AMERICAN UNIVERSITY OF BEIRUT

# A NOVEL ROLE FOR NFATC1 GENE IN PATIENTS WITH BOTH CONGENITAL HEART DISEASES AND EYE DEFECTS

By

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# AN ABSTRACT OF THE THESIS OF

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Title: A Novel Role for NFATC1 Gene in Patients with Both Congenital Heart Diseases and Eye Defects

Valvulogenesis and eye development are among the most intriguing events during development. Complex signaling hierarchies of transcription and growth factors orchestrate such developmental processes. Among these modulators, *NFATC1* (Nuclear Factor of Activated T-cells), VEGF (Vascular Endothelial Growth Factor), *TBX5* (T-box transcription factor), and calcineurin are known to be key players implicated in development. Investigating the role of *NFATC1* and unveiling its downstream targets and co-operators, are crucial in order to understand the pathological context of valvuloseptal and congenital eye defects.

We have recently shown two novel missense (P66L, I701L) mutations in the *NFATC1* gene in one patient with tricuspid atresia. Functional analyses did show a defect in its cellular localization, transcriptional activities and DNA binding activity of the protein. Moreover, previous data form our lab have correlated a defective valvular and septal phenotype of aortic stenosis and atrial septal defect to a heterozygous mutation in the *TBX5* gene. Our preliminary data suggests also that the interaction between *NFATC1*/*TBX5* and *TBX5*/calcineurin boosts the transcriptional activity of VEGF promoter. We screened for mutations in the coding region of NFATC1 in a family with septal and valve defects in addition to severe eye defects and found a previously documented polymorphism (rs62096875) leading to a missense mutation (V210M).

Our results unravel a novel pathway implicating an interaction with Tbx5, probably responsible for the underlying phenotype. We can confidently suggest that NFATC1 plays a major role in congenital valvular diseases and eye defects. We hypothesize according to our result that the vascular endothelial growth factor (VEGF) is a downstream target for both NFATC1 and Tbx5 and is implicated both in heart and eye development.

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# LIST OF ABBREVATIONS

| | |
|---|---|
| AP1 | Activator protein 1 |
| 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 |
| DSCR1 | Down syndrome Critical Region 1 |
| E15 | Embryonic day 15 |
| EC | Endocardial cushion |
| Ec | Endocardium |
| EMT | Endothelial Mesenchymal transformation |
| EGF | Epidermal growth factor |
| ECM | Extracellular matrix |
| HOS | Holt-Oram syndrome |
| Nkx2.5 | Homeobox protein |
| HRP | Horse raddish peroxidase |
| My | Myocardium |
| MEF2C | Myocyte enhancer factor 2 |
| NHR | NFAT homology domain |
| N terminal | NH2 terminal |
| NES | Nuclear export signal |
| NFkB | Nuclear factor kB |
| NFAT | Nuclear factor od activated T-cell |
| EFTFs | Eye field transcription factors |
| OV | Optic vesicle |

# CHAPTER I

# INTRODUCTION

## A. A glance at CHD

From the moment it becomes a primitive muscular tube, the mammalian heart starts to support the normal development of the embryo. Such remarkable feature of functionality is molded in its complex morphogenesis from cell-commitment to valve formation. In this fashion, the process of heart development has offered a prominent scaffold for weaving developmental and molecular knowledge. Congenital heart disease (CHD) is the term encompassing a wide spectrum of defects and anomalies affecting heart development. It is the most frequent form of congenital anomalies affecting newborn babies[1] .Worldwide; CHDs are a major cause for mortality within children. The incidence of CHDs is between 1-2% in newborn children (Figure 1[1]); however the exact number of cases cannot be precisely determined due to the fact that a high number of miscarriages [2] are also caused by major defects in heart formation. In Lebanon, the reported prevalence of CHDs with 11.5/1,000 live births at AUBMC slightly exceeds that reported in literature[3], due the high rate of consanguinity in the Lebanese population. Such high incidence obligates extensive examination in order to determine key genetic, molecular and epidemiological markers for the Lebanese population.

