AMERICAN UNIVERSITY OF BEIRUT

NFATC1 AND PAX PATHWAY IN CARDIAC AND OCULAR DISEASES

by
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AN ABSTRACT OF THE THESIS OF

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The cardiac valvular and septal malformations account for the majority of the congenital heart defects (CHD). Processes that govern the heart development have been studied thoroughly through the last decade, however little is known about specific interactions that lead to the normal heart formation. Nuclear factor of activated T cells (NFAT) signaling and Vascular Endothelial Growth Factor (VEGF) have been shown to be main players in valve formation. NFATC1 knockout mice did not develop aortic and pulmonary valves and died of congestive heart failure. These studies revealed a pivotal role of NFATC1 in heart valve formation.

Our hypothesis is that novel mutation within the *NFATC1* gene lead to defects that affect the protein at different levels so we aim to functionally characterization this novel point mutation (P68L) of NFATC1 at three different levels:
- Translocation of NFATC1 to nucleus.
- Transcriptional Regulation
- Interaction with calcineurin.

We hypothesize that this novel P68L mutation in the NFATC1 gene is disease causing and CHD contributing. We suggest that the mutation will distort the expression of VEGF and disrupt the physical interaction of NFATC1 protein with calcineurin.

Our second hypothesis is related to a previous study have been done in our lab revealed a potential role for *NFATC1* gene in the context of valve and eye development. To understand how this previously documented V210M *NFATC1* mutant affects the heart and the ocular development process. We hypothesize a physical interaction between NFATC1 and transcription factor PAX6 that has been considered as a master gene in eye development.

The newly identified NFATC1 mutation P68L in patient with an atrial septal defect resulted in different biophysical properties than that of the wild type NFATC1 and the P68T mutant that has been found in normal population. P68L and P68T affect the subcellular localization of the NFATC1 protein induced by calcineurin, as well as decreased interactions with calcineurin compared to the wild type NFATC1. Interestingly, P68L exhibited significantly decreased transcriptional activity in vitro compared to other mutant P68T and the wild type.

Future studies will need to confirm the interactions of this mutant with other cardiac co-transcription factors to determine the influence of this mutation on valve-septa formation. In

vivo animal models are the ultimate way to prove that these mutations are directly causing the observed phenotypes.

For the second aim of the study, in characterizing a potential role for NFATC1 gene in the context of valve and eye development. This report comprehends novel *in vitro* finding of interaction between NFATC1 and PAX6. Furthermore, the previously reported mutation V210M in NFATC1 showed an even increased interaction with PAX6 that might explain the severe atrial septal defect and eye malformations seen in patients.

Our hypothetical model would involve the up regulation of VEGF promoter expression in both the developing valves and emerging ocular structures, via the intervention of NFATC1, PAX6. More studies and experiments need to be conducted to decipher the VEGF expression in the presence of PAX6 and NFATC1.

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ABBREVIATIONS

AP1	Activator protein
ALM	Anterior lateral plate mesoderm
ANOVA	Analysis of variance
Ao	Aorta
a	Atria
ASD	Atrial septal defects
AVC	Atrioventricular canal
BAV	Bicuspid aortic valve
BMP	Bone morphogenic protein
CNA	Calcineurin A
CNB	Calcineurin B\
Ca2+	Calcium
CaM	Calmodulin
C terminal	Carboxy terminal
C terminus	Carboxy terminus
CHD	Congenital heart disease
CsA	CyclosporinA
DNA	Deoxyribo nucleic acid
E15	Embryonic day 15
EC	Endocardial cushion

Ec	Endocardium
EFTFs	Eye field transcription factors
EMT	Endothelial Mesenchymal transformation
EGF	Epidermal growth factor
ECM	Extracellular matrix
LV	Left ventricle
NHR	NFAT homology domain
N terminal	NH2 terminal
NES	Nuclear export signal
NFκB	Nuclear factor κB
NFAT	Nuclear factor of activated T cell
My	Myocardium
PAX	Paired box genes
PBS	phosphate-buffered saline
PDA	Patent ductus arteriosus
RV	Right ventricle
SA	Sinoatrial node
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
VSD	Ventricular septal defect

OFT	Outflow tract
OV	Optic vesicle

CHAPTER I

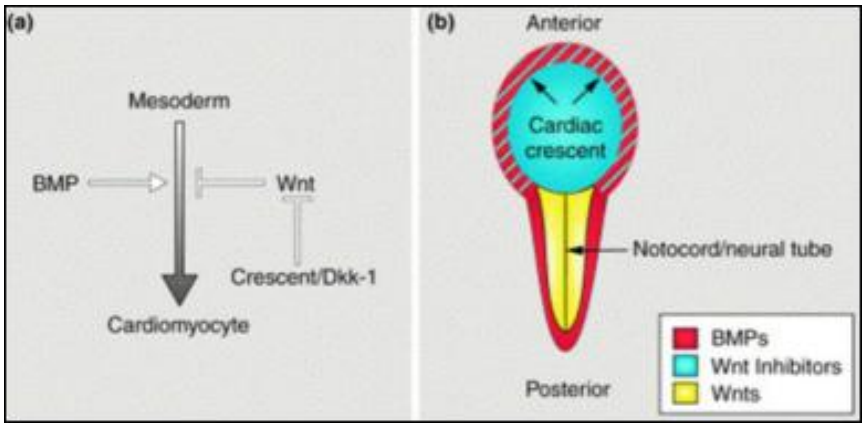
INTRODUCTION

One of the most fascinating processes during the embryonic development is the formation of the heart that incorporates a numerous molecular and morphogenic events. A functional beating heart will be formed due to precise assembly of multiple different cell types. The first heartbeat, early in development, is the most evident sign of life and is essential for embryonic life. The importance of the precise assembly of the embryonic heart is evident in the high incidence of congenital heart diseases, which is one of the major causes of neonatal morbidity and mortality throughout life. (1,2).

A. Cardiac Morphogenesis (Normal Heart Development)

The very first organ will be formed in developing embryo in all the vertebrates is the heart. At about embryonic day 15 after gastrulation in humans, the cells in the anterior lateral plate mesoderm (ALM) become committed to develop into cardiac cells that form a beating cardiac crescent (3). At this stage two types of cells can be distinguished; cells that form the primary heart field derived from(ALM) and cells forming the secondary heart field derived from the dorsal-medial aspect of the cardiac crescent. This cardiac crescent then develops into parallel cardiac primordia that fuse in the midline to form the primitive cardiac tube that is composed of an outer muscular wall referred to as myocardium, and inner endothelial lining referred to as endocardium (4). Between the myocardium and endocardium a

cardiac jelly, made up of glycoconjugates will form later on. Inductive interactions between theses layers are necessary for several steps during heart development (4). The families of signaling molecules that induce this process include, fibroblast growth factor (FGF), bone morphogenic proteins (BMP), Wnt and NOTCH pathways that lead to the development of the primitive heart and are expressed by the adjacent endoderm. Positive and negative coordination of these signals are very crucial to ensure the normal formation of the cardiac crescent (3). For example, the BMP promotes the cardiomyocyte fate, whereas the Wnt mediated signals suppress it (figure 1)(4). Many molecular evidences demonstrate that the cardiac tube forms during the 3rd week but in addition to the folding process some of the chambers and vessels arise as a consequence of fusion of different heart fields, the 1st heart field arises from the cardiac crescent and gives rise to the left ventricle (LV), right and left atria, whereas the 2nd heart field fuses with the cardiac tube and gives rise to the right ventricle (RV) and outflow tract (OFT) from its ventral portion and the atria from the dorsal portion (also referred to as tertiary heart field) (6)



Figure(1): Wnt and BMP signals pattern the crescent(7).

The end result is the formation of a centrally located heart, from cardiac precursors that were bilateral in location. The single cardiac tube has dilatations that constitute the sinus venosus, common atrium, presumptive left ventricle, presumptive right ventricle, and the conus. The truncus is not present in the early heart tube, but is added to the end of the tube from a secondary heart field (2).

Between days 23 and 28, the cardiac loop will be formed after the elongation and the bending of the heart tube. Cardiac looping is the first indication of right–left asymmetry in the embryo, and it is thought to possibly occur because of differential cell growth or altered cell adhesion. Looping of the heart to the right occurs in all vertebrates, suggesting that this is a critical and conserved step in cardiac morphogenesis. Studies of mouse mutants and chick embryos have led to insights into the signals that initiate cardiac looping. The embryonic heart begins to beat concomitantly with the formation of the heart tube and the onset of looping.

A further contribution from migrating cardiac neural crest cells from the neural tube to the OFT and pharyngeal arches enables division of the common trunk into aortic and pulmonary arteries and also gives rise to the coronary circulation. ‘Ballooning’ of specific areas of the tube produces the four chambers of the heart. Meanwhile looping, septation, and spiraling of the tube occurs to establish a heart with normally connected vessels and with 2 separate sides functioning in parallel during the fetal period but able to function in series after birth. (5)(figure 2).

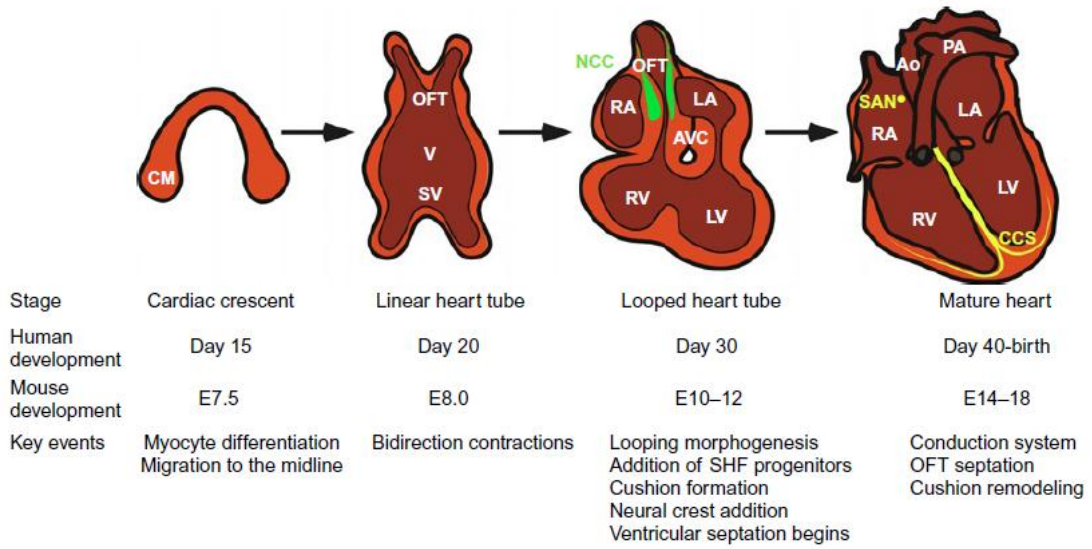


Figure (2): Schematic representations of several stages of mammalian heart development with key events in mouse and human heart development denoted. Mouse development is denoted in embryonic days (E). Ao, aorta; AVC, atrioventricular canal; CCS, cardiac conduction system; CM, cardiac mesoderm; LA, left atrium; LV, left ventricle; NCC, neural crest cells; OFT, outflow tract; PA, pulmonary artery; RA, right atrium; RV, right ventricle; SAN, sinoatrial node; SV, sinus venosus; V, ventricle (3).

The establishment of the functioning fetal circulation will occur by the eighth week of gestation. Molecular studies propose that chamber specification pave the way the actual septation of the heart. The development of the cardiac conduction system occurs prior to septation. The sinoatrial (SA) node, which is the pacemaker of the heart, forms from a caudal region of the straight heart tube. Initially, electrical impulses spread from the SA node throughout the ventricle and across the AV canal region to the ventricles. As the atria and ventricles become electrically isolated, the AV node in a region of the interventricular septum becomes the only pathway of depolarization from the atria to the ventricles. (2).

B. Partitioning the heart: Cardiac Septation and Valve Development

Because the majority of the cardiac malformations found in humans include valve and septal abnormalities, characterization of the molecular mechanisms driving valvuloseptal development is an important area of research (6).

1. Septation:

For the Septation of the primitive cardiac chambers, the AVC and the OFT is necessary to form a four-chamber heart.(7)

The heart continues to develop through the development of the major septa. The formation of the Septa occurs between days 27 and 37, Septum formation is achieved through the formation of endocardial cushions. These are made up of endocardial cells, which delaminate and undergo an epithelia to mesenchymal transformation (EMT). The lumen will be subdivided into two cavities by the endocardial cushions. By the formation of the septum premium, the right and left atria will be developed, whereas the septum secundum will subdividesthe primitive atrium. At the end of the fourth week, the right and left canals, as well as the mitral and tricuspid valves will be formed by the appearance of the endocardial cushions in the atrioventricular canal (9). During the same time, the muscular interventricular septum will be formed by the gradual fusion of the medial walls of the ventricles. The middle third of *bulbus cordis* is comprised by the *conus cordis*, and in the fifth week of development, the conus will be subdivided by the cushions to form the outflow tract of the right and left ventricles, as well as the membranous portion of the interventricular septum (8).

2.Valvulogenesis:

Valvulogenesis is one of the most intriguing events during the embryonic development.by which a fragile gelatinous matrix is remodeled into thin fibrous leaflets capable of sustaining unidirectional blood- flow over a lifetime (6).

a. Valvular Morphogenesis

Heart valves develop at endocardial cushions of the AVC and OFT. Valve morphogenesis begins with the transformation of endocardial cells into mesenchymal cells through EMT (figure 3). Endocardial cushions with mesenchymal cells then elongate and remodel themselves to form primitive valves that gradually mature into thin valve leaflets. The elongation of valve leaflets is accomplished by a combination of cell proliferation at the growing edge and apoptosis at the base of the cushion (11).

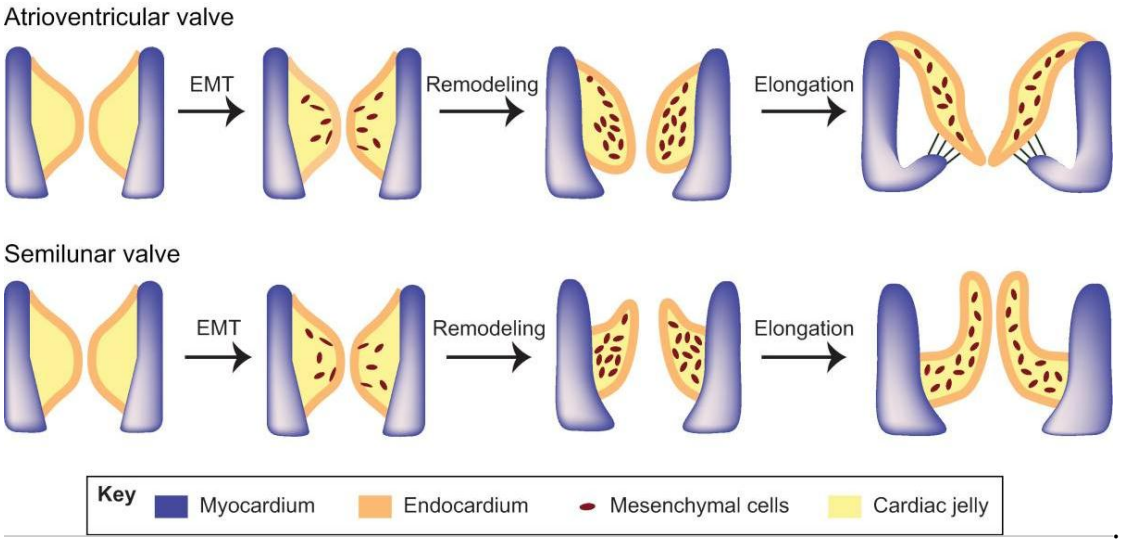
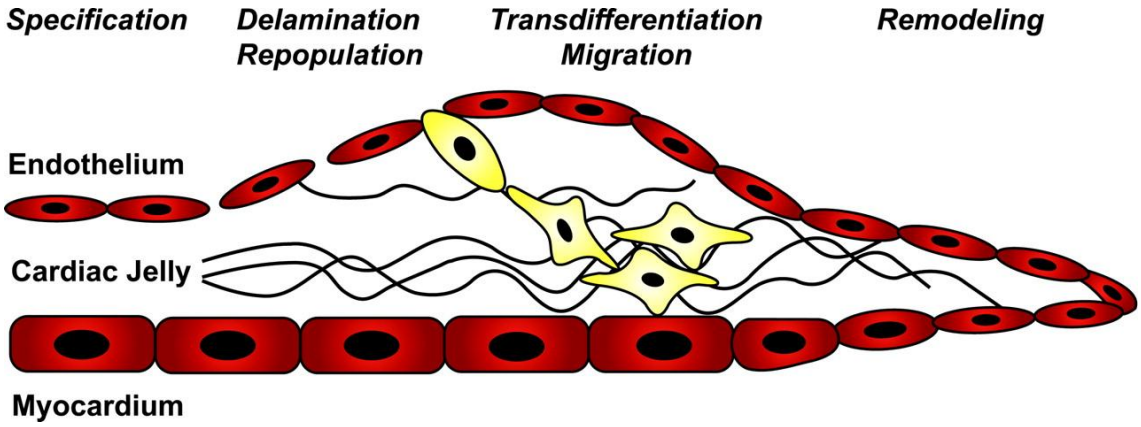


Figure (3): Epithelial to Mesenchymal Transformation during valvulogenesis(27).

