we also found two E2F binding sites at -45 bp and the other at -372 bp upstream the transcriptional start site. We have constructed a shorter full length 924 bp VEGFR-3 promoter consisting of all the putative Prox1 sites and the E2F binding sites. We also constructed a 696 bp promoter which is devoid of the distal Prox1 putative binding sites and a 266 bp promoter which lacks the distal Prox1 and E2F binding sites. A still smaller 130 bp and 90 bp promoters were also cloned into the luicferase reporter vector. **(B)** A full length 2.9 Kb and a smaller 220 bp FGFR-3 promoters cloned into pGL2 luciferase reporter vector were gifted by Dr.Ornitz.

12



HEK 293 cells
N=9, $p<0.05$

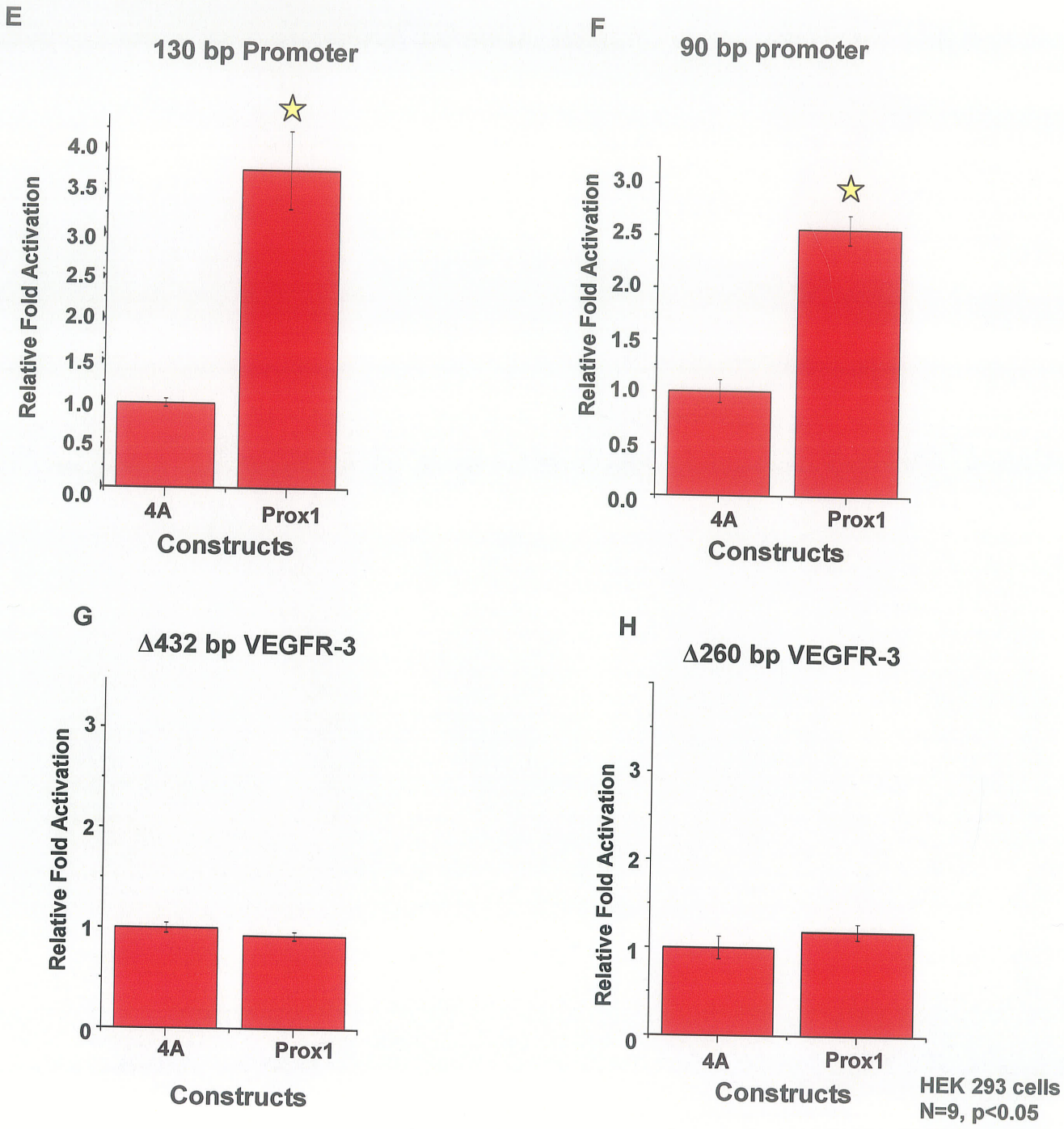


Fig 12: Determination of the minimal *VEGFR-3* promoter region necessary for Prox1 mediated activation: 1.8 Kb (A), 924 bp (B), 696 bp (C), 266 bp (D), 130 bp (E) and 90 bp (F) versions of *VEGFR-3* promoter cloned into pGL3 luciferase expression vector were activated by Prox1 significantly compared to the control. Proximal 432 bp (F) deletion construct of *VEGFR-3* promoter show no significant activation by Prox1 compared to control which indicates the crucial role for the proximal 432 bp of the *VEGFR-3* promoter for the regulation by Prox1. However, Δ266 bp VEGFR-3 promoter show little increase in activation by Prox1 compared to 4A. This increase could be due to the presence of distal E2F binding site in Δ266 bp promoter which is absent in Δ432 bp promoter. Error bars indicate standar errors. ☆ indicate significantly different from control.

4. Prox1 activates *VEGFR-3* by a DNA binding independent mechanism.

Since we have determined that the minimal 90 bp *VEGFR-3* promoter version is also being activated by Prox1, we further studied the molecular mechanism by which Prox1 activates VEGFR-3 using a combination of luciferase reporter assays and western blots. As shown in Fig 12A both wild type Prox1 and Prox1HDPDΔ were able to activate the 1.8 Kb promoter. The Prox1HDPDΔ protein contains no known DNA binding motifs. Similarly, a 266 bp *VEGFR-3* promoter version was equivalently induced by Prox1 and Prox1HDPDΔ (Fig 16B). This shows that, Prox1 does not need to bind to DNA to activate *VEGFR-3* promoter. To further support our result and to study the mechanism of Prox1 mediated activation of *VEGFR-3 in situ*, we examined the expression of VEGFR-3 protein in the HUVECs infected with adenovirus expressing Prox1 and Prox1HDPDΔ for 48 hrs. Consistant to our luciferase data, both Prox1 and Prox1HDPDΔ infected cells showed an increase in the expression of VEGFR-3 compared to EGFP infected and uninfected cells (Fig 15A and 15B).

We also compared regulation of *FGFR-3* promoter, a known Prox1 DNA binding dependent target, to Prox1 mediated regulation of *VEGFR-3* promoter. This comparison not only helps in understanding the divergent roles of Prox1 but also illustrates the functional roles of the various Prox1 domains. As shown in Fig 17A, both Prox1 homeodomain deleted version (Prox1HDΔ) and Prox1 DNA binding domain mutant (Prox1DBD mt), which are incapable of binding to DNA, activated the 266 bp *VEGFR-3* promoter as much as the wild type Prox1. In contrast, both were unable to activate *FGFR-3* promoter (Fig 17B). These

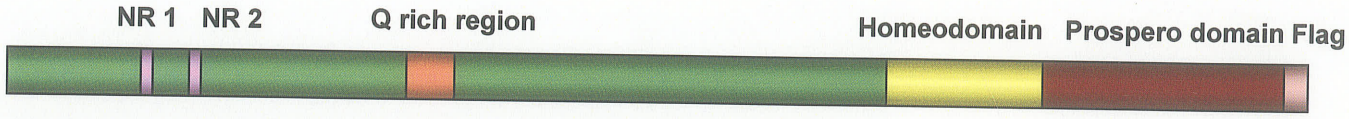
findings support the hypothesis that Prox1 activates the *VEGFR-3* promoter in a DNA binding independent manner.