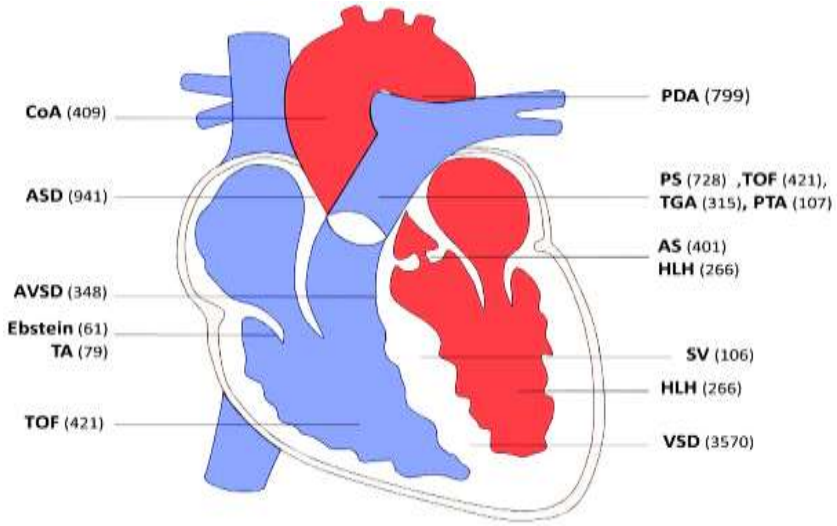



**Figure 1: Locations of heart malformations that are usually identified in infancy, and estimated prevalence based upon the CONCOR database** {van der Bom, 2012 #1149}. Numbers indicate the birth prevalence per million live births. Abbreviations: CoA, Coarctation of the Aorta; AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; Ebstein, Ebstein anomaly; HLH, hypoplastic left heart; MA, mitral atresia; PDA, patent ductus arteriosus, PS, pulmonary stenosis; PTA, persistent truncus arteriosus; TA, tricuspid atresia; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect; SV, Single Ventricle.

Despite the fact that the etiology of CHDs is not fully elucidated, the combinatorial interplay among genetic, epigenetic and environmental factors contributes to this disease (Figure 2). Unveiling the molecular pathways and signaling morphogens implicated in the development of heart is a prerequisite step for translating the genetic basis of congenital heart disease in to therapeutic pathway.

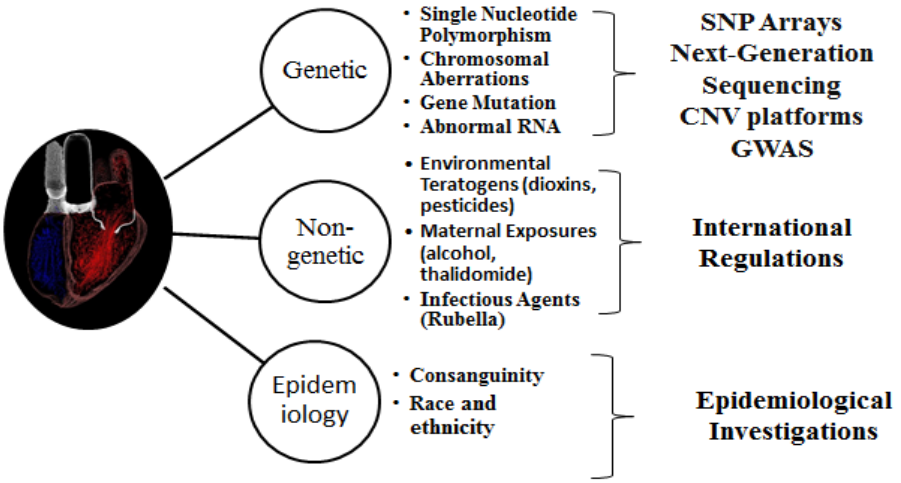



**Figure 2. Etiology of CHD.** Genetic, non-genetic and epidemiological factors contributing to CHDs and the strategies followed to approach each factor. Abbreviations: SNP, single nucleotide polymorphism; CNV, copy number variation; GWAS, genome-wide association studies.

### *B. Cardiac Morphogenesis under light*

Being the first organ to develop, the heart is a dynamic muscular pump that maintains proper flow of blood and sustainable distribution of oxygen and nutrients throughout the body. Studies done so far on models from invertebrates and vertebrates have shown a conserved morphogenetic and molecular program of heart development varying in complexity as it appears with the single peristaltic pump in bilaterians and up to the four-chambered pump in mammals. The progress in the field of molecular genetics was first made due to the use of animal models mainly transgenic mice and secondly to the completion of sequencing of the genome. Breakthroughs in the fields of developmental biology and genetic lineage analyses, have additionally integrated complex molecular signaling cascades and developmental events of fate determination and differentiation in to a unified program controlling heart development.

The building mechanism of the vertebrate four-chambered heart is initiated by a mesodermal cardiac cell specification and a circuit of interaction among a panel of mesodermal and endodermal entities. At embryonic day (E) 15 in humans, progenitor cells of the lateral plate mesoderm respond to specific endodermal signalling cues that induce their cardiogenic fate commitment[4]. Signalling cues governing the cardiac induction process are thought to emanate from bone morphogenic (BMP) and Wnt proteins. BMP inhibitors promote cardiac specification whereas Wnt mediated signals supressit[4]. The cross-talk among bone morphogenetic proteins, fibroblast growth factors (Fgfs) and Wnts underlies the recruitment of genes encoding cardiac transcription factors including NKx2-5, Gata 4/5/6, Tbx5, myocardin and Mef2c[4]. Committed cardiac precursors will then migrate bilaterally to form the two-crescent shaped primordia which will subsequently fuse together to form a beating cardiac tube. The outer and inner layers of myocardial and endocardial cells respectively become evident now, bordering a slimy cardiac grid referred as the cardiac jelly and expressing a set of cardiac-specific transcription factors[5]. Subsequent steps of patterning are required for the formation of the atria and ventricles along the antero-posterior axis of the embryo.