In a normal heart, there are four valves. The atrioventricular valves are found in the right (tricuspid valves) and left (mitral valves) atrioventricular junction. However, the semilunar valves are located at the roots of the aorta (aortic valve) and at the roots of the pulmonary artery (pulmonary valve). The morphogenesis of the atrioventricular and semilunar valves is a precise and complex process that happens concurrently with changing the cardiac morphology (12). As we stated earlier, the early embryonic heart is a single tube of endocardial cells surrounded by primary myocardium. During the looping process, the primary myocardium secretes a hyaluronan-rich gelatinous matrix, the cardiac jelly, forming swellings that project into the lumen at the levels of the atrioventricular junction and the outflow tract (OFT). A subset of myocardial cells lining these regions then secretes factors that activate the overlaying endocardium. These factors include members of the TGF-β family, including TFG-β1–3 and BMP 2,4. Studies on chick embryo show that during the embryonic developmental stage 14 (E9.0 in mouse), activated endocardial cells downregulate cell–cell contacts (PECAM1, NCAM1, DS-CAM) and upregulate cell–matrix adhesions (integrins)(13). Activated endocardial cells then change from a polygonal quiescent epithelial phenotype to spindle shaped migratory cells and begin to invade the hyaluronan-rich cardiac jelly matrix transforming into a mesenchymal phenotype characterized by the expression of α-smooth muscle actin (14). Continued expansion of this mesenchymal population creates the swellings that eventually form valvular and septal structures, dubbed ‘cushions’ owing to their appearance on the myocardial wall and apparent softness. The cushions extend into the lumen driven by increased mesenchymal cell proliferation and matrix deposition, and appose to help maintain unidirectional blood flow (4). Afterwards, cushion cells protrude from the myocardium to form thin and tapered leaflets made of one single endothelial layer. The

leaflet has a central matrix, which is full of collagen, elastin and glycosaminoglycans. These structures undergo extensive remodeling to become the semilunar and the atrioventricular valves(11) (figure 4).



Figure(4): Anatomic overview of heart valve development. During heart valve formation, a subset of endothelial cells overlying the future valve site are specified to delaminate, differentiate, and migrate into the cardiac jelly, a process referred to as endothelial-mesenchymal transformation or transdifferentiation (EMT)(90).

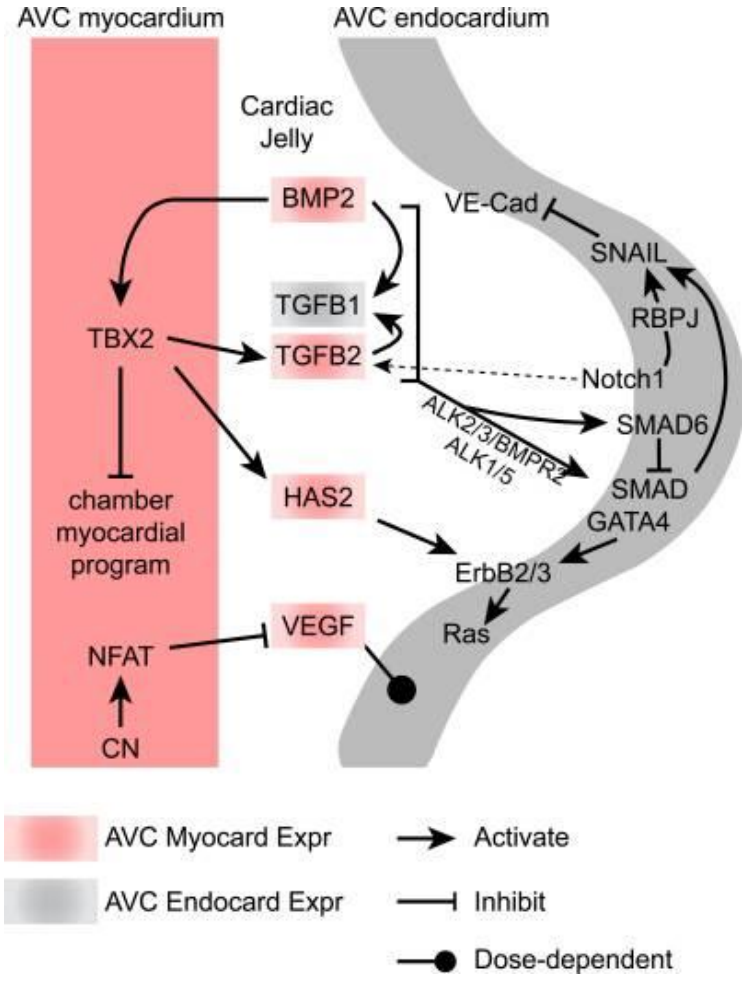
b. Molecular Regulation of Valvulogenesis

Most of the research studies investigating the molecular regulatory events in valvulogenesis focuses on the initial stage, which is during 'endocardial to mesenchymal transformation' or EMT. Numerous transcription factors, extracellular proteins and growth factors at various stages of valve formation have been identified. (6, 15,16).

i. Endocardial to Mesenchymal Transformation (EMT)

Initiation of mesenchymal transformation involves the coordination of numerous growth factor-mediated signal pathways originating from both myocardial and endocardial sources.

These include VEGF, BMP, TGF-β, EGF and NOTCH. One of the critical mediator of EMT is the vascular endothelial growth factor (VEGF), its isoforms are expressed in both the endocardium and myocardium of the AV and OFT valve forming regions in the mouse during the valve forming period (E9–E15) (17). Research from a number of groups has shown that VEGF expression required for proper EMT to occur remains elusive. While 50% reductions in VEGF expression are lethal at E9.5 in the mouse, two- to threefold exogenous overexpression of VEGF also results in severe cardiac abnormalities and death by E14 (18). The cellular source of VEGF is also a determinant of EMT. VEGF secreted from myocardium is an antagonist of EMT(19), and it is blocked by the transcriptional regulator NFATC1(Nuclear factor of activated T-cells cytoplasmic 1)(20). Deficient myocardial or endocardial NFATC1 expression results in hypoplastic cushions with limited EMT, and similar results occur by blocking calcineurin signaling. Experiments in zebrafish, however, show that VEGF receptor signaling is still required for EMT, and is associated with expression of NOTCH1 and BMP-4 (21)(figure 5). These results suggest that some basal level of VEGF expression from the endocardium is required for EMT, potentially to maintain endocardial integrity during the transformation process. VEGF acts on endocardium through its receptor Flk-1 to transmit calcium to calcineurin from the endoplasmic reticulum by the IP3 second messenger pathway. Calcineurin then phosphorylates NFATC1-mediated proliferation and PECAM1 (CD31) expression (22). Another critical source of calcium entry into this pathway is mediated through connexin 45 in endocardial cell contacts. The connexin 45 null mutants do not undergo EMT due to inhibited NFATC1 signaling (23) indicating the importance of NFATC1 in regulating EMT and consequently to the formation of the valve.



Figure(5): Molecular regulation of endocardial cushion EMT(10).

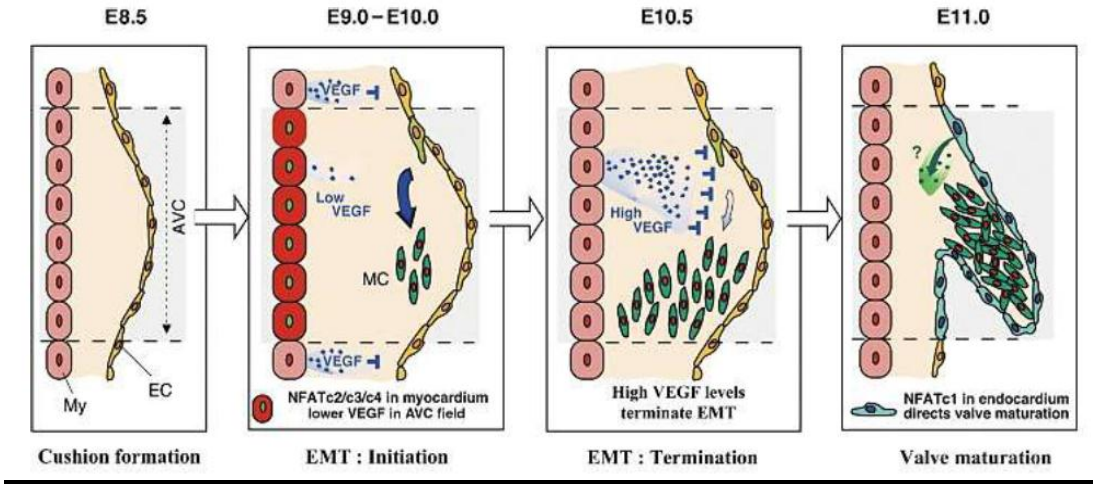
ii. Post-EMT cushion valvular maturation

The fully mesenchymalized cushions that act as primitive valves during early cardiogenesis will not contribute however to the mature valves. These are thin fibrous leaflets with largely fibroblastic cells entombed in a highly organized matrix. The process by which these cushions differentiate into valves is largely unknown, but recent mutant mouse models have revealed important signal pathways that are involved in this process.

Cells of the endocardial cushions, the only defined precursors of valves, elongate, differentiate and remodel their ECM to achieve their final fibrous structure.

Many of the same factors that initiate valvular formation are also involved in its termination, but cross-talk and feedback loops between these pathways are probably responsible for normal valvular maturation.

Many studies suggest that endocardially expressed VEGF may act through NFATC1 to induce endocardial proliferation necessary to accommodate the dramatically increasing leaflet surface area while inhibiting mesenchymal proliferation. VEGF expression is markedly increased in the endocardium and cushion mesenchyme after the onset of EMT in the E9.5 but it is expressed only in the endocardium by E14.5. In addition to its previously described roles, VEGF signaling induces endocardial/endothelial cell proliferation in adult valves mediated by activation of transcription factor NFATC1 (22). By using inhibitors of NFATC1 or calcineurin, it was shown there is a field of endocardial NFATC1 expression, which is necessary for proper elongation and condensation of cushion mesenchyme into thin leaflets, and blocking this expression results in persistent rounded hypercellular cushions (20). Coupled with the fact that high levels of VEGF expression correlate with a lack of mesenchymal transformation (24)(figure 6).



Figure(6): VEGF signaling functions via NFAT transcription factors to control valve morphogenesis (12).

C. Congenital Heart Disease

Congenital heart disease (CHD) is the primary cause of birth defects, and it's the second leading cause of deaths in the first year of life after the infectious etiologies. CHD affects over 1 out of every 100 live births(1). Additionally, about 3 to 4 per 1000 live births will be in need of an intervention in their first year of life (25).

As we described earlier, development of the heart is a very complicated process, so it is susceptible at all stages both to genetic and environmental factors that will contribute to cardiac anomalies. To the date, the etiology of CHD is still not fully understood; That's why it has been considered to be multifactorial, a combination of environmental factors and a genetic predisposition but recently an increasing numbers of specific genes are being identified as main contributor to CHD(26).

Congenital heart defects may be broadly grouped into two major categories:

(1) morphological abnormalities, including developmental defects resulting in structural malformations and (2) functional abnormalities, including cardiac rhythm disturbances and cardiomyopathies. VSD is the most common CHD, followed by atrial septal defect (ASD), and patent ductus arteriosus (PDA). Other cardiac malformations include transposition of the great vessels, truncus arteriosus, and coarctation of the aorta. (1)

Underlying the genetic basis for many of these defects remains elusive, mutations in genes encoding core cardiac transcription factors have been identified to be responsible for some defects in cardiac development.

1. Atrial Septal Defect:

Atrial septal defects (ASDs) are one of the most common form of CHD, it affects over 1 in 1000 live births, and often occurs in association with other congenital malformations specifically the valvular ones(25). The atrial septum is a heterogeneous structure separating two atrial chambers and forms through a complex series of events that is far more detailed than simple fusion of the septa primum and secundum(28). Deficiencies of any of these structures can lead to ASDs. Although ASD refers to a communication between the right and left atria, primum, secundum, or sinus venosus ASDs bear very few anatomic similarities outside of chamber communication.

Atrial septal defects can be anatomically classified into four categories: ostium secundum, which is a defect in the septum primum and it counts for 85% of all ASDs and 10% of all CHD, ostium primum which is a defect in septum secundum and counts for 10% of ASDs, sinus

venosus, which is a defect in right horn of sinus venosus and counts for 5% of ASDs, and coronary sinus , a rare defect in left horn of sinus venosus(29).

ASD can occur solely or associated with other congenital malformations mainly valvular ones. Most ASD cases are sporadic and few familial occurrences with isolated ASDs and defined dominant inheritance patterns were highlighted. A wide panel of transcription factors, structural proteins and receptors have been linked to sporadic and familial cases of ASDs(26). The regulatory panel of genes includes: *GATA4, NKX2.5, TBX5, GATA6, TLL1, MYH6, MYH7, ACTC1, ALK2* and many others (30,31).

D. Transcription factors involved in Septal and Valvular formation:

In the last several years, significant progress has been made in elucidating the transcriptional regulatory hierarchies that control septal and valve development .These studies demonstrate similarities in the regulatory interactions that control endocardial cushion mesenchyme proliferation and gene expression with other mesenchymal progenitor populations in the embryo. As shown in table 1,several transcription factors, that are expressed in mesenchymal valve progenitor cells have been identified like *Twist1, Tbx20, Msx1* and *Msx2* (32). Later stages of valve development share transcriptional regulatory mechanisms with development of osteoclasts, cartilage, and tendons, related to ECM remodeling and compartmentalization (33). NFATC1 is required in osteoclast differentiation, as well as in the transition from proliferation to remodeling of the valve primordia (34,35,36).In addition, Sox9, which is required for cartilage precursor differentiation, and scleraxis, important in the development of tendons, are critical for normal ECM compartmentalization in the developing valves

(37,33,38). Furthermore, PAX3's function is required for the cardiac neural crest to complete its migration to the developing heart and new studies suggest that PAX 6 as well is involved in cardiac progenitor cells differentiation.(15).

Gene	Known downstream targets expressed in valves	Function in valve progenitor/mesenchymal cell development
Twist1	*Periostin, Tbx20, Col2a1, cdh11*	Proliferation/migration/ECM organization/collagen production
Tbx20	*N-myc, Tbx2, Isl1*	Proliferation/differentiation
Msx1	None identified	EMT/cushion formation
Msx2	None identified	EMT/cushion formation
NFATc1	*NFATc1, DSCR1, CtsK*	Cushion endothelial cell proliferation/valve ECM remodeling
Sox9	*Aggrecan, Col2a1, Col11a2, Hapln1*	Proliferation/differentiation
Scx	*Col14a1, tenomodulin*	Differentiation/ECM organization

Table 1: Transcriptional factors associated with valve development(6).

1. NFAT Family

The NFAT (Nuclear Factor of Activated T-cells) family of transcription factors was first identified in the spotlight of T-cell biology, as an easily inducible nuclear factor regulating cytokine induction in T cells (42). They are DNA binding transcription factors that are evolutionary related to the Rel/ NF-kB family (39).Rel family has a highly conserved DNA binding domain, known as RHF (Rel homology domain)(43).

NFAT family consists of five members (NFATC1 to 5) in mammals, all located on different chromosomes with different function and expression (40).NFAT1-4 (or NFATC1-4) respond to calcium change inside the cell and subsequently act on regulating the transcription of

downstream target genes; however, NFAT5 is regulated by signals through integrin receptor and T-cell receptor and is responsive to osmotic stress (41)(table 2). Driven by distinct expression profiles, the resulting protein isoforms fulfill different regulatory functions in the cardiovascular, immune, musculoskeletal and nervous system.

NFAT family member	Alternative names	Regulation	Expression in the immune system
NFAT1	NFATc2 and NFATp	Calcium–calcineurin	Yes
NFAT2	NFATc1 and NFATc	Calcium–calcineurin	Yes
NFAT3	NFATc4	Calcium–calcineurin	No
NFAT4	NFATc3 and NFATx	Calcium–calcineurin	Yes
NFAT5	TonEBP and OREBP	Osmotic stress	Yes

Table 2: The NFAT Family: Members of the NFAT family, their alternative names, regulation, and expression in immune system(44).

The NFAT genes are conserved in their central regions and different at the 5' and 3' ends. In exon 5-7 there is an 80% conserved identity, and a 50 % for the NFAT gene as a whole. There are several alternatively spliced transcripts of NFAT gene in mouse and humans leading to different protein isoforms (Figure 8). Even though NFAT proteins have common regulatory roles in immune, skeletal, cardiovascular, musculoskeletal and nervous systems, their gene expression profile is rather distinct. All NFAT proteins, except NFATc4, are highly expressed in immune system, spleen and peripheral blood lymphocytes. Also NFAT proteins are found in the heart, brain, lung, testis(10).

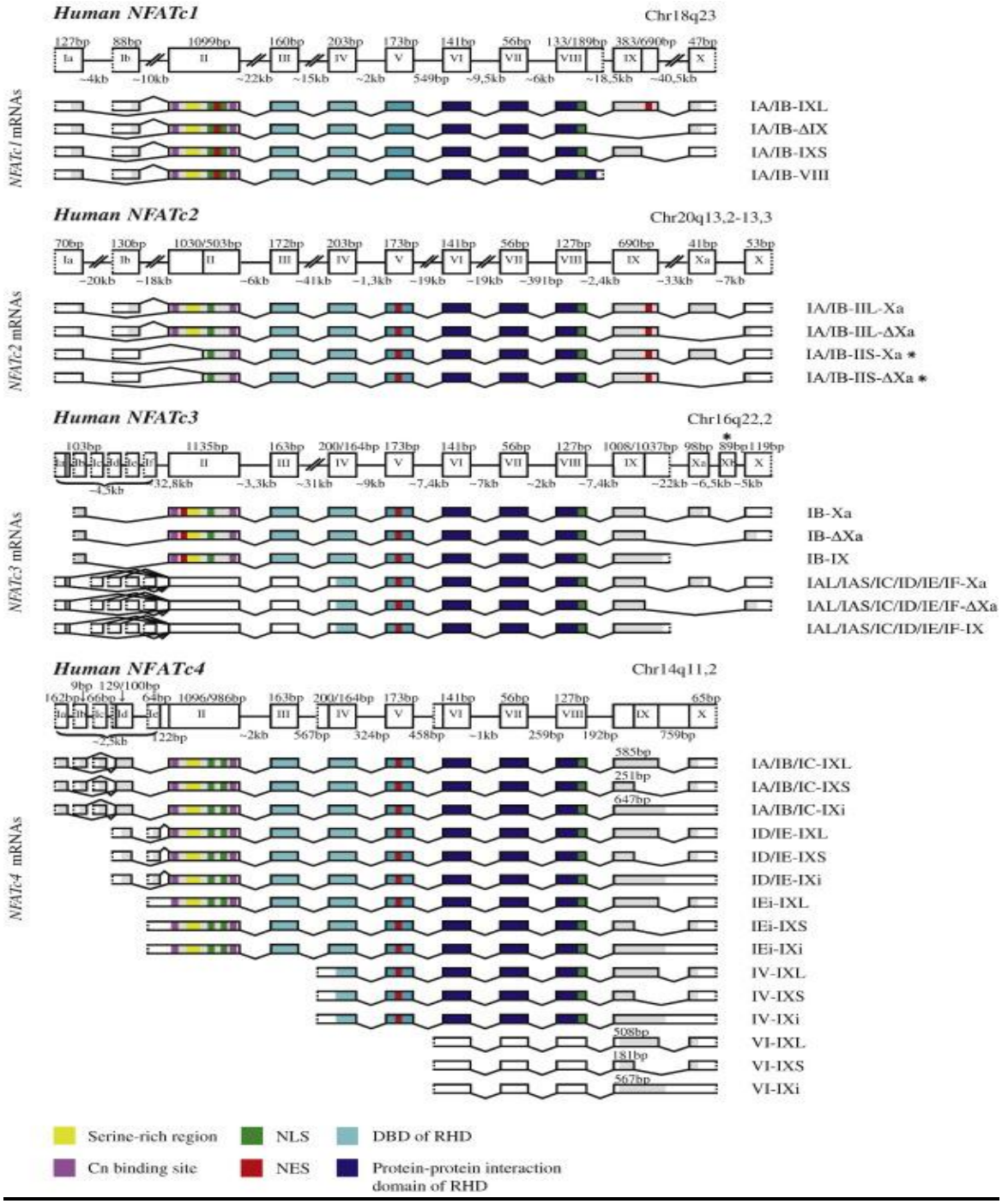


Figure (7): Chromosomal localization and alternative splicing of the NFAT family: illustrates the different isoforms of NFAT family generated by alternative splicing. It also shows the chromosomal localization and the domains of the NFAT family of transcription factors. NES, nuclear export signal; NLS, nuclear localization signal; DBD, DNA binding domain; RHD, Rel homology domain; Cn, calcineurin A.(10)

2. NFAT Domain Structure:

All the classical members of NFAT (NFAT 1-4) are consist of three functional domains, the Rel- homology domain (RHD), which is vital for DNA binding activity and also for cooperative binding with other transcription factors, the regulatory domain or the NFAT homology region (NHR), and the transactivation domain (TAD) (44).