The Prox1 prosperodomain deleted version (Prox1PDΔ) also activated the 266 bp *VEGFR-3* promoter version (Fig 17A) but not as much as the full length Prox1. Thus, indicating that the prosperodomain has a role in the activation of *VEGFR-3* promoter.

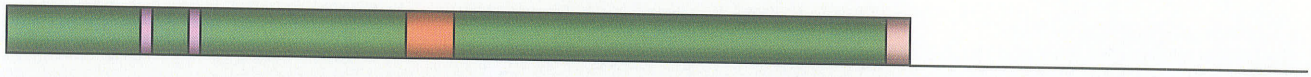
Prox1 nuclear receptor boxes deleted version (Prox1 NRΔ) and Prox1 glutamine rich region deleted version (Prox1QΔ) activated the 266 bp *VEGFR-3* promoter equivalently to wild-type Prox1 (Fig 18A). Prox1NRΔ activated the *FGFR-3* promoter but to a lesser degree than the wild type Prox1 whereas, the Prox1QΔ version didn't activate at all (Fig 18B). Thus, nuclear receptor boxes and glutamine rich region of Prox1 are essential for the full activation of *FGFR-3* promoter but not *VEGFR-3* by Prox1. Among the smaller Prox1 constructs, the Prox1 N-terminal region activated the 266 bp *VEGFR-3* promoter but neither the Prox1 C-terminal, which does not localize to the nucleus nor the NLS tagged C-terminal (NLS C-terminal) activated the *VEGFR-3* promoter (Fig 19A). In the case of *FGFR-3* promoter, none of the smaller Prox1 constructs activated its expression (Fig 19B).

13

A. Wild type Prox1



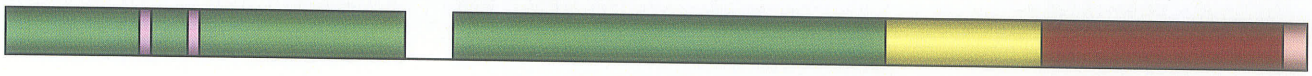
B. Prox1 homeodomain prospero domain deletion



C. Prox1 homeodomain deletion

D. Prox1 prosperodomain deletion

E. Prox1 glutamine rich region deletion



F. Prox1 NR box deletion

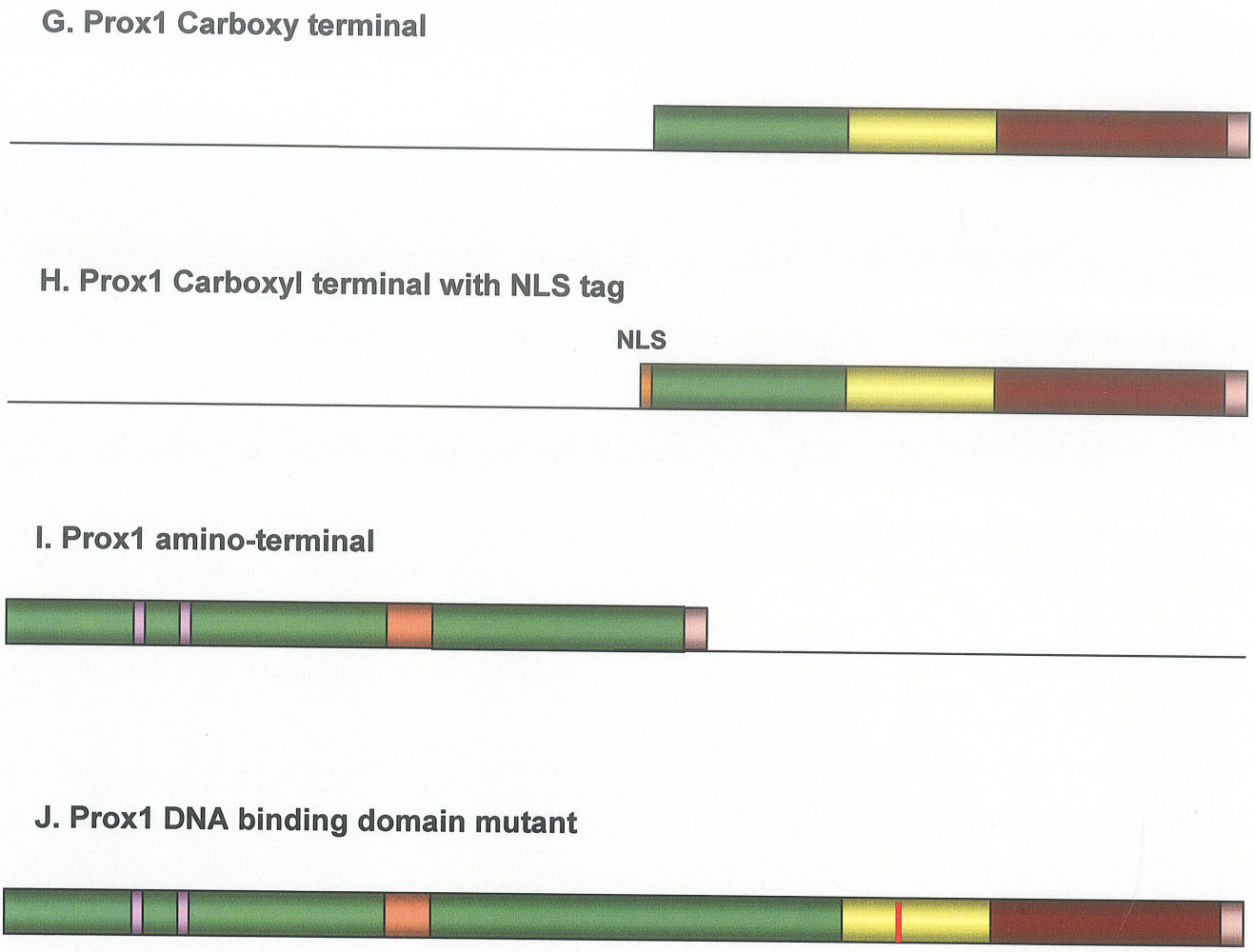


Fig 13: Various Prox1 constructs : Wild type Prox1 (A), Prox1 Homeodomain Prosperodimain deletion (Prox1HDPDΔ) (B), Prox1 Homeodomain deletion (Prox1HDΔ) (C), Prox1 Prosperodomain deletion (Prox1 PDΔ) (D), Prox1 Q rich region deletion (Prox1 QΔ) (E), Prox1 Nuclear boxes deletion (Prox1 NRΔ) (F), Prox1 C terminal (C-ter) (G), Prox1 C terminal with NLS tag (NLS C-ter) (H), Prox1 N terminal (N-ter) (I), Prox1 DNA binding domain mutant (Prox1 DBD mt) (J). All the Prox1 constructs are FLAG tagged at their C-terminus.