Cardiac looping of the heart tube violates the bilateral symmetry of the developing embryo, where the expression of nodal and lefty in the lateral-plate mesoderm early on and the asymmetric expression of the homeodomain protein Pitx2 at later stages of organ development, drives the rightward looping of the heart tube[6]. Pitx2 and other Wnt-dependent pathway effectors play the major role in the global establishment of the Left/Right symmetry of the organs such as: the heart, lungs, liver, spleen and guts[6].

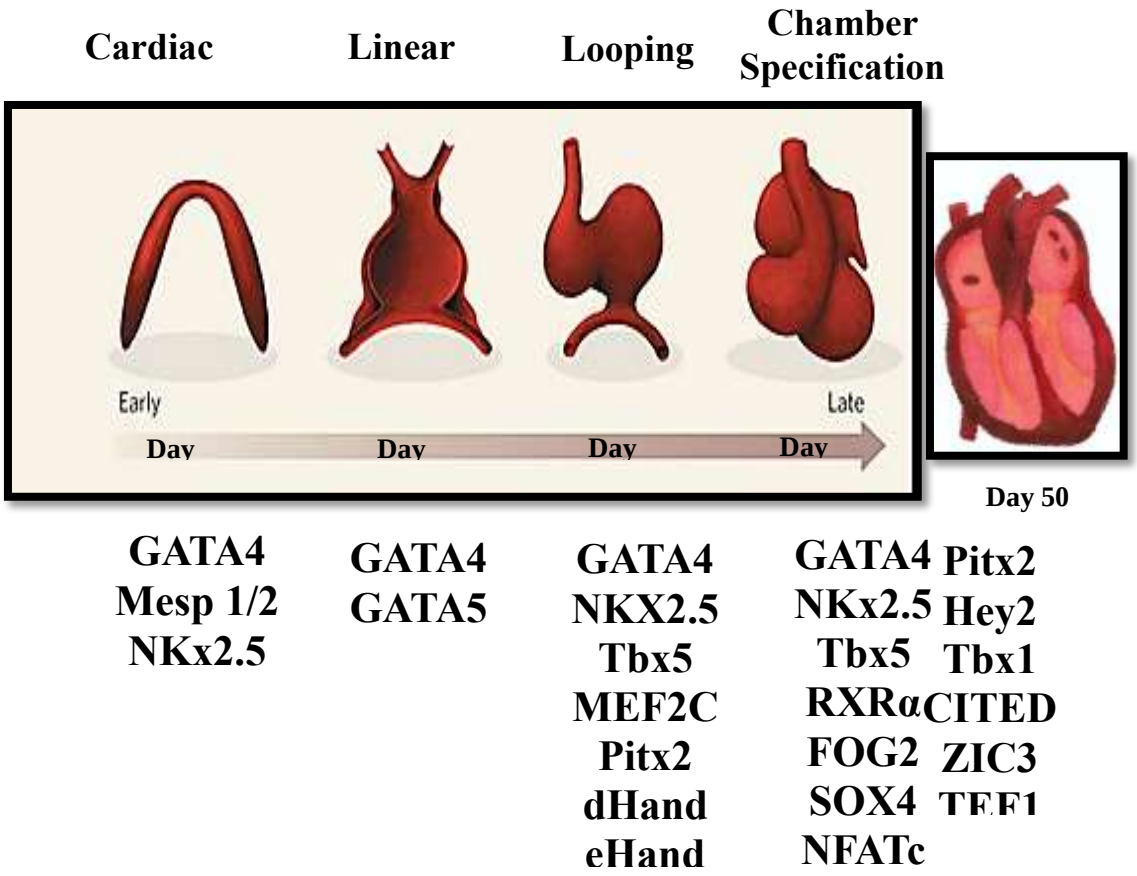



**Figure 3: The developmental stages of the human heart; from a cardiac crescent to a mature four-chambered heart.** Transcription factors implicated in heart development are shown below. Transcription factors are grouped according to their spatial and temporal expression at each stage of development.

Complex changes in the curvatures of the primitive heart tube occur during cardiac looping including chamber formation, trabeculation, septation, and valve formation in addition to the development of the conduction system[7,8]. Driven by a restricted-pattern of gene expression, cells from the cardiac crescent contribute to the formation of the chamber myocardium. Cardiac chambers will then start to expand and curve the elongated heart in the form of trabeculated nodules. The ballooning process of

the secondary chamber myocardium is characterized by a distinct genetic signature for a pool of transcription factors including: Gata4, Tbx5 and NKx2-5[4,7].