The highly conserved DNA binding (Rel homology domain), which has been named due to the distant sequence similarity (~20%) with the Rel DNA binding domain of NF-kB proteins. NFAT binds with DNA in the major groove of 5` half site of the conserved NFAT recognition sequence 5` GGAAAA 3`. There is also another DNA binding site referred to as the Rel-insert region(RIR) that is about 20 amino acids, contacts the 3` half site at the minor groove. These two interactions are very crucial to stabilize the DNA-NFAT contact and allow the NFAT to bind promoter DNA as monomers, which NF-kB is incapable to do. The NFAT consensus recognition sequence is conserved in all the members of the NFAT family. This domain is also required for the binding of NFAT to other transcription factor partners (45).

The NFAT homology domain or the regulatory domain is about 320 amino acids and is located toward the amino terminal end to the NFAT protein. This domain also has the calcineurin docking site, which is highly conserved among all the NFAT family members.

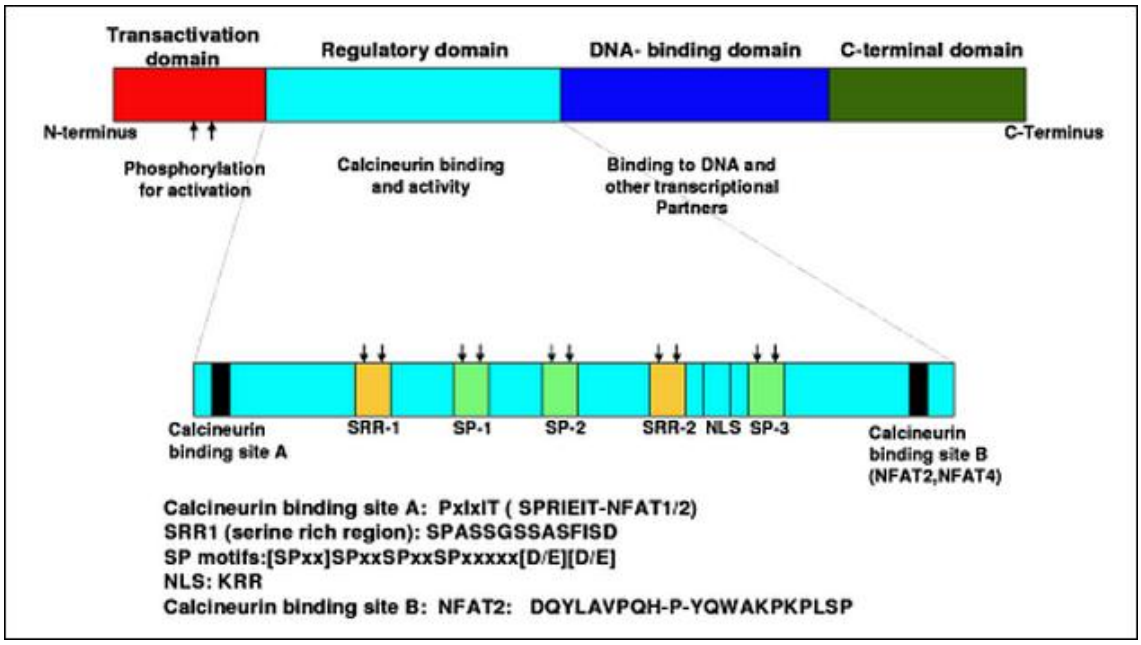


Figure (8): NFAT structure domains(51).

The cellular localization patterns of the NFAT protein is controlled by the reulatiory region that has the conserved nuclear localization sequence (NLS) present in the domain. This domain is very rich in serine residues and can be broken down into different conserved motifs (figure 9). The SRR(serine rich region) motif is essential to maintain NFAT proteins in the cytosol of resting cells, and a constitutive nuclear localization will result by the deletion of this motif (45).

The third important functional domain is the transcriptional activation domain. There are two transactivation domains in NFAT, one at the amino terminal end and the other at the carboxyl terminal end. The N- terminal transactivation domain has been considered to be more significant in determining gene regulation. It is identified to be highly acidic, it's highly susceptible to phosphorylation. This is very rich of serine and threonine residues that are phosphorylated by a host of proteins leading to transcriptional activation. (43)

3. Calcineurin Mediated Dephosphorylation of NFAT:

All known NFAT proteins are dependent upon cytosolic Ca^{+2} flux for nuclear translocation. Increased cytosolic Ca^{2+} leads to activation of calmodulin and ultimately calcineurin, which is a serine/threonine phosphatase.

NFAT proteins can be activated at three different steps: dephosphorylation , nuclear translocation, and DNA binding. In resting cells, NFAT proteins are phosphorylated and located completely in the cytoplasm(43). Ca^{2+}/calmodulin- dependent calcineurin activity regulates NFAT transcriptional activity. Calcineurin or PP2B, also known as protein phosphatase 2B, is a calcium-calmodulin dependent protein phosphatase formed by heterodimerization of two subunits A and B .Subunit A is catalytic and binds to calmodulin, whereas subunit B has four EF-hands, it binds calcium with high affinity and it is known to be the regulatory subunit. Calcineurin dephosphorylates the Ca^{2+} calcineurin sensitive subunits of NFATC, which are Serine-Rich Region (SRR) and Serine-Proline (SP) repeats at the N terminus of the NFAT protein (46). Upon dephopsphorylation of the NFAT, a conformational change of the NFAT will occur. Hence, exposing the nuclear localization sequence, which was previously inaccessible, and the DNA-binding region of NFAT. The dephosphorylated NFATs has higher affinity for DNA and binds precisely to the consensus (A/T) GGAAA sequence (48).

Calcineurin is not only responsible for NFATC dephosphorylation in the cytosol, but the presence of calcineurin is so vital in the nucleus for the full transcriptional activity of NFAT protein (49). Calcineurin has another important role to prevent the export of NFAT from the nucleus to the cytoplasm. In the absence of active calcineurin, NFAT is rapidly translocated

back into the cytoplasm within minutes (50). Cyclosporin A or FK506 are specific calcineurin inhibitors, they block the phosphatase activity of calcineurin and consequently inhibiting the nuclear translocation of Nfat protein(43).Consequently, NFAT proteins will be subject to vain cycling across the nuclear envelope. The activation ends when intracellular free Ca2+ decreases and calcineurin dissociates from the transcription factor, export prevails, thereby ceasing the activated receptor's signal.

Hence, the export of NFAT proteins out of the nucleus is highly regulated. Glycogen synthetase kinase 3 phosphorylates NFAT at the same sites that are dephosphorylated by calcineurin and promotes NFATC1 nuclear export. Thus, NFATC transcriptional activity is regulated at the protein level by a balance between its nuclear import and export, depending on the phosphorylation status (46,49).

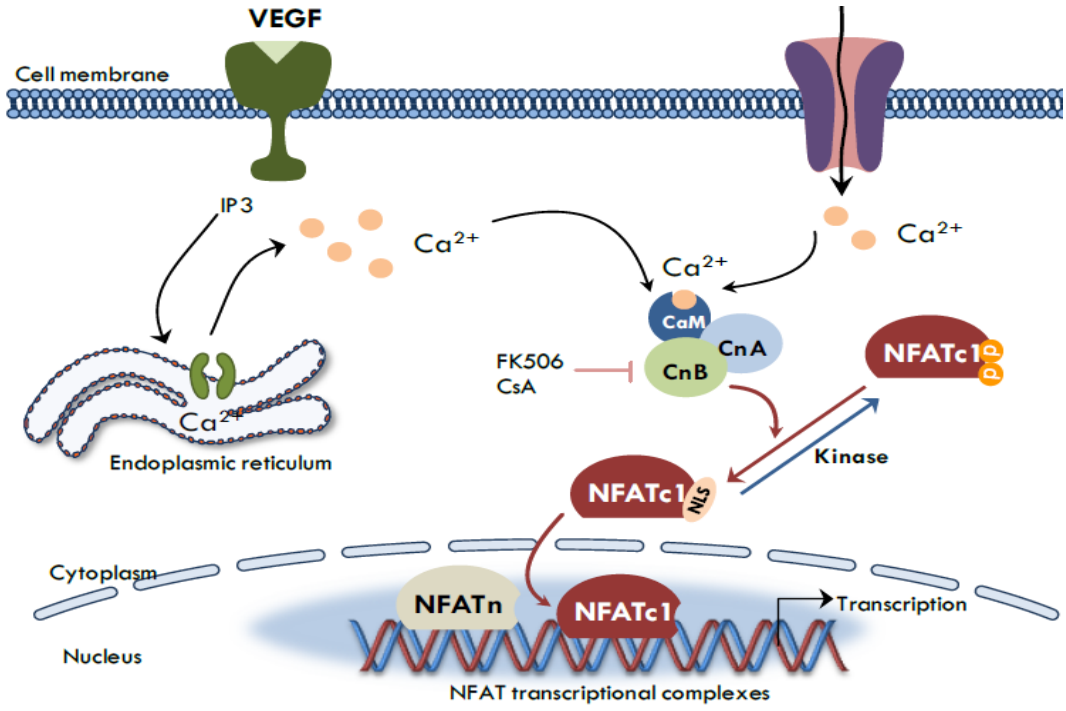


Figure (9): NFAT Signaling pathway(51).

4. NFATC1

Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATC1) also known as NFAT2, is a member of the NFAT family of transcription factors, first purified from bovine thymus and cloned later on. NFATC1 is a master transcriptional activator for homeostasis , and development of heart, brain, immune system and skeleton (34). It contains a Rel homology region DNA binding domain and is regulated by calcineurin.

NFATC1 gene is located on 18q23 spanning approximately 135kbp. It consists of 10 exons, out of the ten exons that make up the gene, only eight exons are coding ones. Exons 6, 7, and 8 are most important for encoding DNA binding domain, while exon 2 and 3 are essential for calcineurin interaction (calcineurin docking sites). Multiple proteins are generated as a result of alternative splicing, and the gene is transcribed into 10 potential spliced mRNAs. The biggest protein size is 930 amino acids. In human tissues, NFATC1 is expressed as two mRNAs of 4.7kb and 2.8 kb. The 2.8 kb is more confined to the muscle, thymus and peripheral blood leukocytes. Nevertheless, the 4.8kb isoform is highly expressed in skeletal muscles but also found in different tissues including thymus, spleen, heart, lung, prostate, testis, small intestine, colon and leukocytes(51) (Figure 11).

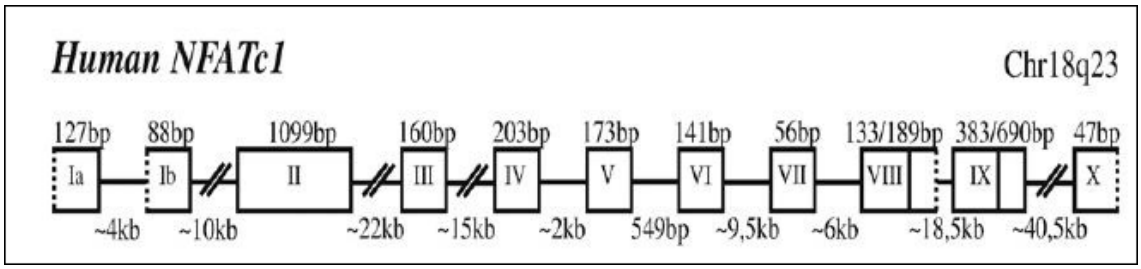


Figure (10): NFATC1 Gene: The picture illustrates the NFATC1 showing the 10 exons, 8 of which are translated (10).

NFATC1 is associated with regulating a broad spectrum of developmental events in the human body.NFATC1 autoregulation has been shown involved in cell-fate decisions in T-cell activation/expansion (51), osteoclastogenesis (50), and hair follicle stem cells (53) .In addition, during embryogenesis, NFATC1, regulated by calcineurin, plays a major role in the endocardial cell-fate decisions during valve development(34,50). It is exclusively expressed in the endocardial cushions; the heart valves and septa early precursors and the *bulbus cordis*.

Many studies done so far on murine embryos, have demonstrated the NFATC1 expression profile during the different stages of heart development. In mice, NFATC1 is found broadly expressed in embryonic heart from day E7.5 till E11.5, but eventually becomes limited to emerging valvular structure until the complete loss of expression at day E13.5. NFATC1 is not detected in adult murine valves (35, 54). "Knocked out" mice models and targeted gene deletions have enhanced our understanding of the prominent role of NFATC1 in valvuloseptal development. *Nfatc1* deficient murine embryos were characterized by defective aortic and pulmonary valves and died of congestive heart failure due to valvuloseptal and ventricular septal defects (35).

While other members of the NFATC family, such as NFATC3 and NFATC4, are expressed in the myocardium of the developing heart (20), NFATC1 is the NFATC family member restricted to the endocardium of the developing heart. Consistently, NFATC1 has a non-redundant function for valve development and embryonic survival, since disruption of the *Nfatc1* gene results in absence of semilunar valves and underdeveloped atrioventricular valves, and *Nfatc1* null embryos die around E13.5 of rapidly progressive heart failure(figure 12) (35, 36).Knockout of other *Nfatc* proteins, such as *Nfatc2*, c3, and c4, has no effect on valve development (39).Further studies have indicated that two waves of NFATC activities are required for valve formation in mice, one in E9.5 myocardium, *i.e.* NFATC3 and NFATC4, for initiation of EMT and the other, NFATC1, in E11.5 endocardium for valve elongation (20).

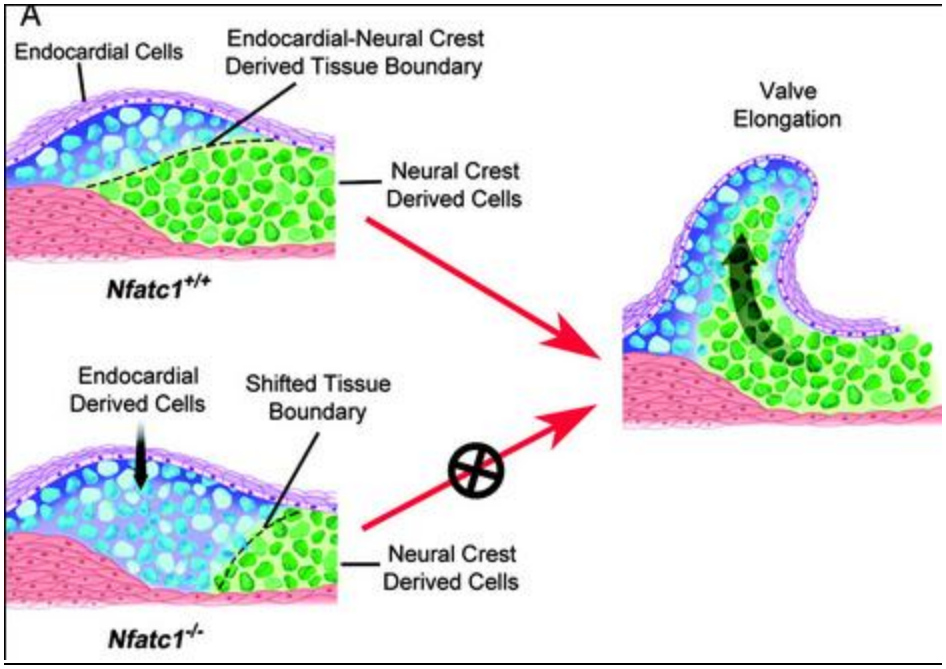


Figure (11): The role of NFATC1 in semilunar valve development(89).

In order to decipher the complex signaling mechanisms behind valve maturation, we have to consider not only NFATC1; other co-operators of transcription and growth factors including: VEGF, GATA5, calcineurin, Tbx5 and others should be considered.

5. VEGF: Vascular Endothelial Growth Factor (VEGF): Mediator of Heart Valve Development:

One of the powerful target for NFATC1 is the vascular endothelial growth factor (VEGF). VEGF has a very critical role in angiogenesis and for the development and differentiation of the vascular system (55).

The human VEGF belongs to the platelet-derived growth factor (PDGF) supergene family; it is located on chromosome 6p21.3 and spans around 14 kb (56). VEGF pre-mRNA is differentially spliced from eight exons to form mRNAs encoding at least six proteins. Due to the alternative mRNA splicing, a single gene is capable to rise several distinct isoforms of VEGF(figure 13), and they are different in their expression patterns as well as their biochemical and biological properties. Thus, The VEGF family consists of seven members that are common in theis VEGF homology domain: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF.