14

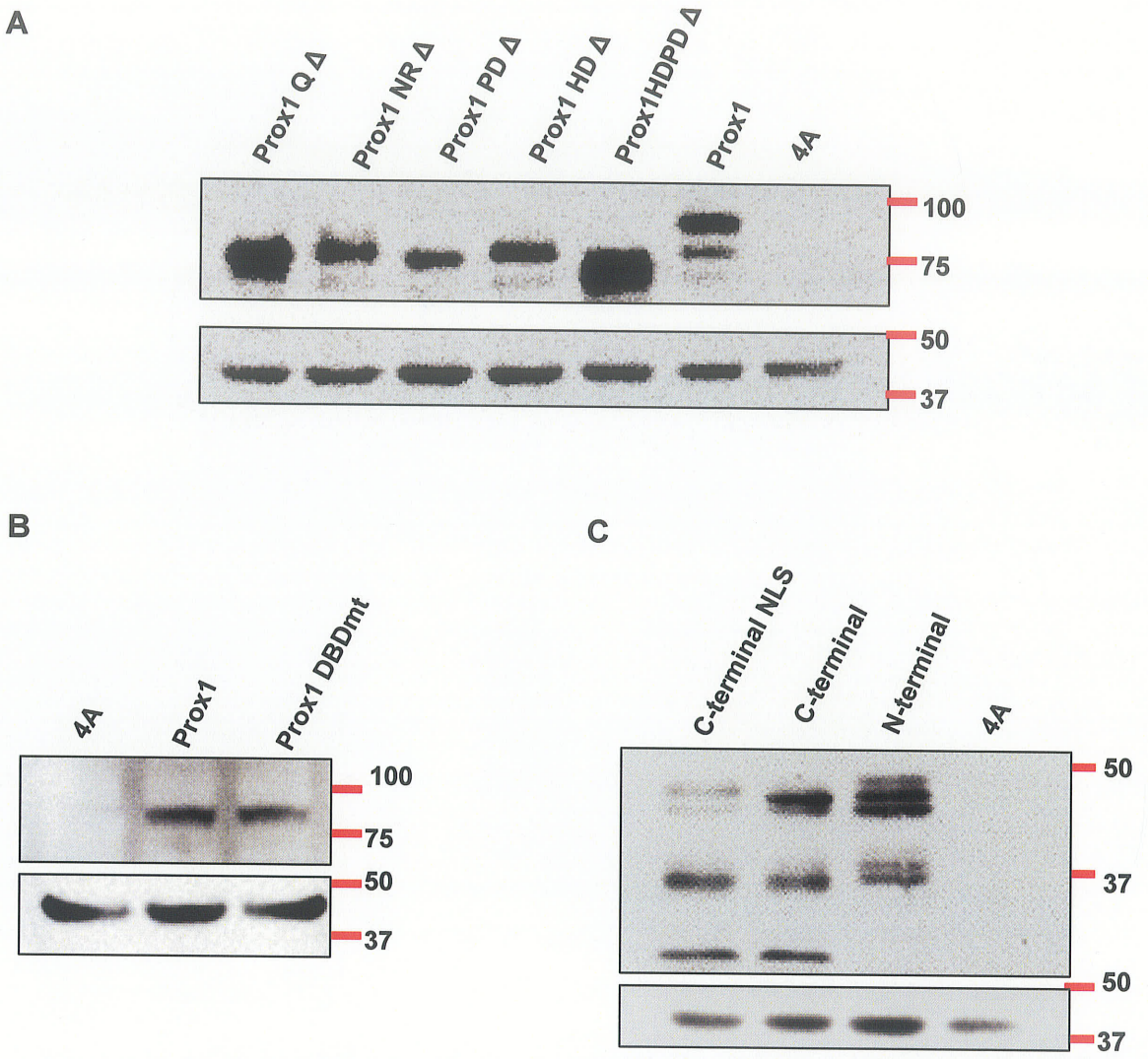


Fig 14: Western blots for the expression of various Prox1 constructs : The protein expression the Prox1 constructs made were checked by western blotting of the 48 hrs post transfection of HEK 293 cell samples (A, B, and C). Prox1 HDPDΔ consistently showed a higher expression levels compared to the rest of the Prox1 constructs. Actin is used as a loading control.

15

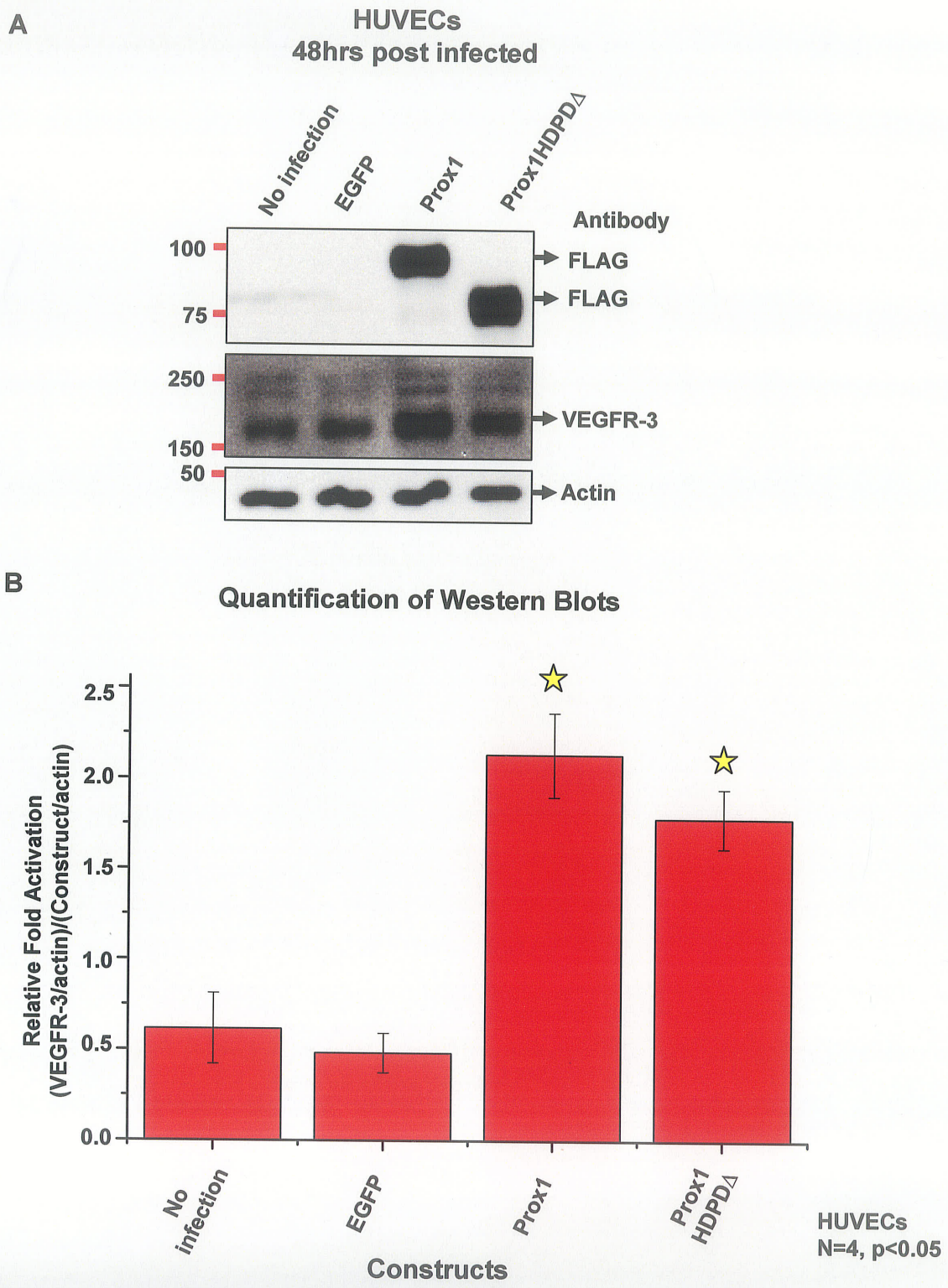


Fig 15: DNA binding independent activation of VEGFR-3 expression by Prox1 in HUVECs : The expression of VEGFR-3 in HUVECs infected with a 50 MOI of AdEGFP, AdProx1 and AdProx1HDPDΔ for 48 hrs was checked using western blot (A) and then the density of the bands on the blots were quantified using the quantity one software (B). The expression of VEGFR-3 in the HUVECs infected with the AdProx1 or AdProx1HDPDΔ was significantly higher compared to the adEGFP and not infected cells. There is no significant change in the expression of VEGFR-3 between Prox1 infected and the Prox1HDPDΔ infected cells. Error bars indicate standar errors. ☆Indicates significantly different from contol.