Concomitant with looping and chamber growth, endocardial cells at the atrioventricular junction delaminate and undergo an epithelial to mesenchymal transformation to form the endocardial cushions. The cushions will then remodel to generate the outflow and atrio-ventricular valves as well as portions of the atrial and ventricular septa. The final step to satisfy the requirements for a mature four-chambered heart is the patterning of a conduction engine of pacemaker cells and Purkinje fibers[4,7].

## C. Valvulogenesis and Septation

### *1. Valvulogenesis: A developmental target*

The process of valve and septa formation is not as simple as it seems to be; a partitioning of the four cardiac chambers by leaflets, flaps and folds. The complex mechanistic and molecular pathways underlying the formation of valves as well as the obscure pathology of valvuloseptal defects, justify its intriguing aspect. Valvular and atrial defects are among the most frequent CHD worldwide, affecting 1% to 2% of the population[9] and accounting for around 20% to 30% of congenital cardiovascular anomalies[10]. Accumulating evidence of the great contribution of transcription factors and cardiac genes to the pathology of valvular defects [10], highlights the importance of studying valve development.

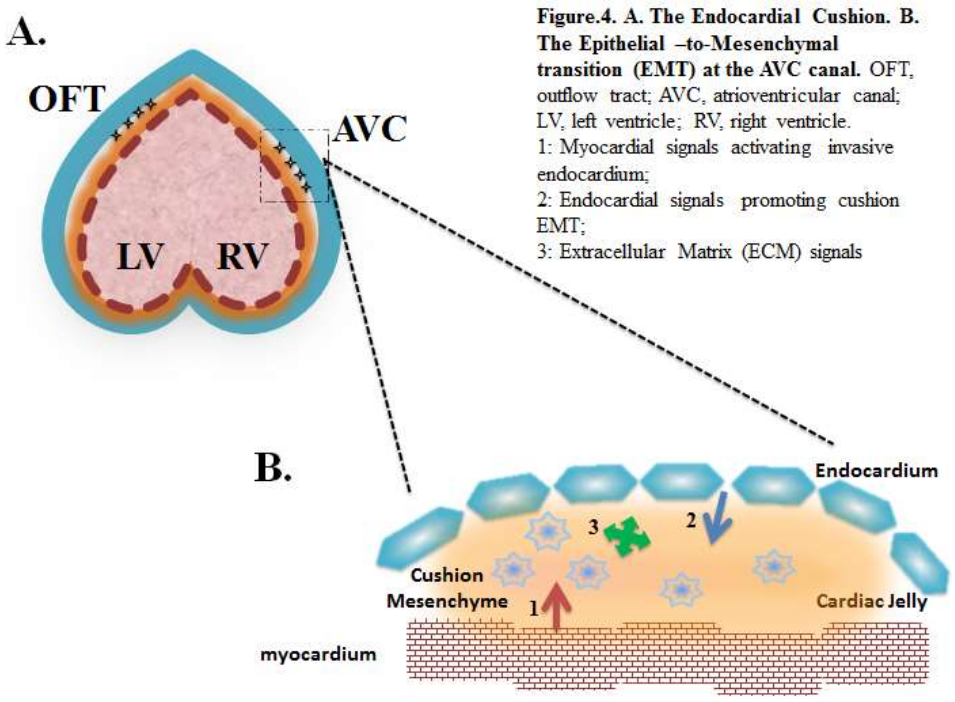



**Figure.4. A. The Endocardial Cushion. B. The Epithelial –to-Mesenchymal transition (EMT) at the AVC canal.** OFT, outflow tract; AVC, atrioventricular canal; LV, left ventricle; RV, right ventricle.
1: Myocardial signals activating invasive endocardium;
2: Endocardial signals promoting cushion EMT;
3: Extracellular Matrix (ECM) signals

### *2. Cushion Formation*

Initially during the early valveless stages of heart development, the rhythmic contractions of cardiomyocytes ensure a unidirectional flow of blood. As form begets function, the developing heart responds to increasing demands of blood flow accordingly. Consequently, complex morphogenetic changes of cushion formation, valve remodelling and septation will then maintain the one-way flow of blood and viability of the embryo. Later on, heart chambers will be gated by highly stratified set of valves; one at the atrioventricular junction (tricuspid and mitral valves), another at the arterial pole (aortic and pulmonary semilunar valves)[10].

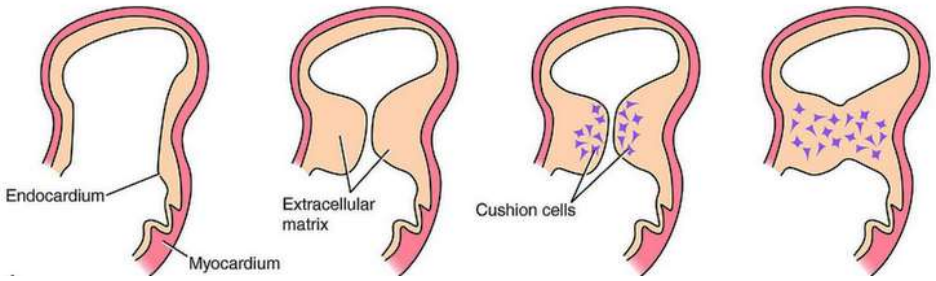



**Figure 5: Cushion tissue formation.** Extracellular matrix is secreted between the endocardium and myocardium at the atrioventricular and outflow tract regions, leading to luminal cushion protrusions.