VEGF A is the major factor of angiogenesis. Two VEGF receptor tyrosine kinases (VEGFRs) have been identified, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (57).

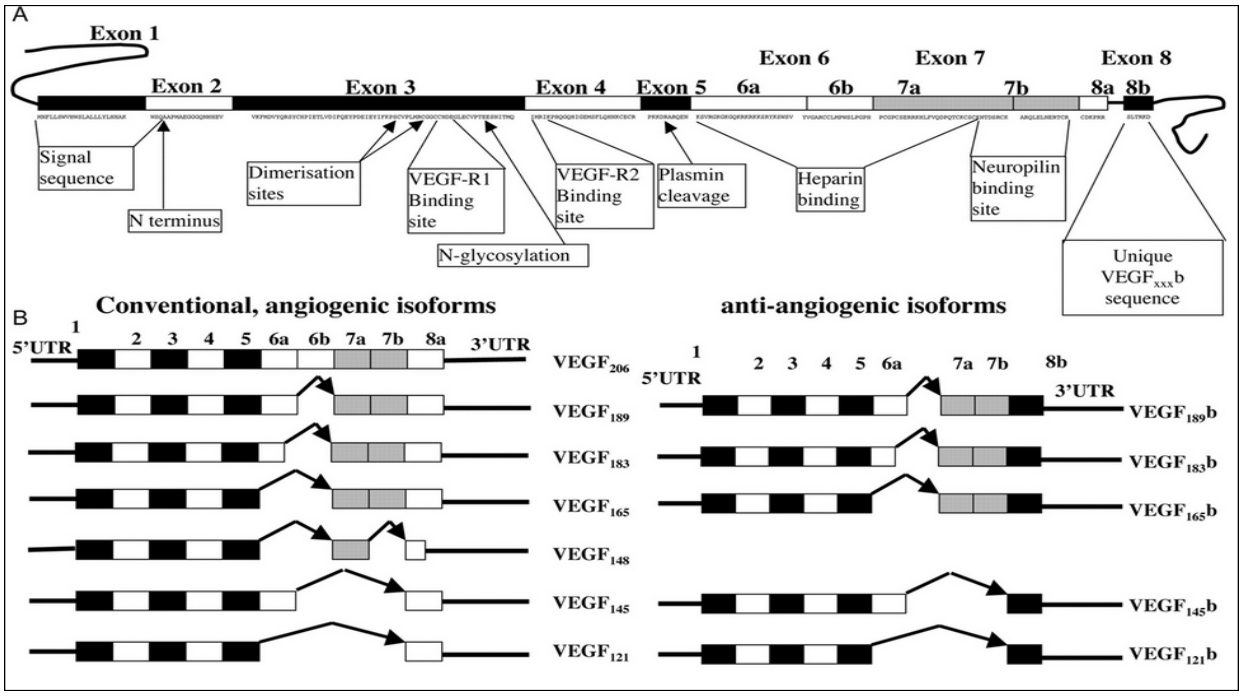


Figure (12): Exonic structure of the VEGF gene and identified splice variants of VEGF(54).

While being best known for its pro-angiogenic role, VEGF signaling is also implicated in the process of valve formation .At early stages of heart development, VEGF is broadly expressed in the entire heart tube. Before EMT proceeds, its expression is restricted to invasive endothelial regions at the AVC (Atrioventricular canal) and OFT, where it promotes the proliferation of endothelial subpopulations and the induction of inactivated endothelial entities in the cardiac cushions. Low levels of VEGF should be maintained in order to allow the progress of the epithelial-to mesenchymal transformation. Interestingly, studies have shown that minimal levels of VEGF are essential during EMT in order to compensate for the loss of the transformed endocardium. VEGF will predominate subsequently to terminate EMT and attenuate mesenchymal proliferation in order to prevent valve hyperplasia(12). Collectively, the proper regulation of VEGF expression is essential for normal EMT.

6. PAX Gene Family:

PAX genes play key roles in the formation of tissues and organs during embryogenesis. Nine mammalian PAX genes (PAX1 to PAX9) are identified, PAX transcription factors are defined by the presence of a highly conserved 128 amino acid DNA binding domain, the paired domain (58). The paired domain is a bipartite domain consisting of two independent subdomains: the amino-terminal PAI domain and the carboxy-terminal RED domain (59, 60). The bipartite paired domain recognizes a bipartite binding site of about 17 nucleotides (61). The PAI domain is generally more conserved and seems to be dominant over the RED domain in the intact protein, which might explain why all PAX proteins seem to interact with similar target sequences. Nevertheless, some differences in specificity have been noticed. Three amino acids (at positions 42, 44, and 47) within the PAI domain are responsible for the difference in the DNA-binding specificities between PAX2/5/8 and PAX6. The amino acids IQN at these positions specify the PAX6 class of transcription factors, whereas amino acids QRH determine PAX2/5/8 specificity (58).

7.PAX in the Heart:

Within the PAX gene family, the PAX 3 gene expression is notified in cardiac neural crest cells (NCC) in mouse embryo. Cardiac NCCs that populate the heart are generated from PAX3-expressing precursors (62). Mutation of *PAX3*, as occurs in $PAX3^{Splotch2H}$ homozygotes, will consequently lead to the development of cardiac anomalies like persistent truncus arteriosus (PTA), symptoms of cardiac failure, and defects

of the aortic arches, as well as to thymus, thyroid and parathyroid defects, eventually leading to embryonic lethality. Between 60% and 85% of *PAX3*^{Splotch2H}homozygotes die at E13.5–E14.5 (63), closely related *PAX3*^{Splotch} homozygotes also die by E13.5 with almost the same spectrum of cardiac defects. NC abnormalities in *PAX3*^{Splotch2H}homozygotes lead to defective excitation-contraction coupling, as referred by a drastic decrease in Ca^{2+} transients. There is a correlation between the presence of OFT septation defects (~60–85%) and defects in other NC lineages, such as reduction or absence of DRGs (63).

E. Eye Development:

The process of eye development is as critical as the process of valvuloseptal development. Heterogeneous and overlapping networks of transcription factors organize the formation of ocular structures at early stages of embryogenesis. PAX6, SOX9, TBX5, BMP4, and BMP7 are few of many candidate transcription factors involved in eye development. Delineating the molecular pathways underlying such biological process is essential in order to explain the genetic basis of many congenital eye defects.

Though the developing eye is believed to be highly specialized extension from the developing neural tube, the formation of major eye structure involves independent coordination of inductive interactions and regional specifications; formation of neural connections between retina and optic tectum; and maturation to a functional eye.

At the day 17th of gestation, the human eye starts to form. The middle layer of the blastoderm, known as the Mesoderm cells, and the outer layer of the blastoderm, referred as ectoderm,

form the eye fields in the neural area of the embryo. Optic vesicles develop in the eye fields and in five days, infold to form the optic cup. At this point, the retina and crystalline lens begin to develop. Meanwhile, a thickness is noticed on the surface of the ectoderm. The lens forms from these thickened cells. By the 32nd day, you can easily identify the lens, and during the next three to three and a half weeks the lens will grow to the size it will be at birth.

The vertebrate eye comprises tissues from different embryonic origins, the iris and the ciliary body are come from the wall of the diencephalon via optic vesicle and optic cup, however, the lens and cornea, derived from the overlying surface ectoderm. A group of transcription factors are dynamically expressed to ensure the correct development of the eye (67). One of the very essential genes for proper eye development is the PAX6, the studies and the analysis of the allelic series of the small eye mouse mutants revealed the PAX6 role during the eye formation. The interaction between the optic cup and the overlaying ectoderm involved the expression of PAX6. In human, mutations in the *PAX6* gene are responsible for aniridia and Peter's anomaly(68).

1. PAX 6: a master control gene for eye morphogenesis and evolution

PAX6 is a highly conserved member of a PAX family. Major sites of PAX 6 expression include the neural tube, the brain and the developing eye. PAX6 is strongly expressed in the ectoderm and neuroectoderm derived tissues of the eye, including the lens, the corneal epithelium and the rim of the optic cup (69, 70). PAX6 is a key transcription factor in lens development. PAX6 contains a paired domain and a second DNA-binding domain, the

paired-type homeobox at the carboxy-terminal end(71). In addition to eye development, PAX6 is also necessary for normal development of the nose, pancreas and the central nervous system.(72,73). PAX6 is first expressed in a broad region of the head ectoderm and later becomes restricted to the presumptive lens placode.(74)

PAX6 contains an internal, paired-type homeodomain (HD), allowing different modes of PAX6 binding to DNA. Two forms of PAX6 proteins are identified in mammals, PAX6 and PAX6(5a). PAX6 has a canonical PD. In contrast, PAX6(5a) contains a 14 amino acid insertion within the PAI domain (figure 14). It has been shown by many studies that PAX6 is crucial for morphogenesis of the eye from its earliest stages and consequent formation of all major ocular tissues(75).

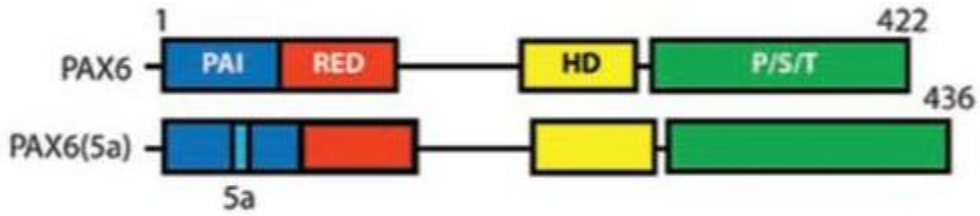


Figure (13): Structure of PAX6 and PAX6(5a) and position of human mutations. PAX6 PD, HD (*yellow*); and transcriptional activation domain (P/S/T, *green*); and subdivision of PD into the PAI (*blue*) and RED (*red*) subdomains. Oligopeptide of 14 amino acid residues (*light blue*) encoded by exon 5a disrupts DNA-binding property of the PD(61).

In humans, a different types of ocular defects can be caused by heterozygous mutations in *PAX6* and as well as subtle changes can occur in the olfactory epithelium and brain (76). Mutations generating truncated PAX6 proteins and deletions of one allele typically result in aniridia. The prominent feature of aniridia is iris hypoplasia, often combined with cataracts, glaucoma, nystagmus, and foveal and optic nerve hypoplasia(77). Missense mutations generating single amino acid substitutions, representing approximately 10% of total

mutations, cause less severe abnormalities (e.g., foveal hypoplasia, Peters' anomaly, congenital cataracts, and autosomal dominant keratitis)(78).A rare case of a human *PAX6* compound heterozygote resulted in anophthalmia and lethal brain defects. Hence, it has been proposed that a *PAX6* gene dosage effect is responsible for phenotypes associated with mutations in one or both alleles of *PAX6*.(79)

2. ***VEGF and Cn/NFATC1 in the eye:***

One of the evolutionary conserved developmental pathway is VEGF - mediated Cn/NFATC1 signaling. The effect of this pathway is well elucidated in cardiac development. Similarly, this pathway has been under intense investigation in the ophthalmic field.

Many pathologic angiogenic cases of retinal dystrophies, neovascular glaucoma, and retinal vascular occlusions, have a profound link with this pathway (61).

VEGF and calcineurin-dependent NFATC1 signaling plays a key role in regulating angiogenic cell behaviours. VEGF can be produced and secreted by minimum five retinal cell types. These include the retinal pigmented epithelium (RPE), astrocytes, Müller cells, vascular endothelium and ganglion cells. Among all the growth factors associated with retinal angiogenesis, VEGF is suggested to be the potent regulator of NFATC1, which is known to act downstream of VEGF. Studies have shown that NFATC1 signaling exerts a pro-angiogenic activity in human retinal microvascular endothelial cells (HRMEC) and shows great sensitivity to FK-506 and ICNA6 inhibitors in both *in vivo* rat and *in vitro* HRMEC models (80).

Calcineurin has also been associated to retinal disorders, especially to pathologic cases of retinal ganglion cells degeneration that lead to glaucoma. Calcineurin is expressed in the retina, brain, and immune cells and known to be implicated in neuronal degenerative and apoptosis. Clinical and basic research studies have reported the observation of a constitutively active truncated form of calcineurin in glaucomic models of mouse and rats with elevated intraocular pressure (IOP) and that FK-506 inhibitor treatment was neuroprotective (81)

It is evident now, that most of our knowledge about the role of VEGF dependent Cn/NFATC1 pathway is weaved by weak genetic extrapolations to pathologic ocular phenotypes seen in research models. More studies need to be done to investigate the downstream targets of VEGF-mediated Cn/NFATC1 signaling to define the molecular genetics of the early stages of eye development.

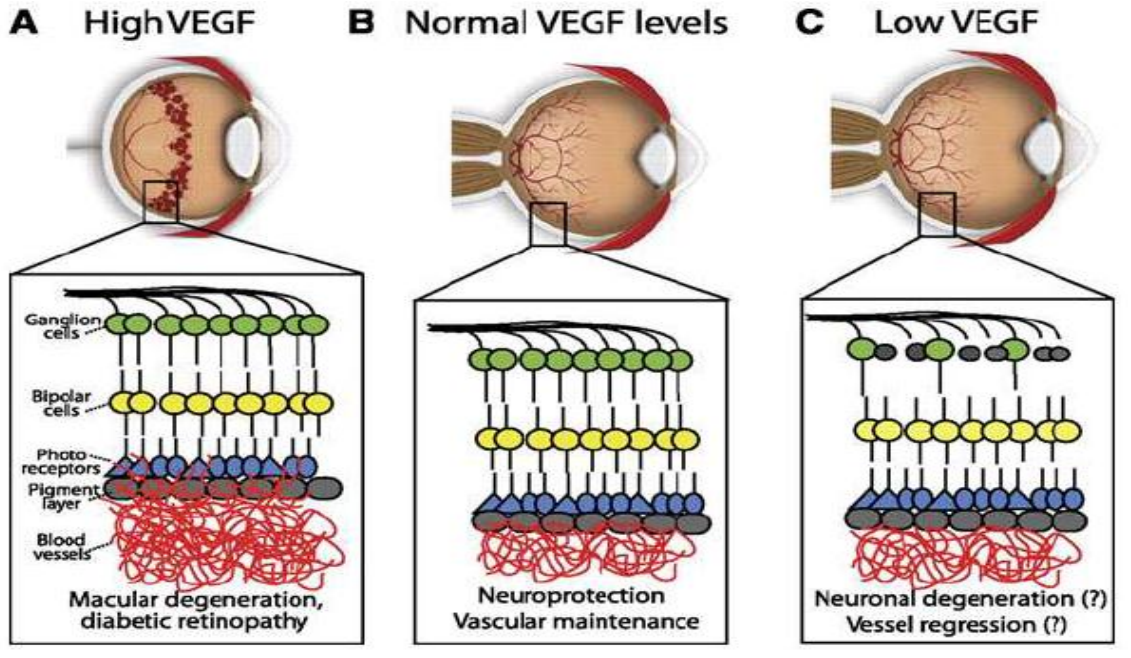


Figure (14): Effect of variable levels of VEGF on retinal integrity(80).

F. Congenital Eye Defect:

Visual loss in infants can be either prenatal or postnatal. All ocular and non-ocular malformations affecting the different tissues and entities in the anterior segment of the eye such as: the cornea, iris, optic cup, and retinal extensions, that become apparent at birth or shortly after, are set under the heading of congenital eye defects. The most common congenital eye defects include: anophthalmos, microphthalmos, and coloboma; congenital cataract, retinal dystrophies such as Leber's congenital amaurosis, infantile glaucoma, and congenital cloudy cornea. In the perinatal period, the following conditions can happen: cortical impairment from birth asphyxia, ophthalmia neonatorum, and retinopathy of prematurity.

Congenital eye defects occur relatively frequently in humans. Worldwide, 20 million children under the age of 16 suffer from cataracts and 1.4 million are blind 1 (68).

Many mutations have been shown to be related to congenital eye defect. The heterogeneous and overlapping expression of transcription factors during embryonic eye development makes it difficult to define a clear genetic rationale behind congenital eye malformations. Studies on animal models revealed many causative genes that cause eye malformations such as:*PITX2, PITX3, PAX6,MAF,FOXC1,LMX1B and CYP1B1*. Mutations in PAX 6 has been reported to have a significant role in developing aniridia and Peter's anomaly can be a result of mutations in the *PAX6* gene(68).

G.Aims of the Study:

Previous works from our lab have highlighted many transcription factor pathways known to be important for normal heart development and discuss how abnormalities in these pathways have been linked to morphological and functional forms of congenital heart defects. Based on the previous results from the work done in our lab, have confirmed a pivotal role of NFATC1 in heart valve formation. Genomic alterations in the NFATC1 locus have been identified in patients with CHD (83) and recently, heterozygous mutations in NFATC1 have been identified in a patient with tricuspid atresia (84).

Our aims are

1) Functionally characterization a novel point mutation (P68L) of NFATC1 at three different levels:

- Translocation of NFATC1 to nucleus.
- Transcriptional Regulation
- Interaction with other transcription factors.

We hypothesize that this novel P68L mutation in the NFATC1 gene is disease causing and CHD contributing. We suggest that the mutation will distort the expression of VEGF and disrupt the physical interaction of NFATC1 protein with calcineurin.

2) To understand how to previously documented V210M *NFATC1* mutant affects the heart and the ocular development process. We hypothesize a physical interaction between NFATC1 and transcription factor PAX6 that has been considered as a master gene in eye development.

CHAPTER II

MATERIALS AND METHODS

A. Site–Directed Mutagenes

After identifying each mutant gene sequence, oligonucleotides encoding for the desired mutation were annealed to one strand of the human NFATC1 gene cloned in thepCEP4 plasmid (Invitrogen). These nucleotides were considered as the forward primer. The second primer was designed in a way that it starts from the same start site of the first primer but extends to the opposite direction. This choice of primers ensures the proper mutant product is formed. Primers were then phosphorylatedand PCR was performed using Site-Directed Mutagenesis kit from FINNZYMES (product code: F-541).

B. Plasmids

Several plasmids harboring the genes of interests were used, the full cDNA of the NFATC1 wt and mutant were cloned the pCEP4 plasmid (Invitrogen). With a FLAG Tag.PAX 6 plasmid was supplied from Dr. Ales Cvekl. NFATC1 mutant and wild type cDNA were sub cloned into pCGN vector with HA Tags.