16

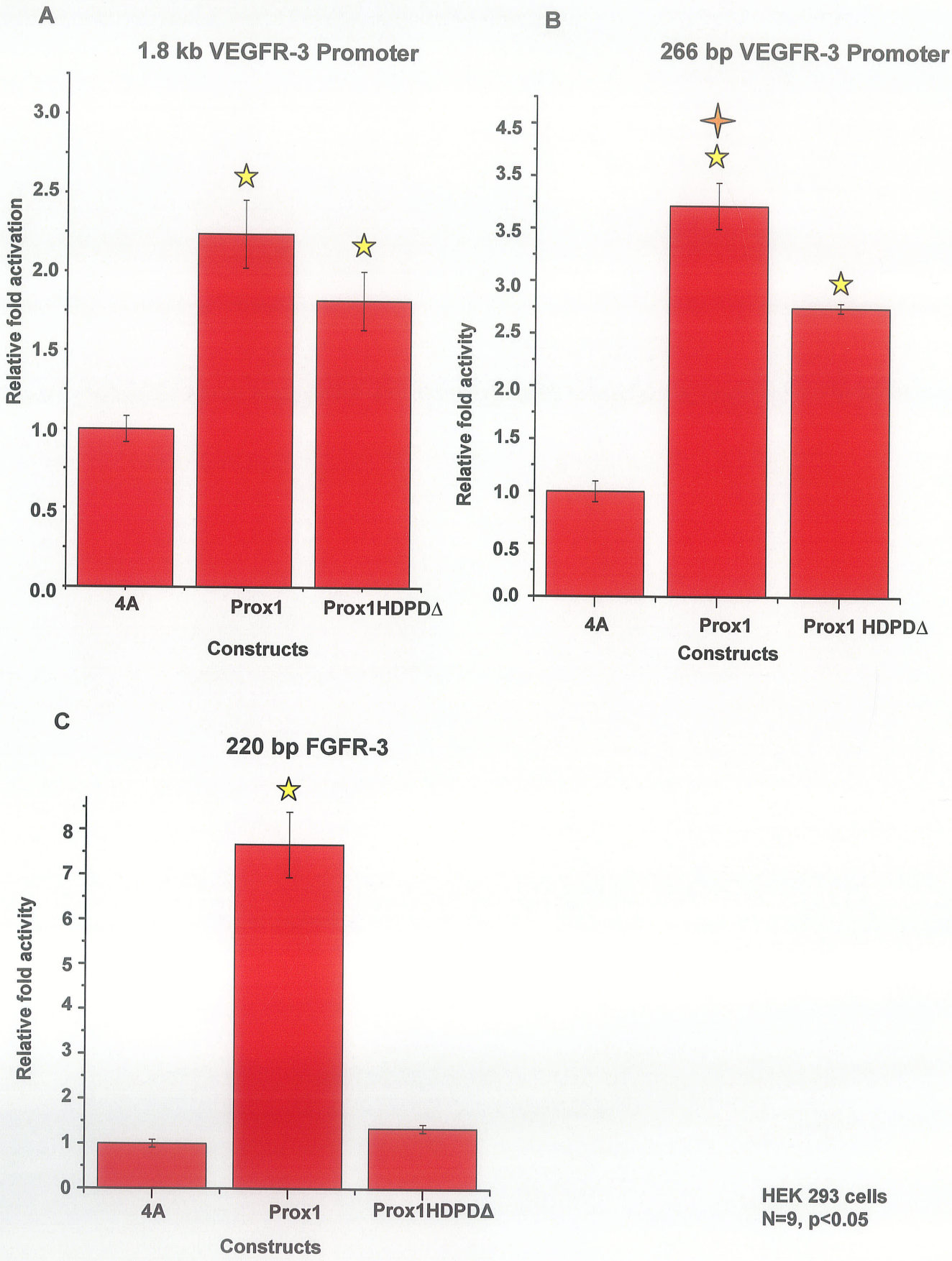

A
1.8 kb VEGFR-3 Promoter
Relative fold activation
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4A
Prox1
Prox1HDPDΔ
Constructs
B
266 bp VEGFR-3 Promoter
Relative fold activity
4.5
3.5
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4A
Prox1
Prox1 HDPDΔ
Constructs
C
220 bp FGFR-3
Relative fold activity
8
7
6
5
4
3
2
1
0
4A
Prox1
Prox1HDPDΔ
Constructs
HEK 293 cells
N=9, p<0.05

Fig 16: Prox1 does not need to bind to DNA to activate VEGFR-3 promoter in HEK 293 cells : Various versions of mouse VEGFR-3 promoter sequences were cloned into pGL3 luciferase reporter vector. Luciferase assay with a full length 1.8 kb mouse VEGFR-3 promoter shows that both wild type Prox1 and Prox1HDPDΔ, which cann't bind to DNA, activated the VEGFR-3 promoter significantly more than the control (A). There is no significant difference in the expression of the full length VEGFR-3 promoter between the Prox1 and Prox1HDPDΔ (A). A similar pattern of expression was observed in the case of 266 bp VEGFR-3 promoter too (B). These results correlate with our results from the western blots of the HUVECs infected with adenovirus. In our studies we have used a 220 bp FGFR-3 promoter which has been shown to be activated by Prox1 by DNA binding dependent mechanism. Prox1 activates the FGFR-3 promoter but Prox1HDPDΔ could not (C). Error bars indicates standard error. ☆ Indicates significantly different from contol.