Immediately after the looping of the heart tube, the cardiac jelly; initially deposited by the myocardium, will protrude in to the lumen at the regions of atrioventricular junction and the outflow tract[11]. Myocardial signals of TGF-β1, BMP2, and BMP4 will turn on the synthesis of a supportive hydrated matrix referred as ECM and turn off the expression of chamber restricted genes at AVC and OFT regions[10] (Figure 6A[12]).

### *3. Epithelial to Mesenchymal transformation (EMT)*

Reciprocal signalling between the myocardium and endocardium, will promote endothelial to mesenchymal transition (EMT) at AVC and OFT regions (Figure 4). Subsequent events of transformation, migration and invasion into the cushion of ECM, is tightly regulated by overlapping myocardial signals of BMP2 and endocardial signals of TGFβ, Notch1 and Wnts[10]. During EMT, activated endothelial cells will respond to inductive signals by first acquiring a spindle-shaped morphology, breaking cell to cell contact and thereby losing their distinctive endothelial baso-apical polarity[13]. Morphological changes are associated with an up regulation of mesenchymal markers

such as α-actin and a down regulation of endothelial markers such as NCAM1, PECAM1 and VE-cadherin[11] (Figure 5B[12]).

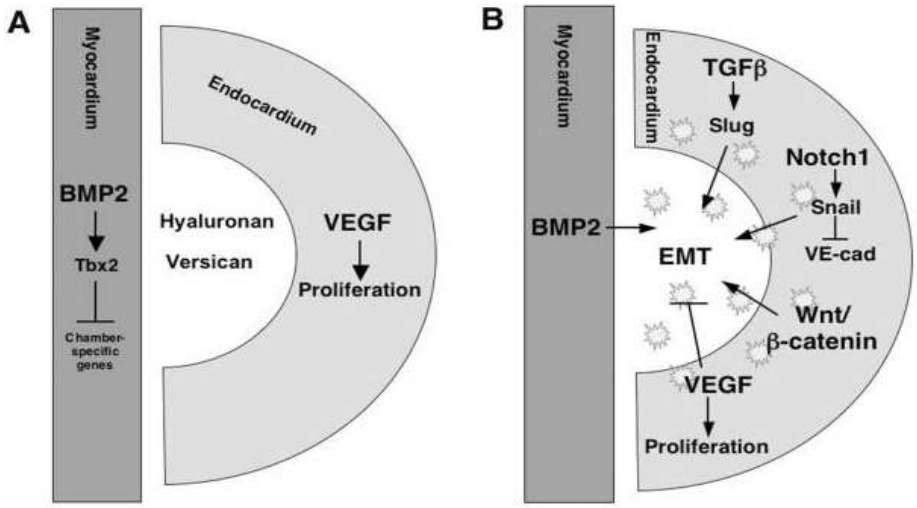



**Figure 6: Molecular regulatory mechanisms underlying the endocardial cushion formation (A) and EMT (B)**

Finally, the mesenchymal transformed endothelial cells will migrate, invade and populate the ECM cushion. Those cells will contribute to the formation of mature valvular leaflets as well as the septation of the AVC canal in to distinct right and left-sided orifices[2].

### *4. EMT: A Molecular Summary*

A field of myocardial-endocardial signalling underlies the epithelial-mesenchymal transit stage during valvulogenesis. Together, the Nuclear factor of activated T cells (NFAT) and Vascular endothelial growth factor (VEGF), are two key mediators of EMT[10,11,14]. At around day 22, myocardial signals of TGF-β, NOTCH, and BMPs initiate EMT[10,14]. The VEGF-driven proliferation of endothelial cells at the AVC

regions is suppressed by myocardial waves of calcineurin-NfatC2/C3/C4 expression, allowing EMT to progress[14](Figure 7[15]). Despite the fact that VEGF is a potent inhibitor of EMT, basal levels of VEGF expression is essential in order to maintain endocardial integrity during transformation[11]. At this stage, the expression of NFATC1 diffuses throughout the whole endocardium and become barely seen as VEGF is up regulated to terminate EMT[14,10,11]. Apparently, VEGF acts as a negative modulator during EMT. However, terminating EMT and attenuating mesenchymal proliferation is indispensable for preventing valvular hyperplasia[16]. The calcineurin-NFATC1 expression is restored and become restricted to the cushion endocardium, where it directs valve remodelling. Overall, it has become evident that a spatial-temporal window of gene expression including NFATC1, calcineurin and VEGF, governs the initiation and progression of heart valve morphogenesis[14]. Additional transcription factors and signalling receptors including RANKL, Connexin-45, and TGF-β may have a promising regulatory role too[10,11,16].