C. Transformation and cloning of constructs in bacteria

The previously obtained constructs are then transformed into E.coli, XL1 blue strain: bacteria stored at -80°C are obtained; in brief 1-2 µg of the plasmids containing our DNA constructs are added to 100 µL of bacteria in eppendorf tubes. The eppendorf tube is then placed 2 minutes on ice, 5 minutes at 37°C(in water bath) then 2 minutes on ice. The transformed bacteria are placed on ampicillin agar plates and streaked, and then they are incubated overnight at 37°C. A single bacterial colony incorporating the desired plasmid is removed with pipette tips, and is then transferred into 15-mL falcon tubes containing 3 mL liquid broth. Minipreps and maxipreps are performed using illustraTM plasmidPrep Midi Flow Kit by GE Healthcare according to the enclosed manufacturer's protocol.

D. Plasmid Construction

1. Digestion
2. Ligation
3. Transformation in bacteria.

E. Cell Lines

HEK293 cells (Human Embryonic Kidney cells) and Hela cells (Human cervical cancer cells) were cultured and maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented

with 10% Fetal Bovine Serum (PAA) (FBS), 1% Penicillin/ Streptomycin and 1% Sodium pyruvate. Incubation was carried out in a humid atmosphere 5% CO2 and 95% air at 37ºC.

F. Transient Transfections:

HeLa and HEK 293 cells were plated 24 hours prior to transfection and transfected with reporter (VEGF) and expression vectors (NFATC1 wild type and mutants, activated calcineurin, PAX 6 and PAX6-5a). Transfection methods are used for a range of applications, including regulation of gene expression, gene function studies, production of recombinant proteins and mutational analysis.

G. Polyethyleneimine (PEI) transfection

Hela and HEK293 cells are plated in 12-well Costar culture plates on cover slips 24 hours prior to transfection. The cells are transfected using the Polyethyleneimine (PEI) transfecting reagent, a commonly used synthetic polycation for gene delivery inside the cells. 5µg of DNA per well are added to 300ul serum free media, 12µl of PEI are then added to the mixture. The mixture is incubated at 32 ºC for 30 minutes on a thermomixer and then kept for 20 minutes at room temperature. The mixture will be added drop by drop over the culture milieu. Three hours post transfection, fresh media is added. After 24 hours, the media is replaced again and cells are ready to be functionally analysed by 36 hours.

H. Luciferase assay

To assay for the transcriptional regulation of VEGF promoter by NFATC1 (wild type and mutant) and/or PAX6 and/or PAX6-5a, Hela and HEK293T cells were transfected with VEGF /Luc as well as NFATC1 protein (Wt, P68L) and/or calcineurin (Cn) and/or PAX6 and/or PAX6-5a (using the PEI method described earlier). Controls were transfected with VEGF/Luc only. The luciferase activity is measured 36 hours post transfection. The transfected cells are first rinsed with PBS 1x and solubilized by 1x 40 lysis buffer and left on the shaker for 30 minutes at room temperature. The cell lysate is then transferred into a 96 well plate (Costar) to which luciferin is added. Luciferin (Promega, Cat # E 1501) is prepared according to the manufacturer's protocol. The signal is read immediately using the Ascent Fluoroscan and the fold activation was calculated by comparing it with that of the reporter alone, and error bars refer to the standard deviation of fold activation. The results are expressed as fold activation and the presented values are the mean +/- standard deviation of three independent experiments carried out in duplicates.

I. Immunofluorescence

Hela cells were plated in 12-well Costar culture plates on cover slips with 100,000 cells/well. Transfections were done on the second day of plating using polyethylenimine (PEI) transfecting reagent. PEI is a synthetic polycation that is commonly used for gene delivery both in vitro and in vivo and which allows high-efficiency nucleic acid delivery based on endocytosis to carry DNA into cells. Immunofluorescence was performed on transfected Hela cells. The cells were first washed for 1 times with PBS 1X (phosphate buffered saline). Then

cells were fixed with 4 % p-formaldehyde for 30 minutes; after washing with PBS, the cells were blocked with 3% BSA/PBT (bovine serum albumin/ phosphate buffer saline Tween) for 1 hour. The primary antibodies Mouse anti-flag (Flag m2 from sigma Aldrich) and rabbit anti-HA (santa Cruz) were used for assessment of subcellular localization of calcineurin , NFATC1 (Wt and mutant), PAX 6 and PAX6 -5a. The primary antibodies were diluted (1:400) in BSA/PBT and added to the cells with an overnight incubation at 4ºC. The cells were then washed in PBT 3 times, and the secondary antibody goat anti-mouse biotinylated or donkey anti-rabbit biotinylated (GE Healthcare) were diluted 1:500 in BSA/PBT. They were added to the cells for 1 hour at RT with shaking. After washing 3 times with PBT, cells were incubated with Streptavidin Texas Red or Alexa fluor (anti-mouse or anti-rabbit) for 1 hour at RT with shaking. Hoechst staining for the nucleus was also performed by applying Hoechst, diluted 1:30 in water, to the cells for 30 minutes. The cells were then washed with PBT and mounted on a circular slide containing an antifading agent (DABCO). The slides were examined using the Olympus BH-2 microscope and captured at x20. The nuclear versus cytoplasmic staining was conducted on three independent experiments. HA-probe (Y=11): sc-805, Oct A- probe (H-5): sc-166355, Anti-rabbit IgG- biotinylated (donkey) RPN1004V, Anti-mouse Ig biotinylated (sheep) RPN1001V1, Alexa Flour 488 goat anti-rabbit A11008, Chromeo 642 Streptavidin : sc-364698.

J. Protein Overexpression

Over expression experiments were performed using PEI transfection. HEK293T cells are plated in 100mm culture plates (Corning) with 70% confluency. 24 hours post seeding, 20µg of DNA is added to 1ml of serum free media and vortexed for
20 seconds. 35 µl of PEI is then added to the mixture to be incubated for 20 minutes at room temperature and then added gently over the culture milieu. 3 hours post transfection, the media is replaced. The cells will be kept for 24 hours and media will be changed after that. Nuclear or whole-cell extraction is to be done 36 hours post transfection.

K. Nuclear Protein Extraction

Nuclear protein extracts from HEK293 cells were obtained according to the following protocol. The cells were first washed with 1X PBS. Then 2 mL of 1X PBS and 20-40 µL of EDTA (chelating agent) were added to the petri dishes to detach the cells. The petri dishes were then placed on the shaker for 10-15 minutes, to allow the detachment of the cells, and then the cells are collected in eppendorf tubes and centrifuged for 90 seconds at 11000rpm (fixed-angle rotor). The supernatant is discarded, and the pellet is resuspended in 800 µL of buffer A (10mM Tris pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.5mM PMSF, 0.5mM DTT and 0.5mM protease inhibitors cocktail) with 1µL DTT (1M reducing agent), 2µL protease inhibitor (cocktail inhibitors) and 1µL PMSF (0.5 M). The contents are mixed by inversion, and then the tubes are placed on ice for 15 minutes. Then, 50 µL of NP40 10% per tube is added, vortexed, and then centrifuged for 90 seconds at maximum speed. A transparent pellet is obtained; the supernatant is carefully discarded, and the pellet is

resuspended in 200 µL buffer C (20mM Tris pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 0.5mM PMSF, 0.5mM DTT and 0.5mM protease inhibitors cocktail). The tubes are then placed on the shaker in a cold room (T=4ºC) for 20 minutes, after which the tubes are centrifuged for 90 seconds at maximum speed, the pellet is discarded, and 30 µL aliquots of the obtained supernatant are prepared, to be stored at -80ºC for future use.

L. Protein Quantification

Protein concentration was measured using a colorimetric assay is based on Lowry protocol (Bio-Rad DC protein assay kit), according to the manufacturer's protocol.

M. SDS-PAGE and Western blotting

Equal amounts of nuclear cell extracts (10 µg protein) were resuspended in 5X laemmlli buffer (1mL glycerol, 0.5mL βME, 3mL 10% SDS, 1.25mL 1M Tris pH6.7 and 2mg bromophenol blue). The samples were then boiled for 3 minutes and run on a denaturing SDS-PAGE(12%) for 1.5 hours then transferred to a Polyvinylidenedifluoride membrane PVDF membrane (Amersham). The membrane was then blocked in 5% non-fat dry milk (klim)at Tris-buffered saline (TBS) 1X for 45 minutes shaking at room temperature.After blocking, the membrane is incubated overnight with primary antibody diluted 1:1000 in 1 % non-fat dry milk at 4ºC with shaking. After being washed three times for 5 min in TBST (TBS, 0.05% Tween 20), the membranes were incubated with the correspondin secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase-conjugated. The membrane was

then incubated with the secondary antibody conjugated with horseradish-peroxidase, Anti-mouse-HRP, diluted 1:40000. Revelation was done using the Western Lightening Chemiluminescence Kit (Perkin Elmer, Cat # NEL 103). The protein bands were visualized by autoradiography.

N. Co-immunoprecipitation assays

Co-immunoprecipitation (Co-IP) of HA- NFATC1 (Wt and mutant) and endogenous Flag-PAX 6 and PAX6- 5a were performed using nuclear extracts of HEK293T cells overexpressing the required proteins. Co-IP reactions were done using ten times the quantity of nuclear extracts loaded in western blot. The beads (Dynabeads® Co- IP Kit (Invitrogen, Oslo, Norway) were captured on a magnetic stand (Invitrogen) and resuspended gently with PBS (1x + 0.001% of Tween 20). After that, the beads were incubated with mouse anti-flag (Santa Cruz) for 1 hour at 4ºC on a rotating platform. The nuclear proteins were then incubated with the antibody complexed beads for 2 hours at room temperature on a rotating platform. The mixture is washed for 2 times with PBS (1x) and proteins were eluted with (1x) SDS. Western blotting was performed with rabbit anti- HA(Santa Cruz), essentially as previously described. Membranes were stripped using a stripping buffer (1M Tris, SDS (10%), β mercaptoethanol) for 30 minutes at 55 ºC in a thermo rotator. The membranes were washed for 3 times with TBT and blocked with 5% non-fatty milk. After that, membranes were incubated overnight with antibody rabbit anti-HA (Santa Cruz) at 4 ºC, washed 3 times with TBT and then incubated with anti-rabbit horseradish

peroxidase-conjugated antibody for 1 hour at room temperature.

O. Statistical Analysis

All data are presented as mean ± standard error. Two-tailed unpaired Student's t tests and ANOVA were used for statistical evaluation of the data. SP SS 18 statistical tool was used for data analysis. A p-value < 0.05 was considered significant.

CHAPTER III

RESULTS

The mutation in the NFATC1 gene will be designated according to the site of the mutation and the change in amino acid. The normal NFATC1will be designated as Wt, while the mutants are designated as P68L,P68T, V210M and P66L. The activated form of calcineurin will be designated as Cn.

A. Case Study

Given the crucial role of NFATC1 in valve development, we screened for mutations in patients with different valvular diseases registered at the congenital heart disease genetic program at the American University of Beirut Medical Center. A next generation sequencing was conducted by Dr.Akl Fahed to sequence all the coding exons (exons 1 to 8) of NFATC1 gene. We found a novel SNP mutation (P68L) in exon 2 of the NFATC1 gene in a patient with an atrial septal defect (ASD),this heterozygote mutation is leading to the substitution of proline with leucine at position 68. These novel mutation was not reported in 150.000 unrelated individuals (ExAC Browser).

B. SNP Prediction Tool:

As shown by the Polyphen - 2software that the P68L mutant is a desease causing mutation nin NFATC1, but the P68T is found to be a benign mutation as it's found in a normal population(Table 3).

The result was as the following:

1.P68L

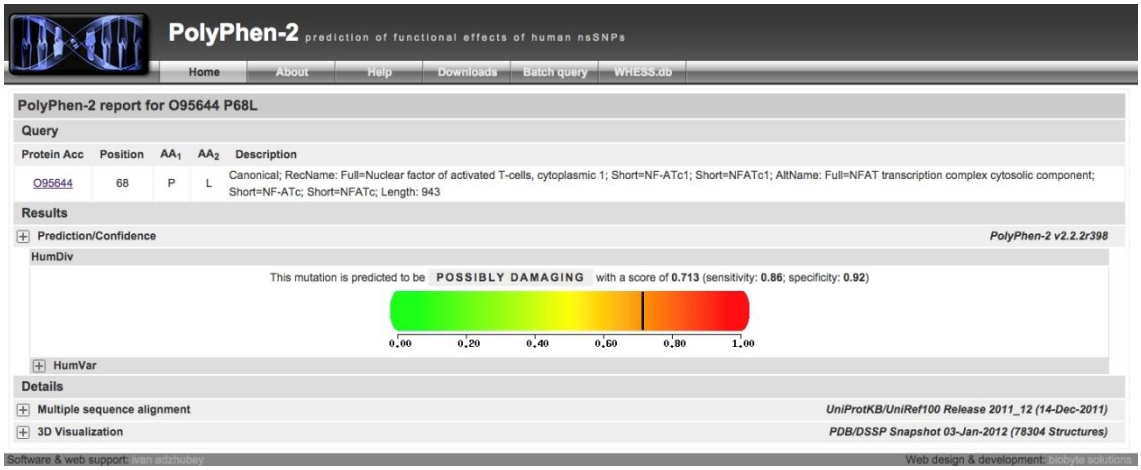
Chr_pos	chr18:77170478
VAR	T
SNP	.
REF	C
QUAL	11706.63
AC	1
AF	0.025
aa Name	p.P68L
Exon Number	2
Gene	NFATC1
vartype	coding-missense
varname	**c.203C>T**
_1KGall.AF	.
EA_AC	0,3000
AA_AC	0,3000
TAC	0,6000
MAF	0,0,0
ID	DH-CHD420
Phenotype	ASD
AD	281,326

2.P68T

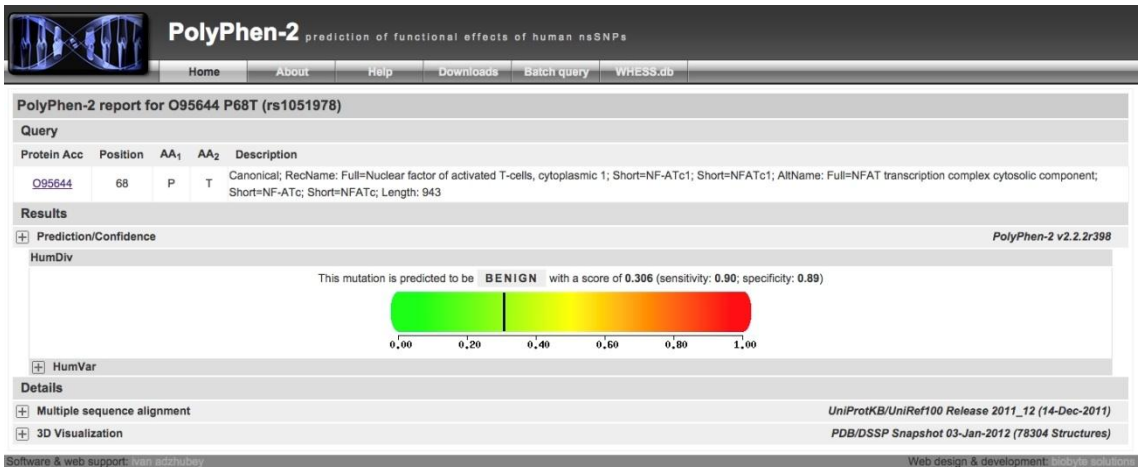
Chr_pos	chr18:77170477					
VAR	A					
SNP	rs1051978					
REF	C					
QUAL	18609.89					
AC	22					
AF	0.0803					
aa Name	p.P68T					
Exon Number	2					
Gene	NFATC1					
vartype	coding-missense					
varname	**c.202C>A**					
_1KGall.AF	0.033					
EA_AC	757,6259					
AA_AC	90,3648					
TAC	847,9907					
MAF	10.7896,2.4077,7.8761					
ID	DH-CHD320	CHD-L17	CHD-L21	CHD-L24	CHD-L28	CHD-L4
Phenotype	VSD PA	CTL	CTL	CTL	CTL	CTL
AD	110,22	312,288	107,149	146,130	125,146	45,40

Table(3): P68L and P68T next generation sequencing results. AC: Allele count ; AF: Allele frequency.

P68L:



P68T:



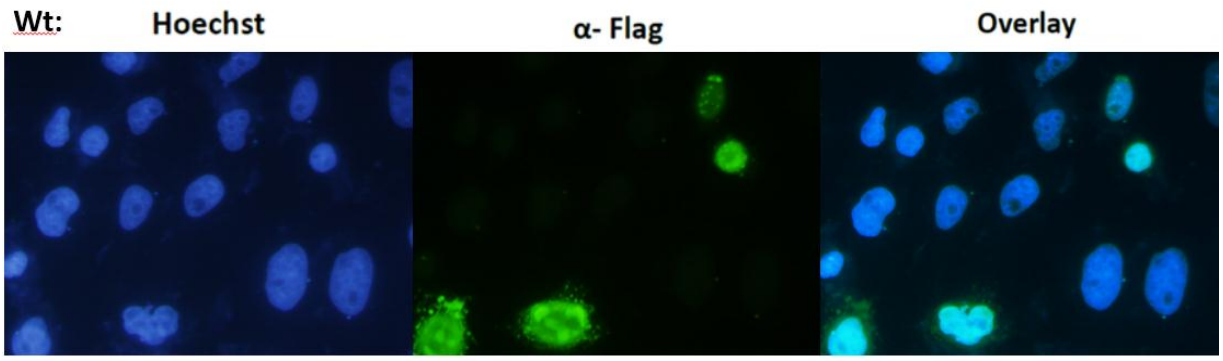
Figure(15): The in silico predictions done by the Polyphen - 2software for the P68L and P68T.