17

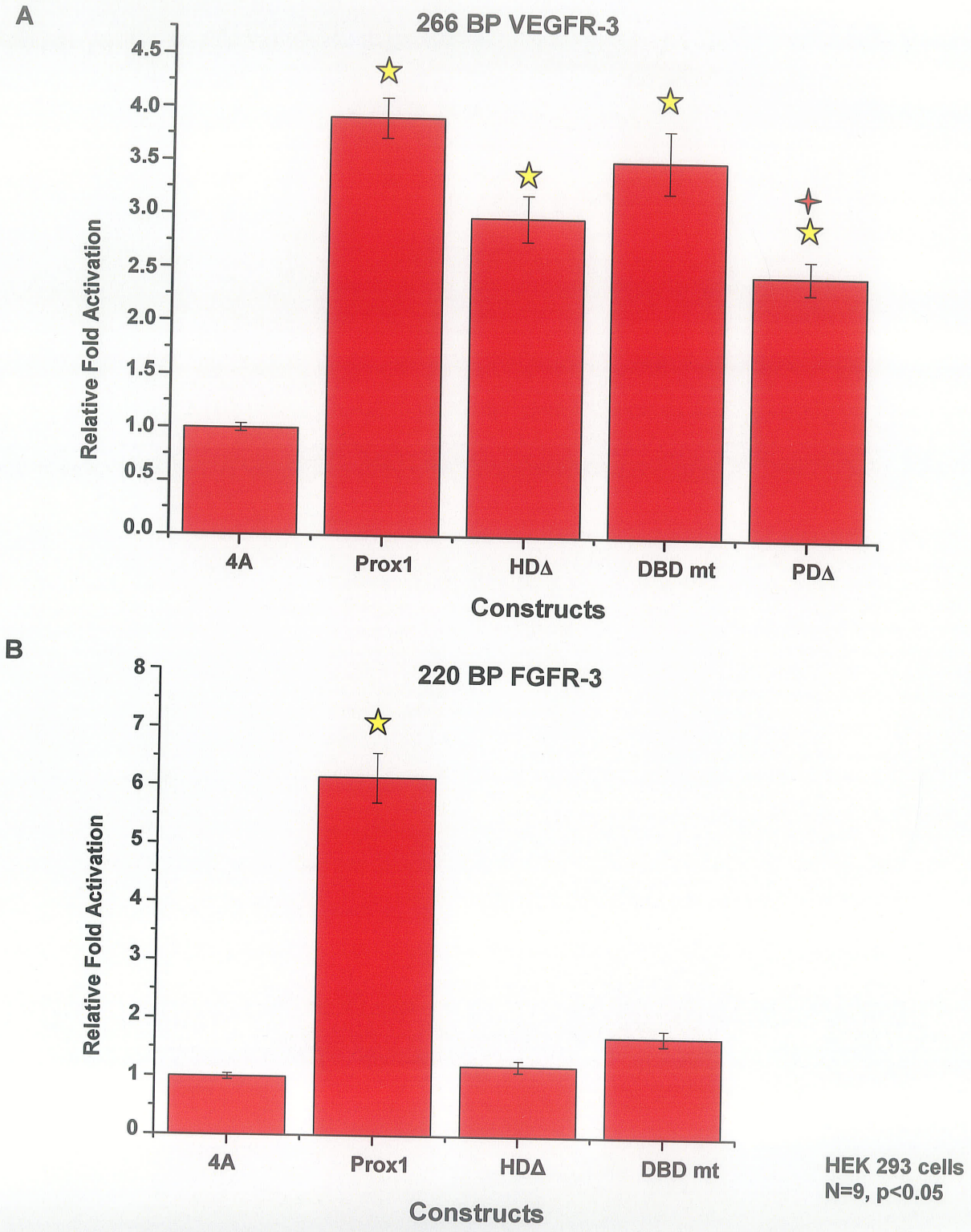


Fig 17: Differential role of Prox1 in the regulation of *VEGFR-3* and *FGFR-3* expreseeion : 266 bp VEGFR-3 promoter and 220 bp FGFR-3 promoter were significantly activated by Prox1 compared to control. However, Prox1HDΔ and Prox1DBDmt which are devoid of DNA binding ability activate VEGFR-3 promoter significantly but not the FGFR-3 promoter. Thus, establishing the DNA binding independent activation of VEGFR-3 and DNA binding dependent activation of FGFR-3 prometer. Error bars indicate standard error. ☆ Indicates significantly different from contol.

18

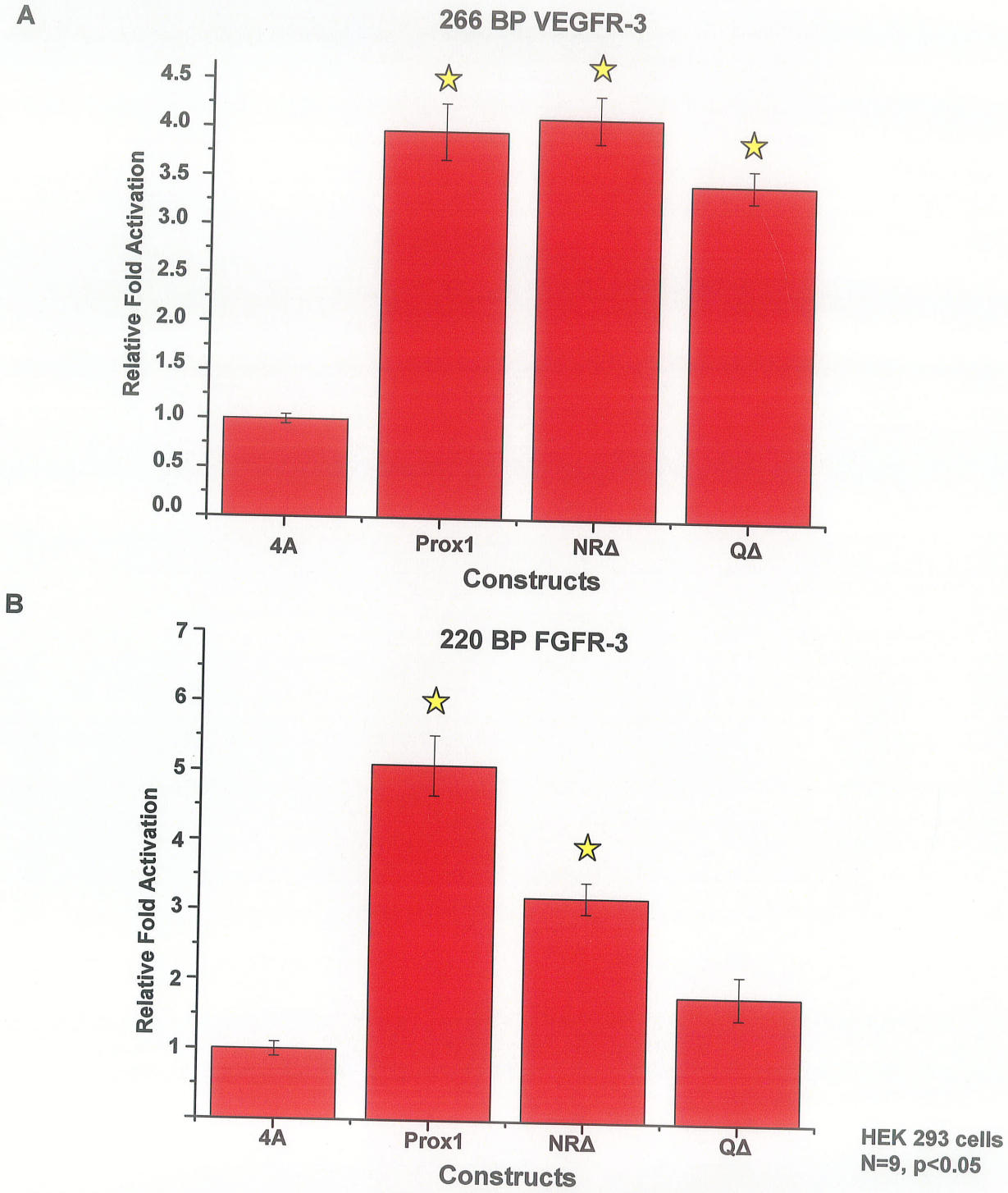


Fig 18: Prox1 NR boxes and glutamine rich region are dispensable for VEGFR-3 expression : Prox1, Prox1 NRΔ and Prox1 QΔ constructs equally activated the 266 bp VEGFR-3 promoter luciferase construct and this activation was significant compared to control (A). In the case of 220 bp VEGFR-3 promoter Prox1 QΔ did not show a significant activation compared to the control where as the Prox1 NRΔ even though activated significantly compared to the control and Prox1 QΔ but did not activate as much as the wild type Prox1 (B). This signifies the critical role of Q rich region in the activation of FGFR-3 promoter. Error bars indicate standard error. ☆ Indicates significantly different from contol.