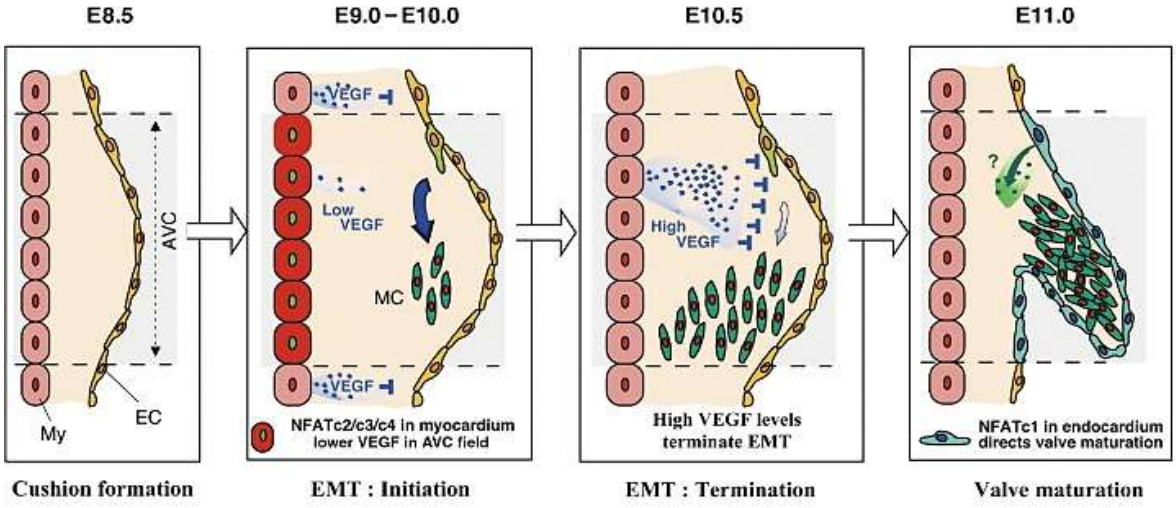



**Figure 7: VEGF and NFAT Family regulating valve formation.** The process of valve formation is initiated by cushion morphogenesis at E8.5. The expression of myocardial NFATC2/C3/C4 supresses VEGF and initiate EMT at the AVC field. High

levels of VEGF are restored at E10.5 to terminate EMT. Valve maturation and remodelling are guided by NFATC1 expression and other unrevealed signals in the endocardium. EC, endocardium; My, myocardium; AVC, atrioventricular canal; MC, mesenchymal cells.

### *5. Post-EMT: The Final Make Over*

During EMT, the endocardial cells and their mesenchymal neighbours maintain their expansion by cell proliferation, while being supported by continuous deposition of ECM[10]. As EMT ceases, the endocardial cushions are well-established and ready to contribute to the future valvuloseptal structures. Cells of the endocardial cushions are the only defined precursors of valves, and they tend to elongate, differentiate and remodel l their ECM in order to attain their final fibrous structure[11] (Figure 8[17]). Cycles of proliferation and cushion mesenchymal apoptosis accompany the process of valve elongation; however the whole process itself is not well-understood[18].

Directly after cushion elongation, mesenchymalized valve primordia remodel in to tapered single-layered endothelial leaflets and cusps. Such thin fibrous threads will be later cushioned by a highly stratified matrix made up prevalently of collagens, elastic fibers, proteoglycans and glycosaminoglycans[10].The early maturation mechanism of semilunar (SL) and atrioventricular (AV) valves progresses along the same pathway in terms of EMT. However, extensive remodelling at late stages of valve development will yield distinct valvular structures in terms of their end morphology. Clusters of branched tendious chordae control the closure and opening of mitral and tricuspid leaflets, whereas pulmonic and aortic roots of the SL valves are radiated by a lamellar elastic matrix of connective tissues[10,11]. At this stage, the differential expression of NFATc1 and VEGF is indispensable.