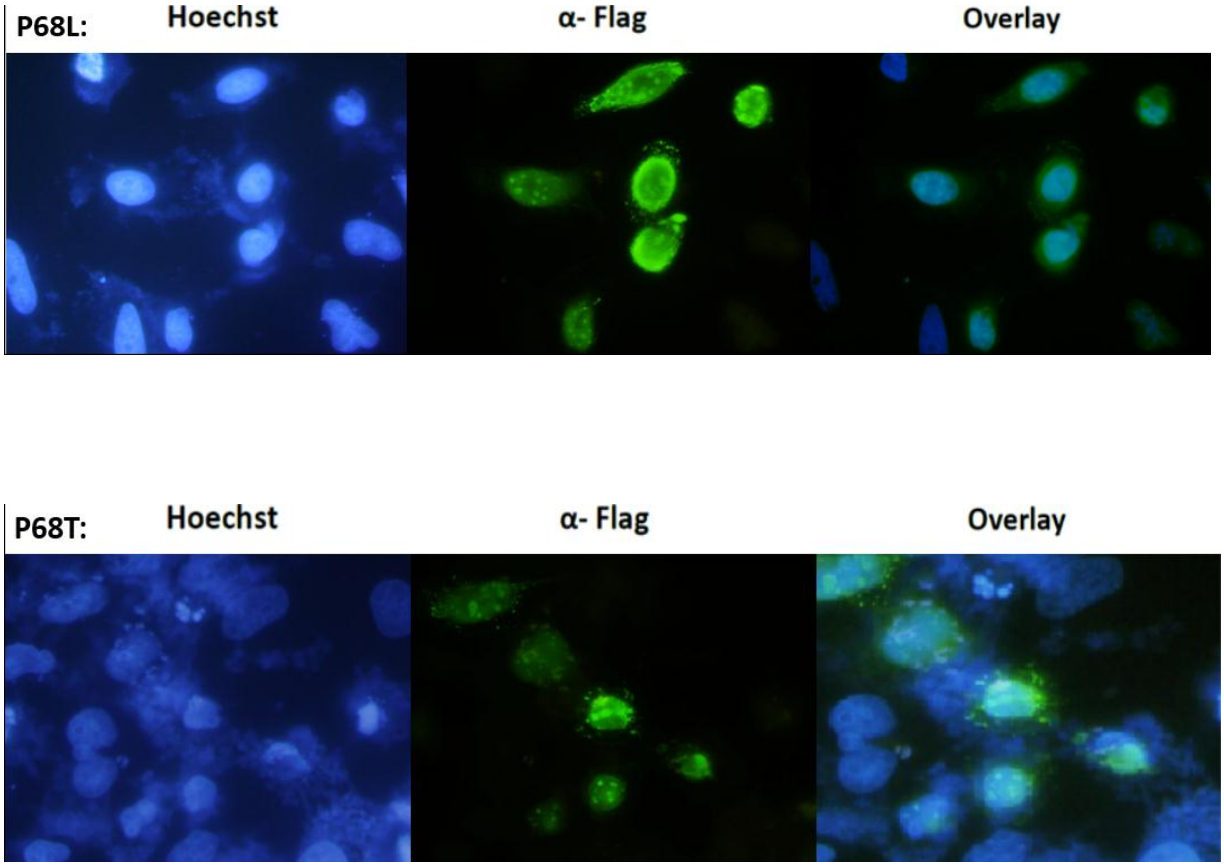
C. Cellular Localization of NFATC1 protein

To investigate the effect of the mutations in NFATC1 gene on the translocation of the protein into the nucleus, immunofluorescence was performed on HeLa cells. Wild type NFATC1 and mutants were transfected into HeLa cells with or without calcineurin.

When cells were transfected without calcineurin, NFATC1was found to be located in the cytoplasm and the nucleus. Mutants P68T and P68L (Figure 16) were also found to be located in the cytoplasm. Calcineurin, as expected, was localized inside the cytoplasm (Figure17). When cells were transfected with calcineurin and NFATC1, we used two different antibodies to be able to differentiate between the two proteins. All NFATC1 mutants were able to translocate to the nucleus but the translocation efficiency in the mutants were less compared to the wild-type Wt NFATC1 (Figure18).

1. ***NFATC1 Wt ,P68L and P68T***





Figure(16): Cellular localization of NFATC1 proteins in HeLa cells via immunofluorescence (NFATC1 alone) Wt, P68L and P68T were found to be located in the cytoplasm as compared to the Hoechst stain. The localization of NFATC1 ,P68L and P68T proteins was visualized using anti-Flag antibody (green color). Nuclei were visualized using the Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x60.

2. Calcineurin

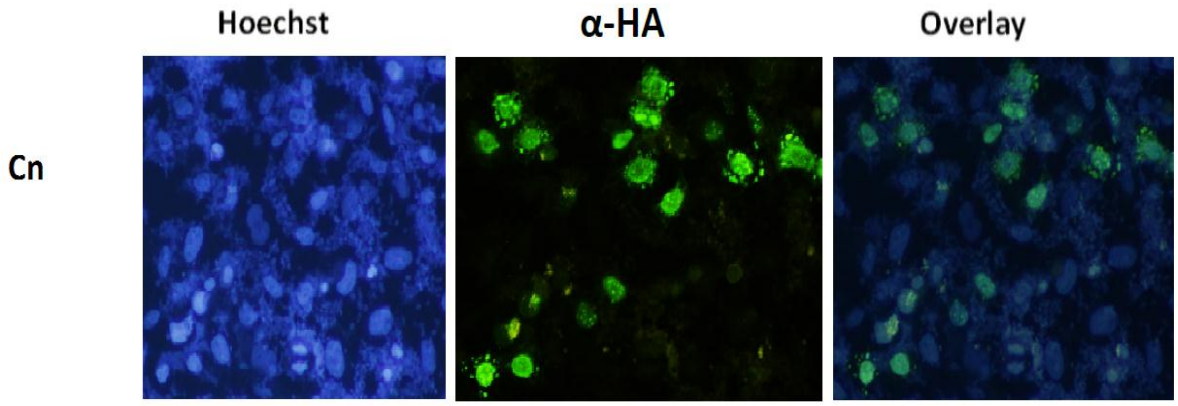
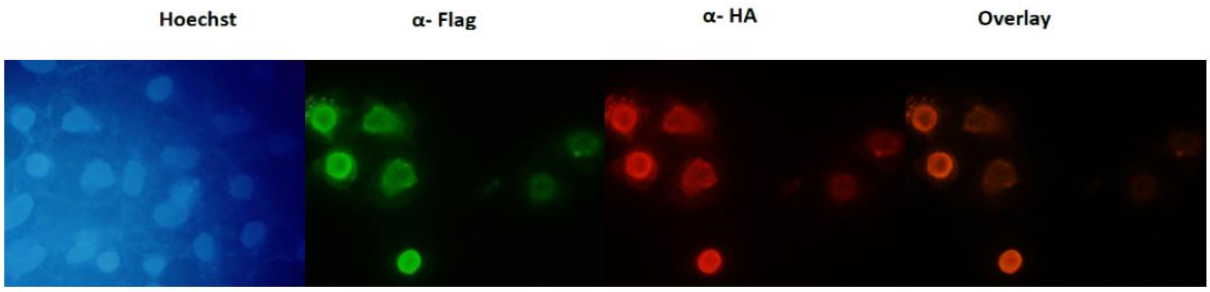


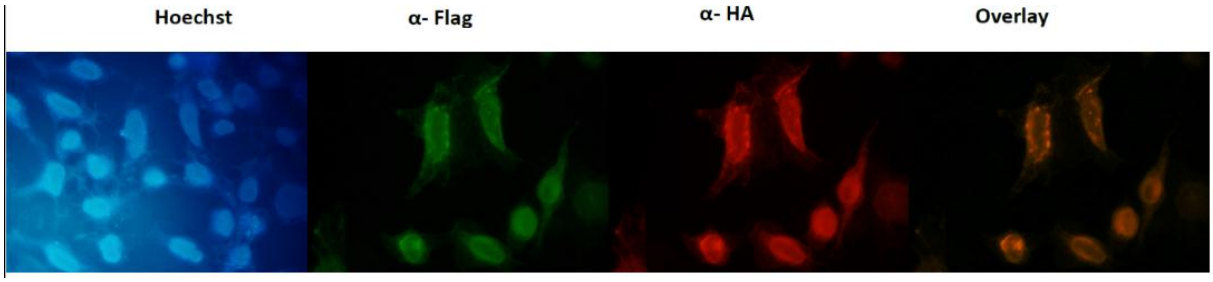
Figure (17): Cellular localization of calcineurin proteins in HeLa cells via immunofluorescence. Cn **(**Calcineurin) was found to be located in the cytoplasm as compared to the Hoechst stain.The localization of calcineurin was visualized using anti-HA antibody (green color). Nuclei were visualized using the Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

3. NFATC1 and Calcineurin

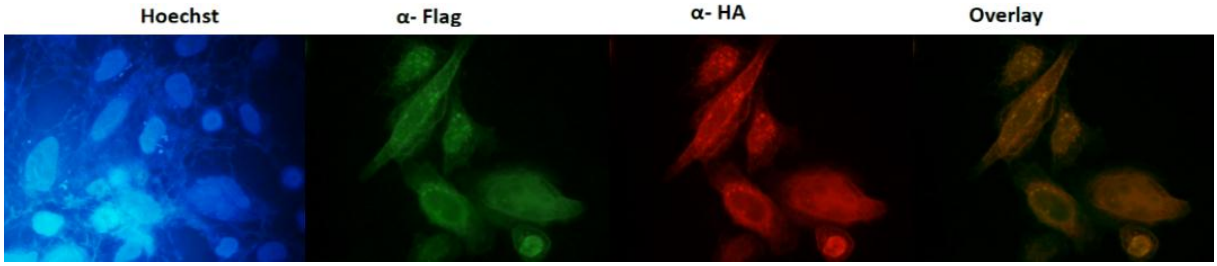
Wt:



P68L:



P68T:



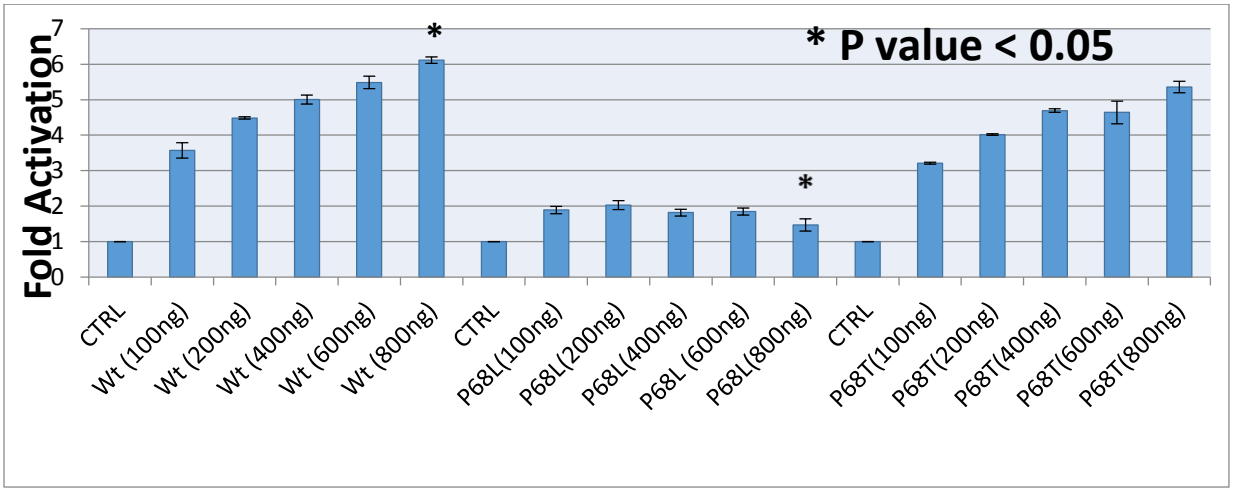
Figure(18): Cellular localization of NFATC1 proteins and calcineurin in HeLa cells via immunofluorescence Wt co-transfected with calcineurin were found to be located in the nuleus as compared to the Hoechst stain. P68L and P68T co-transfected with calcineurin was found to be mainly in the cytoplasm compared to the wild trpe.The localization of NFATC1 ,P68L and P68T proteins was visualized using anti-Flag antibody (green color). Calcineurin was visualized using anti-HA antibody(red).Nuclei were visualized using the Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x60

D. Regulation of NFATC1 Gene Expression:

The vascular endothelial growth factor (VEGF) is a potent regulatory target for NFATC1. Bioinformatics tools were used to determine the potential NFATC1 binding sites on the VEGF promoter. Conserved NFAT binding sites was found in the mouse 1.5 Kb VEGF promoters.

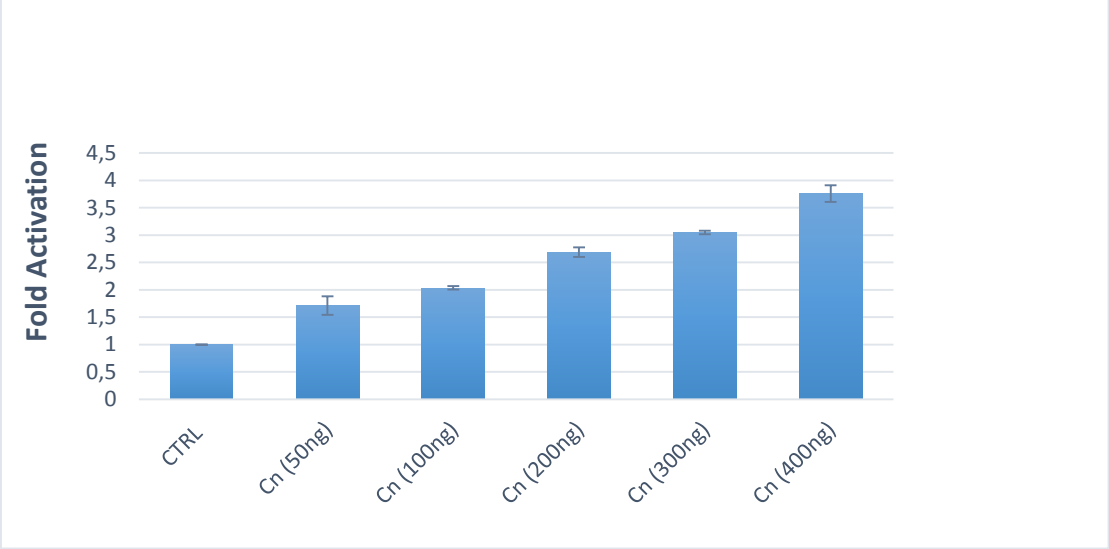
In order to assess the impact of the mutation on the regulatory function of NFATC1 protein, transactivation assays were done. HeLa cells were transiently co-transfected with 1.5 µg of the 1.5kb promoter (VEGF/luc) /well and increasing concentrations of NFATC1 Wt , P68T and P68L mutant with or without activated calcineurin.

1.NFATC1



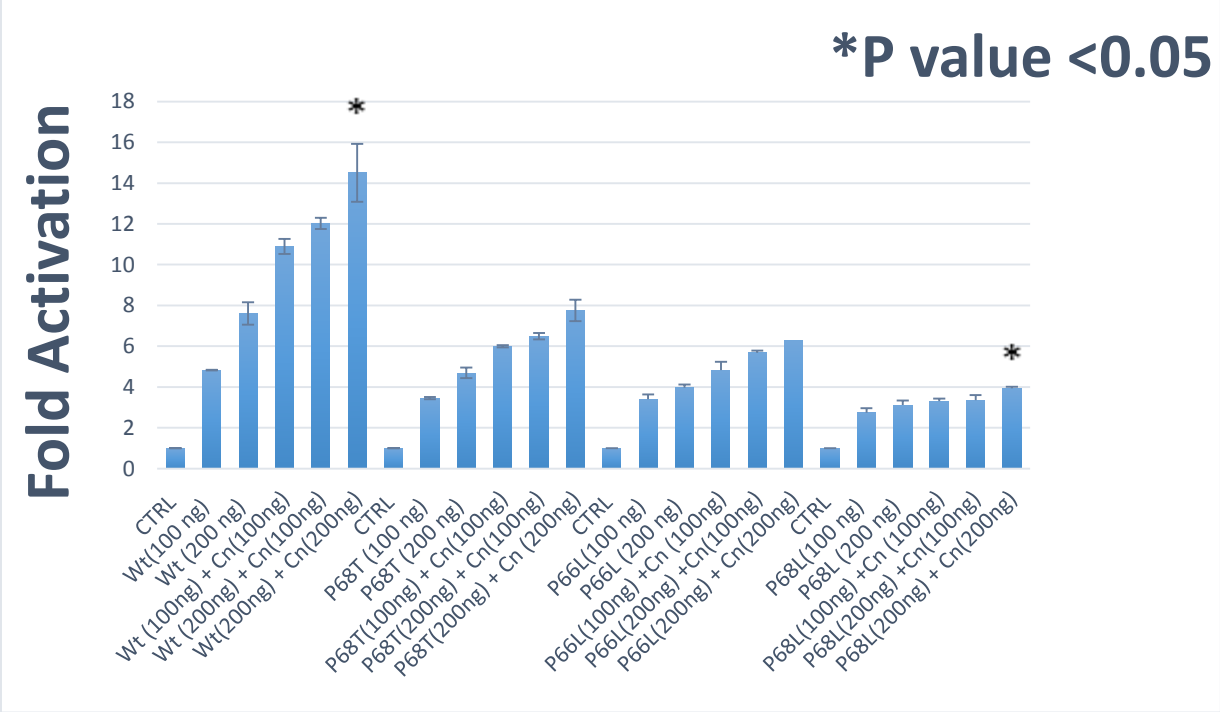
Figure(19):Transcriptional Activity of NFATC1 Wt and mutants (P68L , P68T) on VEGF promoter. NFATC1 Wt , P68L mutant and P68T mutant (100ng, 200ng, 400ng, 600ng and 800ng) dose response.

2.Calcineurin



Figure(20) : Transcriptional regulation by different doses of Calcineurin proteins in HeLa cells (VEGF/luc): Hek cells were transfected with increasing doses of calcineurin

3.NFATC1 and Calcineurin



Figure(21)**: Transcriptional regulation by different NFATC1 proteins in HeLa cells (VEGF/Luc)**: HeLa cells were transfected with 1.5µg of (VEGF/luc)/well and increasing concentration of NFATC1 wild-type and mutants with or without Cn. After cell lysis, luciferase activity was assayed.