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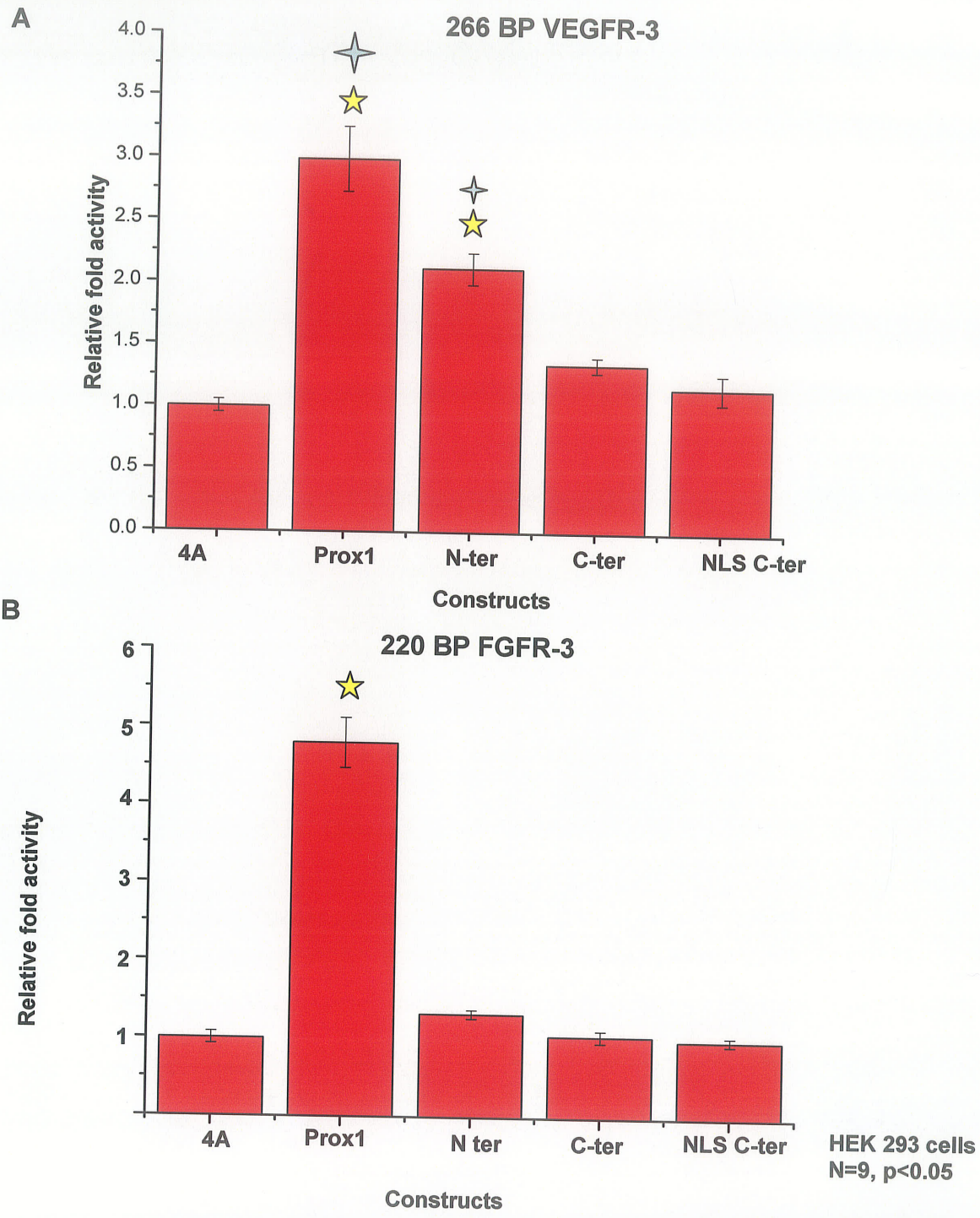

Fig 19: Carboxyl-terminal of Prox1 is not necessary for the activation of VEGFR-3: Neither the Prox1 Carboxyl- terminal nor the Carboxyl-terminal version of Prox1 carrying the artificial nuclear localization tag at its N-terminus were able to activate the 266 bp VEGFR-3 promoter significantly compared to control (A). Amino-terminal version of Prox1 activated the 266 bp VEGFR-3 promoter but was not equivalent to the full length Prox1 (A). However none of these constructs were able to activate the 220 bp FGFR-3 promoter (B). The N-terminal even though carries the glutamine rich region can't activate the FGFR-3 promoter since it lacks the DNA binding ability. Error bars indicate standard error. ☆ Indicates significantly different from contol.

5. SP1 transcriptional factors are dispensable for activation of VEGFR-3 promoter by Prox1.

Using MatInspector program, we searched for the presence of various transcription factor binding sites present in the VEGFR-3 promoter. We found numerous SP1 transcription factor binding sites within the proximal 226 bp VEGFR-3 promoter. SP1 transcription factors, which bind to the GC rich regions, are shown to play important role in the recruitment of the initial transcription factors to the TATA less promoters (Safe et al., 2005). To check if Prox1 has a role in recruiting SP1 transcription factors to the VEGFR-3 promoter and thus enhance the activation of the promoter, we performed luciferase assay to see if SP1 synergistically increased VEGFR-3 transcription. As shown in the Fig 20, we did not observe any increase in the activation of the VEGFR-3 promoter in the presence of Prox1 and SP1 as compared to the control HEK 293 cells.

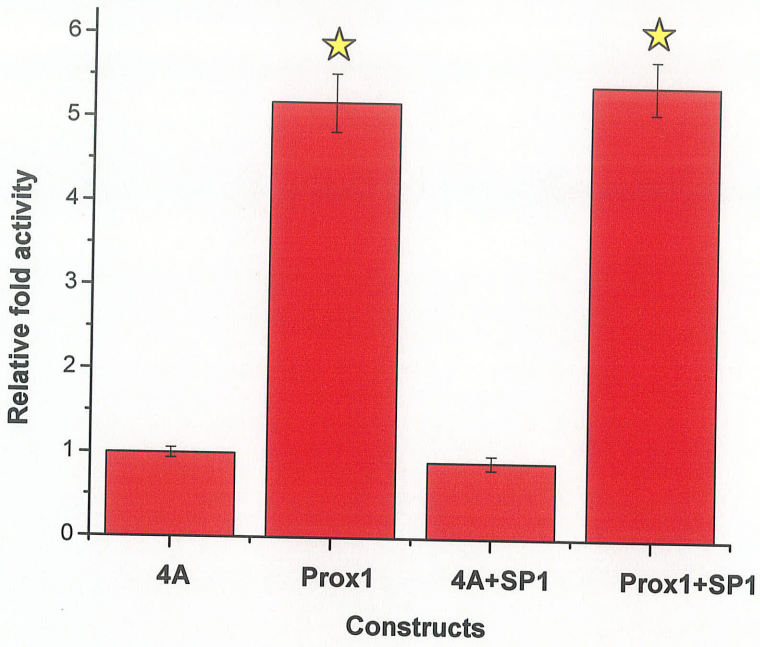

Fig 20: Role of SP1 sites in the activation of 266 bp VEGFR-3 : A luciferase assay was performed to check the role of SP1 transcription factos in the activation of the VEGFR-3 promotor. We used 266 bp VEGFR-3 promoter for the luciferase assay. SP1 cotransfected with control vector pCMV-4A or Prox1 didn't show any significant effect compared to pCMV-4A alone transfected cells or Prox1 alone transfected cells respectively. Thus, showing that SP1 transcription factors may not have a crucial role in the activation of the VEGFR-3 promoter by Prox1. Error bars indicate standard errors. ☆ Indicates significantly different from contol.

Discussion

Prox1 has been shown to act as an activator (*FGFR-3* and *βB1-Crystallin*) where its DNA binding ability is a necessity (Shin et al., 2005; Cui et al., 2004). It has been shown to be a co-repressor (*CYP7A1*) by binding to nuclear receptors (LRH-1 and HNF4α) with its LXXLL motifs (NR boxes) (Qin et al., 2004; Song, 2006). Similar to our laboratory's previous results with *CyclinE1*, where Prox1 has first been shown to act as a co-activator, we report the DNA-binding independent activation of *VEGFR-3* expression. This Prox1 mediated activation of *VEGFR-3* expression also needs the N-terminal region and the role of prospero domain. However, the role of the prospero domain, which has been thought to play a role in the activation of *CyclinE* expression, is not as clear.

Adenoviral expression vectors, which render an efficient delivery and very high expression of the proteins, were constructed. HUVECs infected with adenovirus expressing Prox1 and Prox1HDPDΔ separately showed a time dependent expression of the respective proteins. AdProx1 infected cells showed an instant expression of Prox1 at 24 hrs (Fig 10A) and showed a lesser expression at 72 hrs (Fig 10C). This could be due to the degradation of the excessive protein through the proteosomal degradation pathway. Contrastingly, AdProx1HDPDΔ infected cells showed a delayed expression of Prox1HDPDΔ. At 24 hrs (Fig 10A), Prox1HDPDΔ showed a very less expression compared to Prox1 but Prox1HDPDΔ showed a very high expression compared to Prox1 at 72 hrs (Fig 10C) and both Prox1 and Prox1 HDPDΔ showed an equivalent

expression at 48 hrs (Fig 10B). This striking difference in the expression pattern of Prox1 HDPDΔ, compared to Prox1, could be due to the loss of the degradation signal present in the homeodomain and thus leading to the accumulation of the protein.