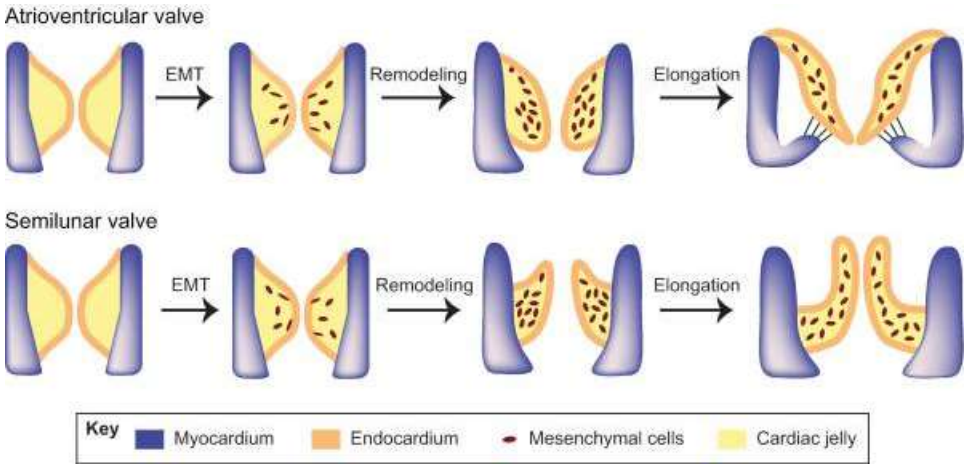



**Figure 8: Valvular maturation and final remodelling.** Valve primordial cells tend to proliferate, elongate, and remodel their ECM in order to attain a mature fibrous structure.

Cushion valve primordia of the endocardium show high proliferative activity; however no evident cell cycling is detected at later stages of valve remodelling and maturation. This restricted phase of cell growth is critical for proper cusp and leaflet remodelling, where increased ECM deposition ensures the emergence of rigid and elastic valves[10]. Less is known about such transition phase of decreased proliferation and elevated ECM protein synthesis[10]. During endocardial cushion development and subsequent stages of remodelling, cycles of mitosis and cell death are balanced. In this sense, apoptosis plays a crucial role in regulating the size and the structure of the endocardial cushion mass, by which regional cell death targets specific undifferentiated regions of the AV mesenchyme[19].

### *6. Valvuloseptal Defects: From Birth to Adulthood*

The majority of congenital heart defects arise from the abnormal morphogenesis of the valvuloseptal tissues; the membranous septa and valves. Being at

the exit of the four chambers and the result of a multistage developmental event, valvuloseptal structures are highly susceptible to errors. In this fashion, valves may have wrong sizes, have malformed leaflets, or even misattached fibrous leaflets. Congenital valvular defects encompass tricuspid valve regurgitation, tricuspid valve atresia, pulmonary atresia, pulmonary stenosis, bicuspid aortic valve, Ebstein's anomaly, atrial and ventricular septal defects, and severe AVC malformations.

**Tricuspid valve regurgitation** is a valvular disorder describing a leaky valve as it fails to seal tightly, causing a backward flow of blood each time the right ventricle contracts. Tricuspid regurgitation usually results from an enlarged ventricle, stenotic pulmonary valve, or any other condition that strains the blood flow to the lungs such as: pulmonary hypertension. It is usually associated with Ebstein's anomaly.

**Ebstein's Anomaly** is a rare congenital valvular defect characterized by an inferior displacement of the septal and posterior leaflets of the tricuspid valve towards the right ventricle. Its prevalence is around 5.2 in 100,000 births and it is accompanied with additional congenital anomalies in 38.8% of cases including secundum atrial septal defects, bicuspid aortic valve, ventricular septal defects, and pulmonary stenosis[20]. Valvular anomalies can affect both infants and adults and contribute to high rates of mortality in newborns. Other valvular defects may cause future complications that may persist and be later diagnosed as adult valvular defects.

### *7. Interatrial Septation: The Walls of the Heart*

As we reach the fourth week of intrauterine life, the heart remodels its inner curvature into a dual-channelled pump, with four chambers and two sets of valves. Between the fourth and seventh week of gestation, different cardiac structures, initially

modelled by cardiac looping and chamber maturation, engage into position to allow septation. In this process, the roof of the common primitive atrium develops a deep depression along the midline of the outflow tract, forming a sickle-shaped membrane known as septum primum[21]. This mesenchymal-capped shelf will extend toward the endocardial cushions leaving a large temporary opening between its lower free edge and the endocardial cushions referred as ostium primum[5,21,22]. Progressively, the latter will be obliterated by the fusion of superior and inferior extensions of the endocardial cushions. Before the complete closure of the ostium primum, small fenestrations appear in the upper central part of the septum primum, which merge to form another opening, the ostium secundum. The ostium secundum remains patent and affords for the free blood access between the two atria. At this stage, the mesenchymal aspect is not evident anymore, as the surrounding myocardial cells start to invade mesenchymal regions[22]. By the end of the fifth week, a new cresenteric fold invaginates from the superior part of the common atrium and converges towards the endocardial cushions as the septum secundum. The septum secundum enlarges to cover the ostium secundum; however it remains as an incomplete partition in the atrial cavity, resulting in an oval-shaped passageway known as foramen ovale. Finally, a complete fusion of the septum primum to the septum secundum will close the oval foramen and forms the definitive interatrial septum.

### *8. Atrial Septal Defects (ASD)*

Septation is not as simple as has classically been described; a mere partitioning of heart cavities by folds and shelves. In this sense, appreciating the molecular pathways

underlying the formation of septal structures is indispensable in order to explain defective septal phenotypes.