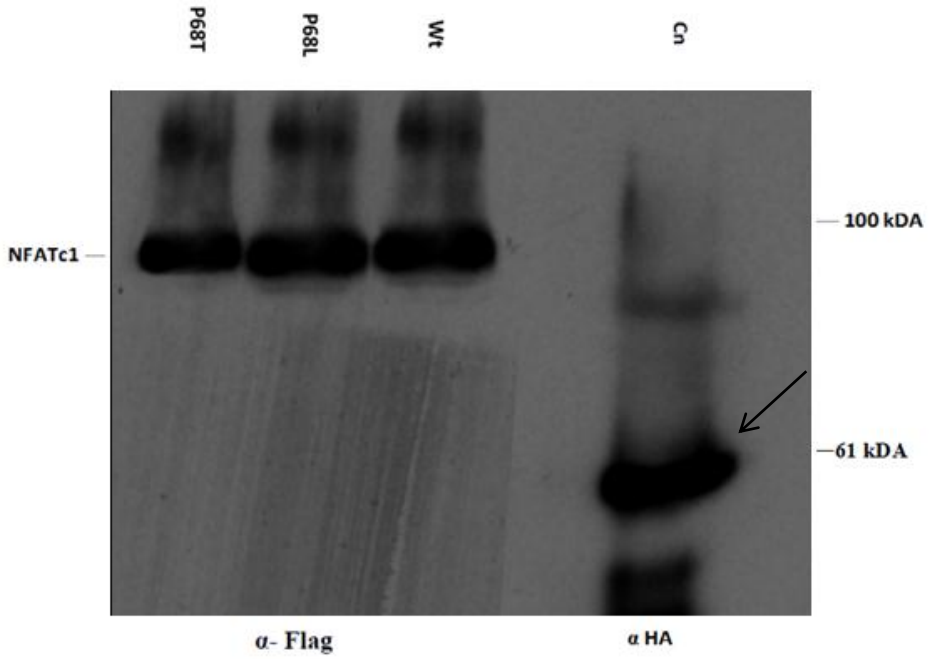
Luciferase assay was done to assess the effect of the P68L mutation on the transcriptional regulation of VEGF. Calcineurin alone induced a dose-dependent increase in transcriptional activity with a maximum of 3.7 folds. The results showed that NFATC1 Wt is a relatively weak activator of the VEGF promoter with a maximum fold increase of 6.1 fold. Upon co-transfection with calcineurin (200ng), the activation of VEGF promoter activity increased reaching around 14.5 folds. Interestingly, P68L mutant showed a declined transcriptional activity with a maximum fold increase of 2.2 and when this mutant was co-transfected with calcineurin, the activation was about 3.9 folds compared to 14.5 fold caused by the Wt NFATC1.

The results are represented as fold actication over the VEGF promoter transfected with the empty vector, The experiments are represented at least 3 times in duplicates.

E. Protein-Protein Interaction

Western blotting was performed to confirm the presence of our over-expressed proteins and to relatively assess equal concentrations of the proteins to be used. As expected, the obtained proteins were found to be over-expressed by the cells.

The obtained protein extracts (nuclear extracts) were used to perform co-immunopercipitation assay.



Figure(22): Western Blot of NFATC1 and calcineurin extracts. NFATC1 (Wt and Mutants P68L and P68T) and calcineurin extracts from HEK293 cells were resolved on an SDS-PAGE prior to co-immunoprecipitation. Western blot showed equal amounts of overexpressed proteins as visualized by anti- α-Flag and anti-HA antibodies. Nuclear extracts from mocked transfected HEK293 cells are referred to as Ctrl.The arrow in the figure refers to the calcineurin.

As shown in figure (24) the overexpressed NFATC1 proteins were aligned to bands of the same size, and thus this confirms that the mutations that occurred did not affect the size of the coding sequence, and no aberrations or extensions arose.

Furthermore, the physical interaction between NFATC1 (Wt , P68L and P68T proteins) and activated form of calcineurin was assessed *in vitro* using CoIP. As expected, NFATC1 Wt interacts physically with calcineurin. Interestingly, the P68L protein showed a drastic decrease in physical interaction with calcineurin of around 80% less ineraction compared to NFATC1 Wt. A decrease of almost 75% was observed in NFATC1 mutant P68T physical interaction with calcinurin compared to NFATC1 Wt.(Figure 25).

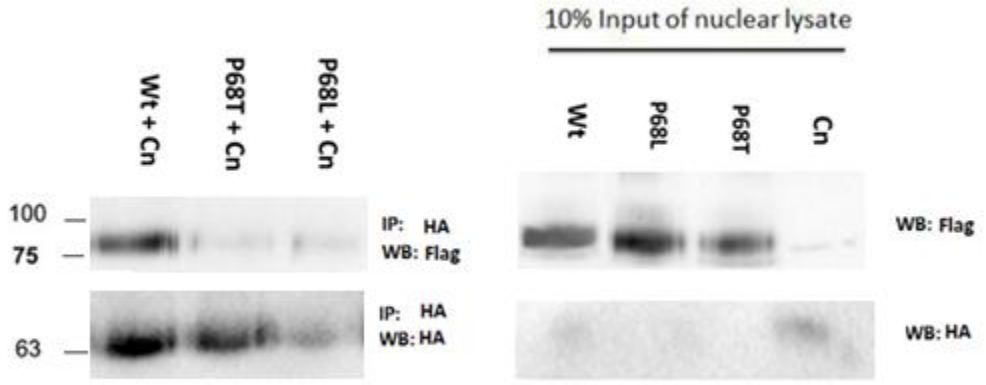
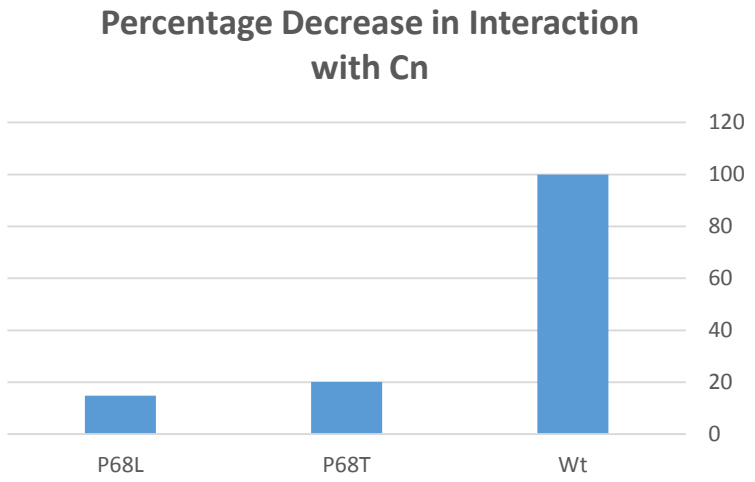


Figure (23): Co-immunoprecipitation assay showing the physical interaction between NFATC1 and calcineurin. Physical interaction between Flag-tagged NFATC1 (Wt,P68L and P68T mutants) and HA-tagged calcineurin is demonstrated. Ten times the quantity of proteins loaded for western blot was used for immunoprecipitation. Nuclear lysates of NFATC1/Cn complexes were immunoprecipitated with HA- antibody and NFATC1 proteins were visualized with western blot via α-Flag antibody. Membrane stripping and subsequent western blot analysis was performed with HA- antibody in order to detect calcineurin proteins. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD).



Figure(24): A percentage decrease in interaction of NFATC1 mutants with calcineurin.

F. NFATC1 and PAX

In order to assess the subcellular localization of PAX 6 and PAX6-5a,immunofluorescence was performed on HeLa cells. PAX 6 and PAX6-5a were transfected into Hela cells. (Figure 28). To understand , where the direct physical interaction is taking place, we co-transfected PAX6 with Wt and V210M. The results showed that the interaction was happening in the nucleus (Figure 29).The same reults were obtained for PAX6-5a.

1. Cellular Localization of PAX6 Proteins:

a. PAX 6 and PAX6-5a :

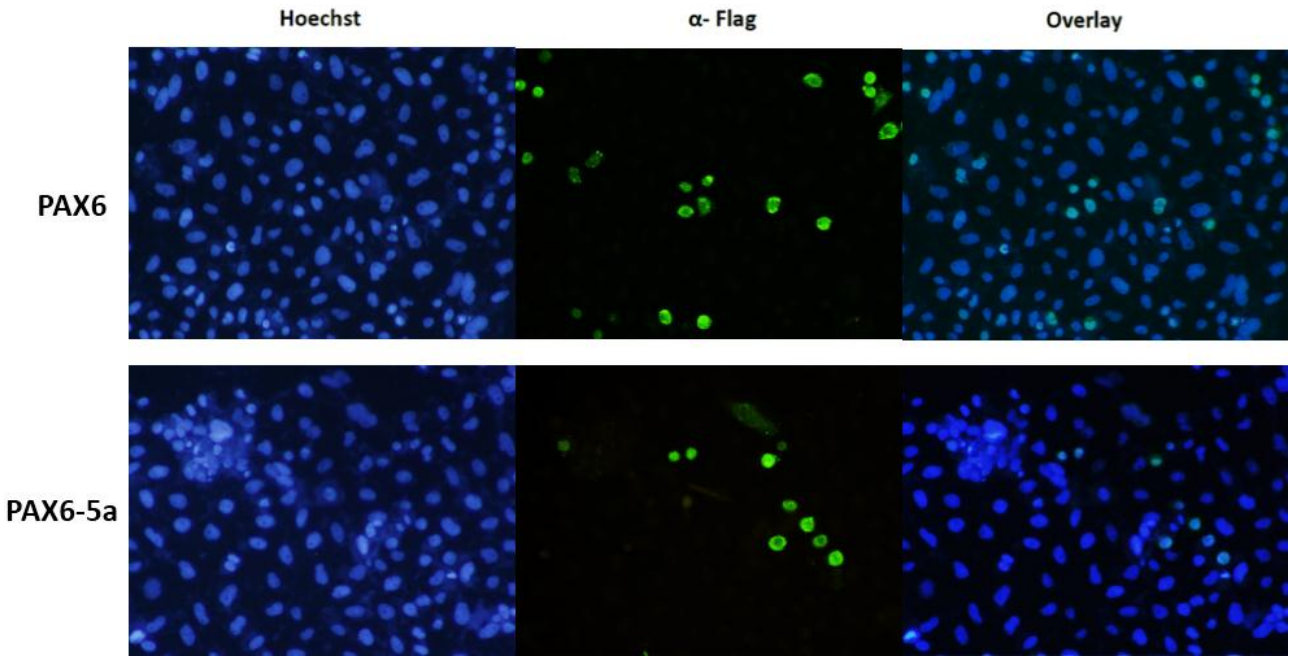
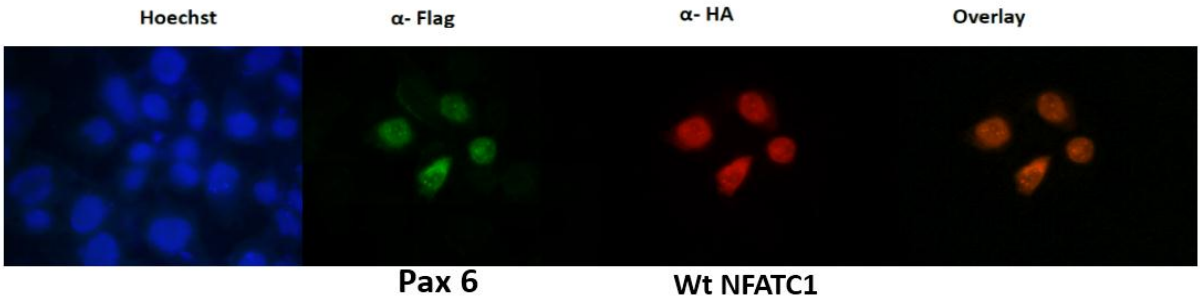


Figure (25): Cellular localization of PAX6 and PAX6-5a proteins in HeLa cells by immunofluorescence. PAX6 and PAX6-5a were found to be located in the nucleas in comparison to the Hoechst nuclear stain. Images were taken with a fluorescence microscope; Magnification is 20x

b. PAX 6 + Wt



c. PAX 6 + V210M

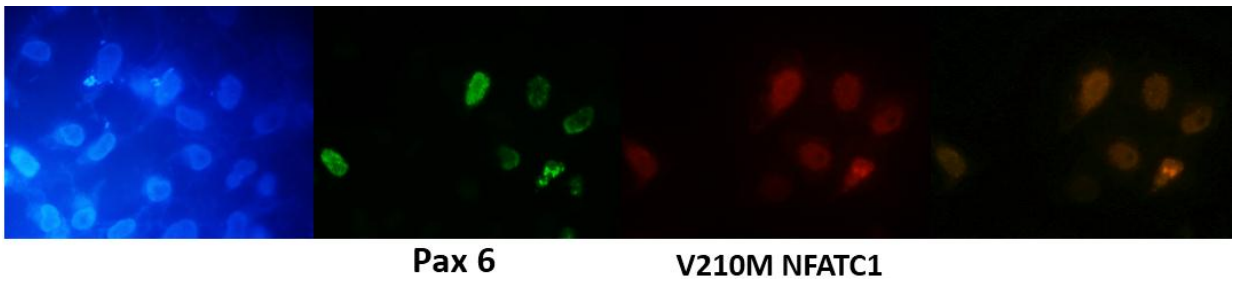
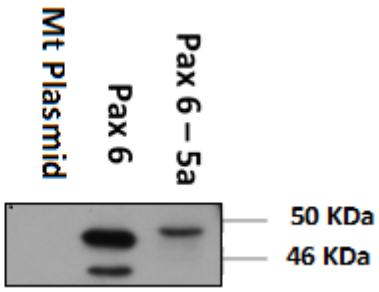


Figure (26): Cellular localization of PAX 6 and NFATC1 proteins in HeLa cells via immunofluorescence Wt and V210M were found to be located in the nucleus as compared to the Hoechst stain when cotransfected together.

2. Protein- Protein Interaction

In order to investigate the effect of the V210M mutation on the physical interaction between NFATC1 and other transcription factors regulating the early stages of valve and eye development, co - immunoprecipitation (CoIPs) assays were done. For this aim, the expression vectors encoding NFATC1 Wt, V210M mutant, PAX6, and PAX6-5a, were transiently transfected in HEK293 cells. Nuclear extractions and western blots were done to

retrieve the target proteins. Protein-protein interaction was then assessed by co-immunoprecipitation assays.



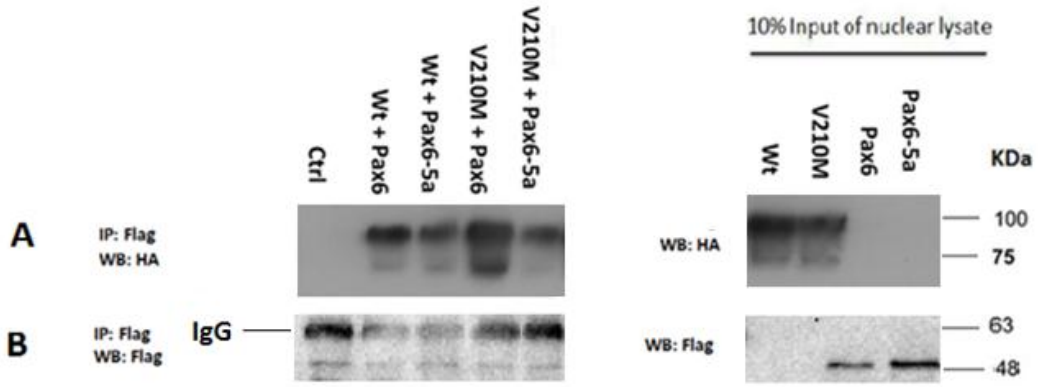
Figure(27): Nuclear protein expression of over expressed PAX6 and PAX6-5a constructs in HEK293: 10 µg of mt plasmid were taken from **PAX6 and PAX6-5a** nuclear extracts of HEK293 cells.

Immunoprecipitation assay showed a novel physical interaction between NFATC1 Wt and V210M mutant with PAX 6 and its isoform PAX6 -5a. V210M mutant has even showed an increase in physical interaction of around 20% when immunoprecipitated with PAX6 proteins and 12% increase in the physical interaction when co-immunopercipitated with PAX6-5a.

- ***NFATC1 and Pax6 :***

Furthermore, PAX6 ,PAX6-5a and NFATC1 interaction on the VEGF promoter was also studied by luciferase assay. HeK cells were transfected with PAX6 alone, PAX6- 5a alone, Wt NFATC1 alone, V210M alone, a combination of PAX 6 with Wt NFATC1, and a combination of

the PAX6-5a with WtNFATC1. Same strategy of transfection was followed but NFATC1 was replaced with the mutant V210M. Increasing amount of PAX6 activated the promoter VEGF in a dose response fashion; such that the maximum activation for the PAX 6 was 10 folds and for the PAX6-5a was 6 folds as shown in figure (23).



Figure(28): Physical **interaction between NFATC1 and PAX6 and it's isoform PAX6-5a.** Physical interaction between HA-tagged NFATC1 (Wt,P68L and P68T mutants) and flag-tagged (PAX 6 and PAX6-5a) is demonstrated. Ten times the quantity of proteins loaded for western blot was used for immunoprecipitation. Nuclear lysates of the proteins were immunoprecipitated with flag- antibody and NFATC1 proteins were visualized with western blot via α-HA antibody. Membrane stripping and subsequent western blot analysis was performed with flag- antibody in order to detect PAX 6 and PAX6-5a proteins. The picture A was taken by xomat and the B past was taken by chemidoc. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD).