We have looked manually for the Prox1 putative binding sites C(a/t)(c/t)NNC(t/c) and (T)AAGACG in the 1.8 Kb mouse *VEGFR-3* promoter (Fig 11) region upstream of the transcription start site. We have found six such putative sites, two sites in the distal promoter region between -847 to -758 and four sites in the proximal region -218 to -33. *FGFR-3*, a known Prox1 direct target regulated by a DNA binding dependent mechanism, also has four similar putative binding sites in the proximal promoter region between -220 and -79 bp (Fig 11). This led us to propose the hypothesis that Prox1 activates *VEGFR-3* expression by directly binding to the DNA. Our luciferase assay with the full length 1.8 Kb mouse *VEGFR-3* promoter showed that both Prox1 and its DNA binding deficient version Prox1HDPDΔ activated the promoter equivalently (Fig 16A). Quantitative analysis of the western blots from whole cell lysates of HUVECs infected with AdEGFP, AdProx1 and AdProx1HDPDΔ showed a consistently parallel result to our luciferase assay reports (Fig 15). This finding supports the DNA binding independent activation of the *VEGFR-3* expression *in situ* and *in vivo*.

The full length 1.8 Kb *VEGFR-3* promoter (Fig 11) also contained two potential E2F binding sites at -372 to -365 and -50 to -40 bp upstream the transcription start site as detected by MatInspector. Luciferase assays with various *VEGFR-3* promoter versions to identify the crucial region sufficient for the

Prox1 mediated activation shows the activation by minimal 90 bp version (Fig 12F). Even though all the *VEGFR-3* 5' deletion promoter constructs we made were activated by Prox1, we observed a difference in the fold activation between the constructs. The 1.8 Kb, 924 bp and 696 bp promoter constructs showed a 2 to 2.5 fold activation (Fig 12A, 12B and 12C), 266 and 130 bp promoter versions showed an induction of 3.5 to 4 fold (Fig 12D and 12E) but 90 bp version again showed a 2.5 to 3 fold induction (Fig 12F). The rise in the fold induction by 266 bp and 130 bp constructs could be due to the absence of distal E2F site or could be due to the loss of inhibitory role by the deletion of distal regions. The 90 bp promoter still contain two putative Prox1 binding sites and one E2F site. However, the Prox1 binding sites are likely dispensable as we have observed the DNA binding independent activation of the *VEGFR-3* by Prox1. This leaves us the E2F binding site at -50 to -40 bp. Like *CyclinE1*, we predict that the activation of *VEGFR-3* is via the removal of the repression activity of the HDAC mediated through the pRb/E2F binding. In the near future we will construct a 90 bp *VEGFR-3* promoter construct with a mutant E2F binding site which would determine the role of the E2F mediated repression in the *VEGFR-3* expression.

In order to establish the region of Prox1 essential for the activation of the VEGFR-3 promoter, we did similar luciferase assays with a 266 bp *VEGFR-3* promoter and a 220 bp *FGFR-3* promoter, which is used as a control to determine the functional roles of the various Prox1 constructs. Similar to Prox1HDPDΔ, Prox1 versions (Prox1 HDΔ, and Prox1DBDmt) that are devoid of the DNA binding ability showed no significant difference in the activation of the

266 bp *VEGFR-3* promoter compared to that of Prox1 (Fig 17A). Contrastingly, none of these constructs (Prox1HDPDΔ, Prox1 HDΔ, and Prox1DBDmt) were able to activate the 220 bp *FGFR-3* promoter (Fig 17B). This establishes the two divergent mechanisms of Prox1 mediated regulation, a DNA-binding dependent activation of the *FGFR-3* promoter and DNA binding independent activation of the *VEGFR-3* promoter.

Like *CyclinE1*, Prox1PDΔ was able to activate the *VEGFR-3* promoter but is significantly less than wild type Prox1. Prox1HDPDΔ, which is also missing the prospero domain, activates the 1.8 Kb *VEGFR-3* promoter in our luciferase assay and expresses equivalent amount of VEGFR-3 at protein level in the HUVECs compared to that of Prox1 (Fig 16A and 15). The divergence in activation could be due to the inefficient localization of the Prox1PDΔ construct in the nucleus as the extreme C-terminus of the prospero domain was involved in masking the nuclear export signal. The lower expression of Prox1PDΔ was also thought to be another cause for the lesser induction in the activity of the *CylinE1* promoter but in our western blots we observed an equivalent expression Prox1PDΔ as that of Prox1. This suggests that Prox1PDΔ has a less significant role in the activation of the *VEGFR-3* promoter than for the *CyclinE1* promoter. However, we have observed significantly less activation of the 266 bp *VEGFR-3* promoter by Prox1HDPDΔ than Prox1 and thus leaving to an ambiguity in the role of prospero domain in the recruitment of the other co-factors responsible for activation (Fig 16B).

The other significant domains of the Prox1 were the two NR boxes (NR1

and NR 2) and the glutamine rich region (Q rich) present at the N-terminus of the Prox1. The NR boxes consisting of the LXXLL motifs were crucial in the repression of the cholesterol 7-α-hydroxylase expression by interacting with the AF2 domain found in nuclear receptors such as LRH-1 and/or HNF-4α (Qin et al., 2004; Song, 2006). However, a functional role for the Q rich region of Prox1 has not been established until now. Neither the Prox1NRΔ version nor the Prox1QΔ version showed any difference compared to Prox1 in the activation of a 266 bp *VEGFR-3* promoter (Fig 18A). However in the case of a 220 bp *FGFR-3* promoter, the Prox1NRΔ version showed a significantly lower activation compared to the Prox1 and Prox1QΔ didn't activate at all (Fig 18B). Thus, for the first time, we report a crucial role for the glutamine rich region and NR boxes of the Prox1 in activation of *FGFR-3* expression and that in contrast these regions are dispensable for the activation of the *VEGFR-3* promoter. The N-terminal Prox1 construct (N-ter) was able to activate the *VEGFR-3* promoter but not equivalently to that of wild-type Prox1 (Fig 19A). It was unable to activate the *FGFR-3* promoter likely because it cannot bind to the DNA as it lacks the homeodomain and prospero domain (Fig 19B). The two other smaller constructs C-terminal and NLS tagged C-terminal Prox1 versions were neither able to activate the *VEGFR-3* promoter nor the *FGFR-3* promoter thus showing a crucial role for N-terminal region of Prox1 in binding to other protein partners for activation. The Prox1 C-terminal version consists of just the homeodomain and the prosperodomain. The nuclear localization signal of the Prox1, which targets Prox1 to the nucleus, has been predicted to be present in the N-terminal region

of the Prox1. Since the C-terminal version of Prox1 is devoid of the NLS, the expression was not observed in the nucleus but was seen in the cytoplasm. So, an artificial NLS tag was attached to the homeodomain end and its expression was observed to be in the nucleus by immunocytochemistry. These luciferase results show that the activation of *VEGFR-3* promoter by Prox1 is DNA-binding independent and Prox1 N-terminal region is crucial for the activation. The lower activation of the *VEGFR-3* promoter by Prox1 N-terminal compared to Prox1 could be due to the subtle interruption in the conformation of the protein and thus weaker interactions with its co-activators. In the case of *FGFR-3* promoter activation, Prox1 not only needs to bind to DNA but also require the NR boxes and the Q rich region of the Prox1 for interacting with other protein partners.