3. Regulation of PAX6 Gene Expression

a. NFATC1 and PAX6 , PAX6-5a

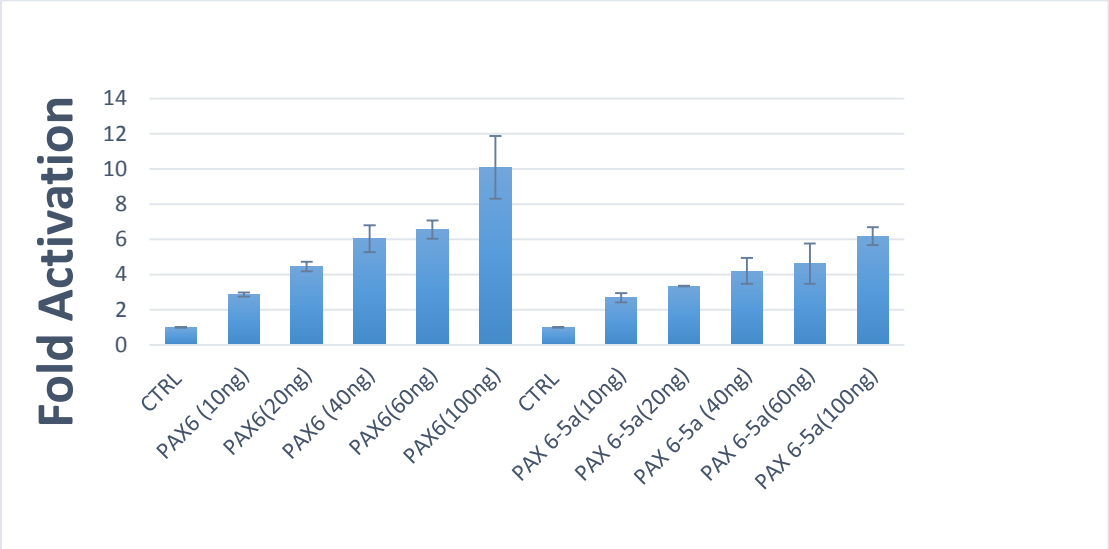
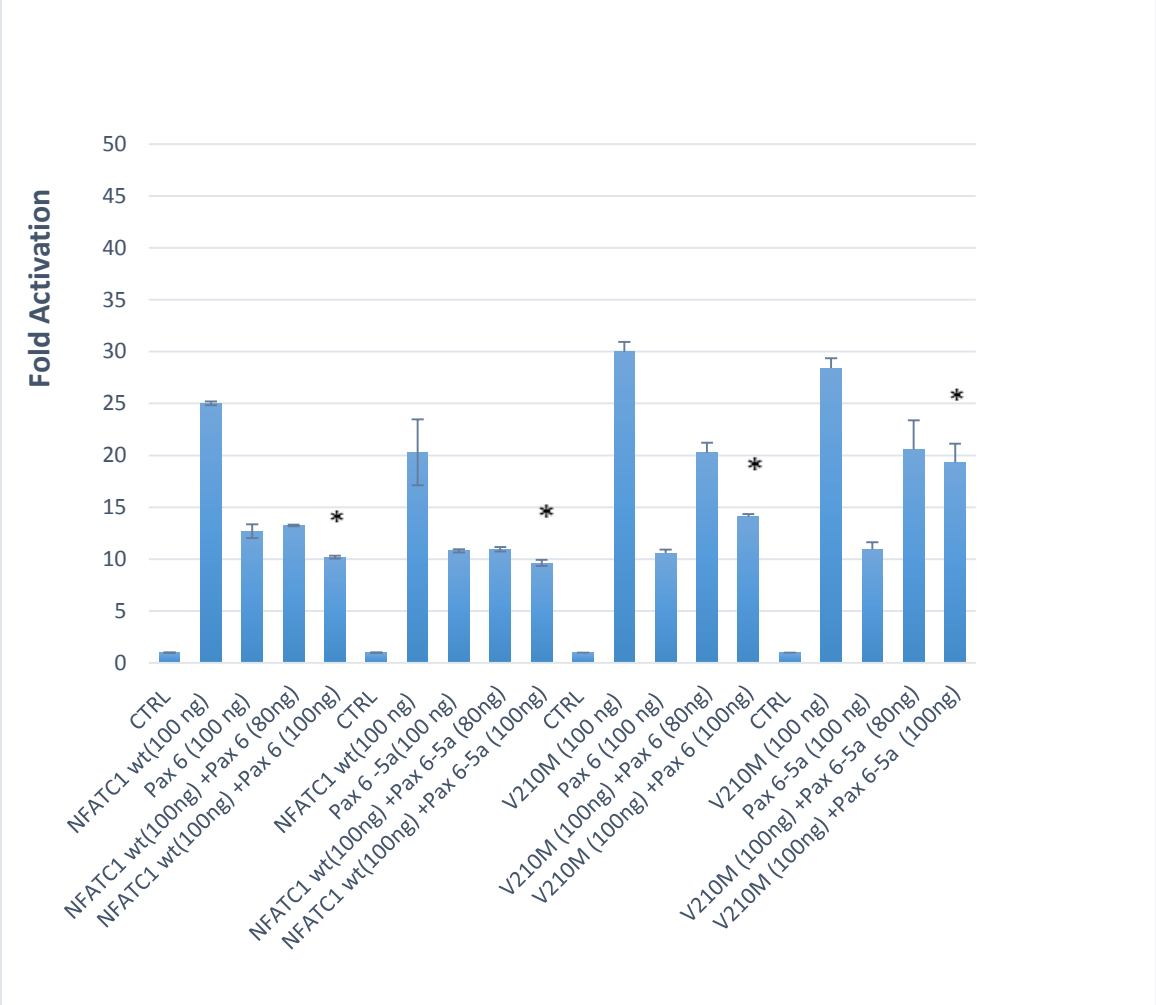


Figure (29) : Transcriptional regulation by different doses of PAX protein and its isoform PAX6-5a proteins in Hek293T cells (VEGF/luc): Hek cells were transfected with increasing doses of PAX 6 and PAX6-5a.



Figure(30): Transcriptional regulation by NFATC1 (Wt, V210M)and PAX6, PAX6-5a proteins in Hek293 cells (VEGF/Luc).

The results are represented as fold actication over the VEGF promoter transfected with the empty vector, The experiments are represented at least 3 times in duplicates.

CHAPTER IV

DISCUSSION

Cardiac morphogenesis is a complex multi-stage process, and the molecular basis for controlling distinct steps of this process remains poorly understood. What is evident till now is that there exist a panel of transcription and growth factors taking the lead in such developmental processes. Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATC1) is one of the key players during valve development; it is associated with and expressed in several other tissues throughout the mammalian body. During heart valve morphogenesis, remodeling of the primitive endocardial cushions into mature valve leaflets is critical for advanced heart development and function. This process requires calcineurin/NFATC1 signaling in the endocardium following EMT (20, 35). However, the regulatory pathways that govern NFATC1-dependent valve remodeling have not been defined. Any mutation affecting the regulatory hierarchy implicated in valve morphogenesis will be reflected as a defective phenotype.

Screening, by DNA sequencing, of samples obtained from patients suffering from congenital heart defects registered in AUBMC, led to the discovery of a novel mutation (P68L) in the NFATC1 gene in one patient with atrial septal defect (ASD).

So the primary aim of this study was to characterize the effects of this novel mutation (P68L) in the NFATC1 gene down at the molecular level, and whether and how they contribute to the pathological effects associated with valve- septa formation defects. Calcineurin is a calcium-dependent phosphatase which is known to mediate the dephosphorylation of the

NLS proximal phosphate group of NFATC1, thereby unmasking its NLS and subsequently permitting its translocation into the nucleus and regulating its downstream targets. So to characterize the effects of these NFATC1 mutations would be on three distinct levels: the first is to compare the mutant forms of NFATC1 with the wild type in terms of subcellular localization, the second is to compare the Mutants to the wild type at the level of interaction with calcineurin and the third is to assess the ability of the mutants to regulate transcription in comparison with the Wt NFATC1.

The second aim of the project was based on the previous work from our lab (Hadla Hariri Thesis 2014) that revealed a potential role for NFATC1 gene in the context of valve and eye development. The study revealed an unreported expression of NFATC1 in the eyes through in vitro finding of interaction between NFATC1 and TBX5. These findings unleash a probable role for another candidate transcription factors that orchestrate the cardiac and ocular developmental processes. One of the many transcription factors that has a crucial role in the eye development is the PAX 6. Hence, we hypothesize a direct physical interaction between PAX6 and NFATC1 that regulate endothelial cell proliferation and ocular structure modeling through a common signaling pathway implicated in valvuloseptal and eye development.

A. NFATC1 Translocation to the Nucleus:

Our results have confirmed that in presence of Cn, WT NFATC1 is able to translocate to the nucleus. To assess the effects of the mutations in NFATC1 on its location in unstimulated cells, we transfected Hela cells with Wt NFATC1 or the Mutants each in a different well. All of the NFATC1 proteins (Wt and mutants) were found to be located in the cytoplasm and some of the NFATC1 were located in the nucleus or in the nuclear membrane due to the

presence of endogenous calcineurin(Figure 20) suggesting no effect on the normal localization of NFATC1. Calcineurin, as expected was found also in the cytoplasm (Figure 21). To screen the effect of the mutation on the translocation of NFATC1 to the nucleus, Hela cells were transfected with Wt or Mutants NFATC1 along with calcineurin. Wt NFATC1 was able to translocate normally to the nucleus in presence of Calcineurin\confirming previous studies about the ability of Cn to provoke NFATC1 translocation to nucleus (Figure 22). NFATC1 mutants also translocated to the nucleus in presence of Cn; P68T,and P68L (figure22)

The normal translocation of our novel P68L mutated protein into the nucleus indicates that the calcineurin - based N terminus dephosphorylating events are probably kept undisturbed. This normal pattern of translocation raises additional intriguing questions, leading us to further investigate other molecular levels.

B. NFATC1 Interaction with Calcineurin:

Following the results of the luciferase assay, it was a must to perform a co-immunopercipitation to assess the physical interaction between the mutants and activated-form of calcineurin**.** Our *in vitro* results have revealed a decreased physical interaction between NFATC1 mutants (P68L and P68T) and activated -form of calcineurin compared to the Wt. The aberrant decrease in the interactive functions of the mutated protein can be correlated to the locus of the mutation that might affect the calcineurin docking site.

C. **One Single Amino Acid Change; Two Different Effects.**

Since NFATC1 is a transcription factor, its main role is in regulating transcription. Starting from this fact, assessment of gene regulation by NFATC1 was our main goal. We studied the effect of the mutations on regulation of transcription through luciferase assays.

Vascular Endothelial Growth Factor (VEGF), a central regulator of angiogenesis and vasculogenesis, activates NFATC1 in human valve endothelial cells (89). VEGF is a significant down-stream transcriptional target of NFATC1 during valvulogenesis. Many studies have revealed the up regulation of VEGF in the atrioventricular field (AV) of the heart after the onset of endocardial cushions. Moreover, another study has also revealed a crucial role for VEGF in mediating hypoxia- driven malformations in heart septation (24). To date, there are no reported defective septal phenotypes related to VEGF mutations. However, in our case the mutation affecting NFATC1 has been seen to cause an atrial septal defect. This may hint at an unknown regulatory pathway downstream of NFATC1.

The results of the luciferase assay performed on VEGF promoter revealed a drastic decrease in activation of the promoter by the mutant P68L in comparison to the Wt and the P68T NFATC1 (Figure23). This could be connected to the fact that the patients suffered from abnormalities in the valves and septa. However, the exact mechanism through which this decrease in activation is leading to these defects is yet to be determined. To make sure that calcineurin is not activating alternative pathways leading to the activation of the VEGF promoter; cells were transfected with increasing doses of calcineurin. Wt NFATC1 activation was more prominent in the presence of calcineurin and there is no significant change in the fold activation of the P68T mutant, but interestingly the novel mutant didn't show any increase in the activation even in the presence of Calcineurin. What we can postulate from

these obtained results, P68L might interrupt the interaction of NFATC1 with other transcription factors that are necessary for the efficient transcription of the target genes. As the NFAT proteins are considered to be relatively weak in terms of their DNA-binding affinities. A group of proteins referred to as NFATn tend to complex with NFAT to compensate for the weak binding efficiency. Some of these NFATn that are previously described is the AP-1 transcription factor family and GATA family (Figure 24)

D. PAX 6 and PAX6-5a Subcellular Localization:

To elucidate and further investigate the previously reported direct physical interaction between NFATC1 and PAX6, we were interested to depict the subcellular localization of the PAX6 and asses where the interaction is taking place. Thus, we transfected Hela cells with Wt NFATC1 or the Mutant each in a different well and as well as the PAX 6 and PAX6-5a each separately. Our results reconfirmed the presence of the PAX6 and its isoform PAX6-5a in the nucleus. To screen the effect of the interaction between NFATC1 and PAX6 on subcellular localization of these proteins, Hela cells were co-transfected with Wt or Mutants NFATC1 along with PAX6 and PAX6-5a. NFATC1 and PAX6 were located in the nucleus, consequently ,we can postulate that the direct physical interaction between NFATC1 and PAX 6 is happening in the nucleus.

E. The V210M: Gain of Function Mutation:

The second part of the study was to characterize a previously reported mutation

(V210M) in the NFATC1 gene and show its association with other transcription factors that control valvuloseptal defects and severe eye malformations.

The fact that NFATC1 is a weak transcription factor necessitates the intervention of other collaborators of tissue-specific and ubiquitously-expressed transcription factors in order to boost the transcription of downstream genes.

PAX 6 is essential in eye development and a previous study showed that *null* mutant mice deficient for PAX6 exhibit the absence of ocular structures (85). To date, there is no study correlating PAX 6 with cardiac development. PAX 3 is the only gene in the PAX family that contributes to the cardiac development that is expressed during the migration of the cardiac NCCs.

A previous study on NFATC1 and PAX6, didn't detect any direct association of PAX6 with NFATC1, suggesting that PAX6 interacts with NFATC1 indirectly by utilizing other transcriptional mediators (85).

On the contrary to the previous study, we are the first to describe a novel *in vitro* direct physical interaction between NFATC1 and PAX6 and its isoform PAX6-5a.

Our results revealed an increase in the physical interaction between NFATC1 mutant (V210M) and PAX6 and its isoform PAX6-5a, in two patients with ASDs and severe ocular defects.

Collectively, we could postulate that the interaction between PAX6 and its isoform PAX6-5a and NFATC1 in addition to other unknown transcription factors regulates endothelial cell proliferation and ocular structure modeling through a common signaling pathway implicated in valvuloseptal and eye development.

This direct physical interaction between those cardiac and ocular expressed transcription factors NFATC1 and PAX 6 suggested the hypothesis that their functional interaction might be involved in both valvuloseptal and ocular entities.

F. PAX6 and NFATC1 Transcription Activity:

While PAX6 acts mostly as a transcriptional activator, there are several reports of transcriptional repressor activity, which is independent of the PAX6-transactivation domain (87). It has been shown a reverse correlation between the expression level of *PAX6* and *VEGF* (88).

Luciferase assay was performed to assess the transcriptional regulation of VEGF by PAX 6 and it's isoform PAX6-5a, Hek293 cells were co- transfected with NFATC1 (Wt or mutants) alone, PAX6 alone, PAX6-5a alone, a combination of two of these proteins. Our data have supported that PAX6 activate VEGF promoter, The activation of VEGF promoter by PAX6 could affect the ocular development, as it has been shown previously that a misexpression of PAX6 or VEGF can result in defective eye formation**.** This result provoked us to study if the interaction between PAX6 and NFATC1 would activate VEGF promoter. As shown in figure (28) PAX 6 was not able to attain the same amount of activation in the presence of NFATC1. Although the co-immunoprecipitation results between NFATC1 and PAX6 lead us to hypothesize a higher VEGF expression in the presence of NFATC1 and PAX6. The observed suppression in NFATC1 expression could be due to the introduction of this potent transcriptional activator, which is the PAX6, might paradoxically suppress the transcription of gene. This so-called 'squelching' is thought to result from endogenously expressed NFAT or PAX proteins within the cell.

G. Conclusion and Future Perspectives:

The results obtained by our studies have opened up a new dimension of strategies that could be followed ultimately leading to the discovery of the specific reasons underlying valvulogenesis defects.

The newly identified NFATC1 mutation P68L in patient with an atrial septal defect resulted in different biophysical properties than that of the wild type NFATC1 and the P68T mutant that has been found in normal population. P68L and P68T affect the subcellular localization of the NFATC1 protein induced by calcineurin, as well as decreased interactions with calcineurin compared to the wild type NFATC1. Interestingly, P68L exhibited significantly decreased transcriptional activity in vitro compared to other mutant P68T and the wild type. Future studies will need to confirm the interactions of this mutant with other cardiac co-transcription factors to determine the influence of this mutation on valve-septa formation. Furthermore, NFATC1 binding to its consensus region on VEGF promoter should be assessed by gel shift assay to further confirm it direct binding to compare the binding of the different NFATC1 mutants with the wild type.

The characterization of a vivo model of NFATC1 mutants will greatly help link the mutations in NFATC1 to valvular defects observed in patients. NFATC1 knock in mice in which the different mutations are reproduced, will help understand the effects of the mutations. The knock-in mice will enable us to look at other downstream targets of NFATC1 such as VEGF, DEGS and CyclinD1.

For the second aim of the study, in characterizing a potential role for NFATC1 gene in the context of valve and eye development. This report comprehends novel *in vitro* finding of interaction between NFATC1 and PAX6 and hints for unregistered candidates implicated in

the regulation of PAX6 during the early stages of eye development. Furthermore, the previously reported mutation V210M in NFATC1 showed an even increased interaction with PAX6 that might explain the severe atrial septal defect and eye malformations seen in patients. Our hypothetical model would involve the up regulation of VEGF promoter expression in both the developing valves and emerging ocular structures, via the intervention of NFATC1, PAX6. More studies and experiments need to be conducted to decipher the VEGF expression in the presence of PAX6 and NFATC1.

To some extent, the depicted molecular scenario can be extrapolated to visualize the *in vivo* events that have contributed to this defective valvuloseptal and ocular phenotype. Though, genetic variants and environmental factors should be also taken into consideration. An *in vivo* knock-in mice model for NFATC1 mutant will help decipher the direct effect of the mutation on the valvular and ocular structures, thus offering a clinical model for valvular and ocular diseases. Nevertheless, the *in vitro* characterization assays will help delineate the regulatory pathways underlying congenital defects and cite novel molecular markers shaping our valvular and ocular entities.

H. **Expectations and Limitations:**

Limitations are inevitable in any research project.

- We are working on immortalized cells such as HEK293 and HeLa cells protein overexpression and luciferase reporter assay and they are quite far from valvular and ocular profiles.
- The *in vitro* system cannot mimic perfectly the *in vivo* system, and might affect the accuracy and where multiple pathways can be forcedly down / up regulated upon

overexpression and induction; thus the actual effects arising are not quiet representative of the *in vivo* outcomes of such mutations.

- *In vivo* studies done via knock in / outs mice models are essential to validate our hypothetical model.
- The presence of endogenous NFAT and calcineurin in cell lines
- We need to check whether this novel mutation P68L is de nove or inherited, thus we need to sequence the whole family members.

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