In the 266 bp *VEGFR-3* promoter, we have found several SP1 transcription factor binding sites. Like *FGFR-3*, *VEGFR-3* is also a TATA less and CAAT less promoter (Iljin et al., 2001). SP1 transcription factors are thought to play a vital role in the activation of such TATA less promoters by recruiting the initial transcription machinery. The studies on the *FGFR-3* in the *Drosophila* SL2 cells, which are devoid of SP1 function, show that the SP-1 sites play a crucial role in the activation of the *FGFR-3* promoter (McEwen and Ornitz, 1998). In our luciferase assay, co-transfection of the Prox1 along with the SP-1 transcription factor in HEK 293 cells did not show any difference in induction compared to that of Prox1 alone (Fig 20). This finding shows that the SP1 transcription factor does not play a role in the activation of the *VEGFR-3* promoter.

Regulation of the TATA less promoters even though is not yet clear, many

studies show the vital role of initiator sequence (Inr) in regulating such promoters (Javahery et al., 1994). The Inr consensus sequence (YYANT/AYY) along with the E boxes (CANNTG consensus sequnece) has been shown to play an important role in recruiting the TFII-1 on to the *VEGFR-2* promoter which is again a TATA less and CAAT less promoter (Jackson et al., 2005). The 90 bp VEGFR-3 promoter shows a similar Inr consensus sequence (**TCACTCC**CAG**CCTA**$^{+1}$**GAGC**) and one E box consensus sequence (**CCGCTG**) at -51 to -57 bp upstream of the transcriptional start site. So, like many other TATA less and CAAT less promoters the regulation of VEGFR-3 promoter might also have a crucial role for the Inr sequence and the E box. However, the possible role of Prox1 in recruiting or enhancing the initial transcriptional machinery is still need to be studied. Since, the 90 bp VEGFR-3 promoter consists of an E2F binding site and a p300 binding site, Prox1 could possibly be enhancing the transcription by either removing the pRb/HDAC3 mediated repression or by promoting the histone acetylase activity of p300/CEBP (Fig 21).

In this study we were able to show both DNA binding dependent and DNA binding independent mechanisms of Prox1 mediated regulations. We were also able to show *in situ* and *in vivo* that the activation of the *VEGFR-3* by Prox1 does not need to bind to DNA and the N-terminal region is crucial for the activation of the *VEGFR-3*. However, we still have to determine if *VEGFR-3* is a direct target of Prox1 or if Prox1 activates other target which in turn activates the *VEGFR-3* expression.

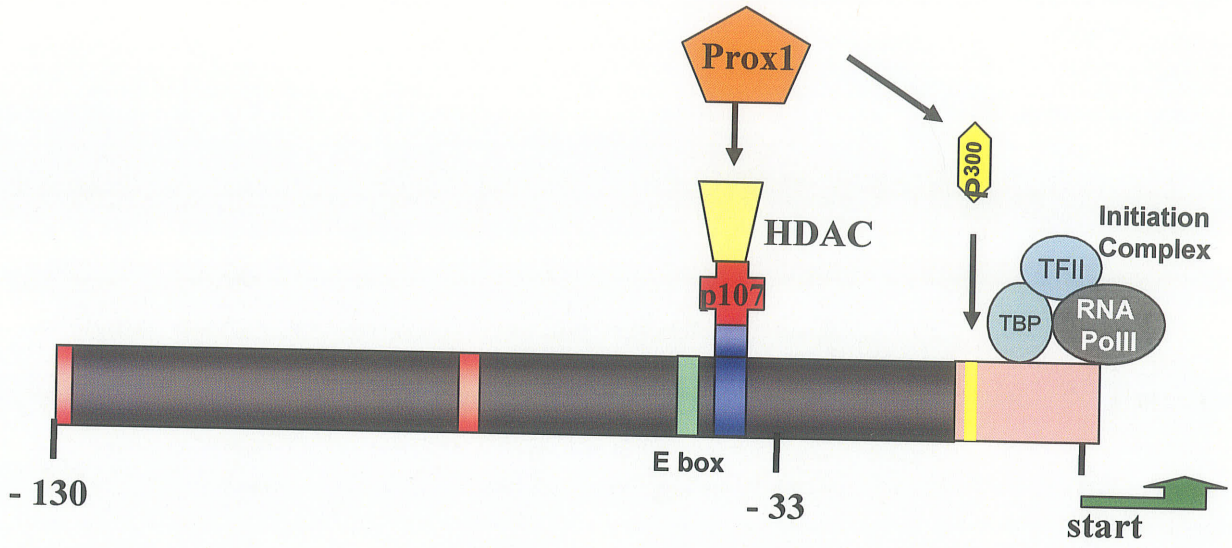

Fig 21: Probable mechanisms of Prox1 mediated activation of *VEGFR-3* promoter: 130 bp VEGFR-3 promoter consists of Inr sequence, P^{300}, E2F and putative Prox1 binding sites. Since our results show that Prox1 activates VEGFR-3 promoter by a DNA binding independent mechanism Prox1 sites could be dispensable. We propose two mechanisms of activation of VEGFR-3 by Prox1. Prox1 has been shown to interact with HDAC3 and the presence of E2F binding site in the minimal 90 bp promoter that is activated by Prox1 indicates the role of Prox1 in regulating the VEGFR-3 promoter by removing the HDACs and thus promoting the transcription. The other mechanism could be through the induction of histone acetylase activity of P300 by Prox1. Either of these mechanisms could enhance the recruitment of basal transcription machinery and thus activate transcription. Like other TATA less and CAAT less promoters, in which Inr consensus sequence recruits the initiation transcription factors, VEGFR-3 also consists of such consensus sequence. The initial transcription machinery consists of RNA polymerase II, TFII and TBP etc. TFII-1 has been shown to interact with Inr sequence of VEGFR-2 and it is also shown that TFII either directly binds to the Inr or by binding to the TBP and thus binding to Inr. SP1 transcription factors are also found in the VEGFR-3 promoter and these could be helping in stabilising the intiation transcription factors but is not directly involved in the activation.

Conclusions

In this study we were able to

1. Determine that the proximal 432 bp is necessary and sufficient for the regulation of *VEGFR-3* promoter by Prox1. Even the minimal 90 bp *VEGFR-3* promoter is activated by Prox1.
2. Contrast the differential regulatory mechanism of Prox1 mediated activation of *VEGFR-3* and *FGFR-3* promoters.
3. Demonstrate that Prox1 does not need to bind to DNA to activate *VEGFR-3* promoter both *in vivo* and *in vitro*.
4. Illustrate that the Prox1 mediated activation of the *VEGFR-3* promoter does not necessarily need either the nuclear receptor boxes or the glutamine rich region.
5. Show that the amino terminal region is important for the activation of *VEGFR-3* promoter.
6. Provide evidence that Prox1 not only need to bind to *FGFR-3* promoter by a DNA binding dependent mechanism but also needs the glutamine rich region as well as the NR boxes for the full activation.
7. Show that the SP1 transcription factors are dispensable for the Prox1 mediated activation of *VEGFR-3* promoter.

Future directions

1. In the near future we are constructing the E2F mutant 90 bp VEGFR-3 promoter into the pGL3 luciferase reporter. This will enable to determine the role of the pRb/HDAC in the regulation of *VEGFR-3* promoter.
2. Chromatin immunoprecipitation (ChIP) assay to determine if VEGFR-3 is a direct target of Prox1 or indirect target. It will also enable to recognize the region on VEGFR-3 where the Prox1 is recruited.
3. To further support the DNA binding independent mechanism of activation of VEGFR-3 by Prox1 an electro mobility shift assay (EMSA) could be performed.
4. Co-Immunoprecipitation of Prox1 and HDAC3 to check if Prox1 is interacting with HDAC3 directly.
5. To further confirm the HDAC role use HDAC inhibitors NaBu or Trichostatin A.
6. Mutation studies in the Inr sequence to study the regulatory mechanism of Prox1 in recruiting the initial transcriptionary machinery.

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