Atrial septal defect is one of the most common forms of congenital heart disease (CHD) accounting for 30% to 40% of all adult CHDs[23]. The abnormal through-and- through shunt of blood between the atria can lead to pulmonary hypertension, right ventricular failure and drastic signs of cyanosis in case of right-to-left shunt. Based on the anatomy and developmental stage, atrial septal defects are categorized. Ostium secundum defect is the most frequent type of atrial septal defects, accompanied usually with mitral valve prolapse with a ratio of 2:1 preponderance in female versus male infants[24].

Atrial septal defect was described to 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. Atrial septal defect has been also a remarkable feature of many complex syndromes such as the Holt-Oram syndrome; an autosomal dominant disorder linked to mutations in *TBX5* gene[6,25]. To date, a wide panel of transcription factors, structural proteins and receptors has 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[6,23,26].

**D. Regulation of Valve Morphogenesis: Key Players and One Target**

Regulatory circuits of transcription factors lie at the heart of gene regulation during normal heart development. Starting from early stages of induction and epithelial-

to- mesenchymal transition to cushion formation and mature valve remodelling, a web of transcription, ligand and receptor focal points controls each developmental event.

| 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: Candidate transcription factors implicated in valvulogenesis.** Many gene-encoding transcription factors have defined functions during valve development and known downstream targets.

The first genetic link to valvular defects was established with Jagged-1 and Fog-2, where both were linked to isolated cases of pulmonary stenosis and tricuspid atresia[27,28,29]. Lately, mutations in several genes encoding transcription factors such as: Nfatc1, sox9, Calcineurin, Vegf, Twist1, Tbx20, Msx1, and Msx2 (Table 1[30]) have been associated with valvuloseptal defects. In this report, we will recapitulate the decisive roles of the NFAT family of transcription factors, VEGF, calcineurin, and Tbx5 in normal valve development.

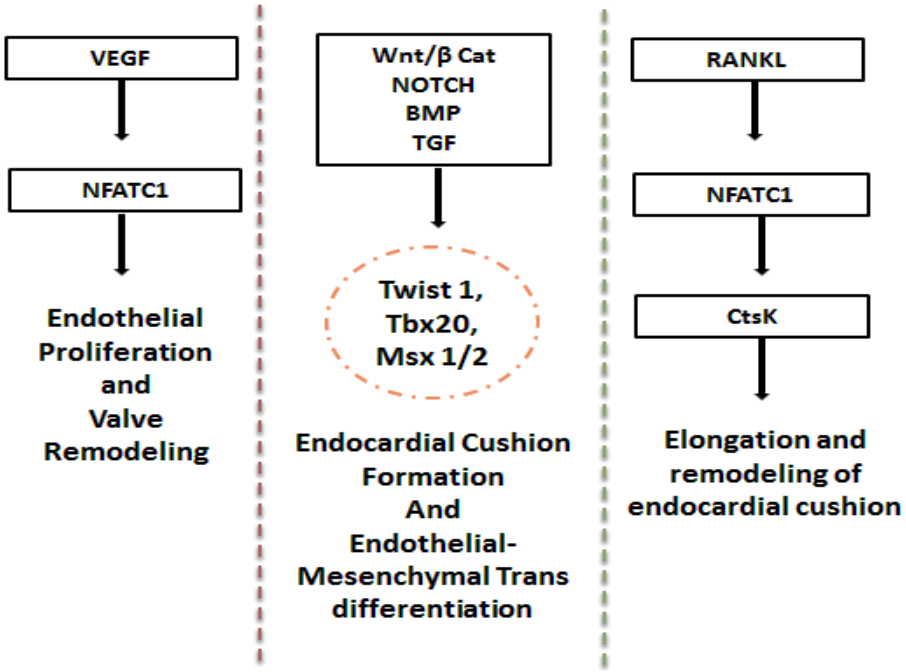



**Figure 9: Regulatory Signalling pathways and transcription factors implicated in valvulogenesis**

### *1. Vascular Endothelial Growth Factor (VEGF)*

VEGF is a potent angiogenic growth factor, known to its crucial role in promoting vascular endothelial cell growth. It belongs to the platelet-derived growth factor (PDGF) supergene family and spans around 14 kb of chromosome six of the human genome. The pre-mRNA alternative splicing of the 8-exons VEGF gene yields six different isoforms of VEGF that will be recruited into activity as disulphide linked-homodimers[31]. Alternate splicing conveys important functional consequences on the splice variants, where they differ in their heparin-binding affinity and amino acid number due to the splicing of exon 6 and 7 and their angiogenic potency due to the splicing of exon 8. VEGF-A is the major factor of angiogenesis, who acts through two tyrosine kinase (TK) receptors; VEGFR-1(Flt-1) and VEGFR-2 (KDR-Flk-1) and tends to regulate endothelial lineage proliferation and migration[31]. As VEGF signals

transduce, complex networks of kinases and signal transduction molecules are turned on and fluxes of intracellular calcium ions are set free. The calcium ion is a highly versatile messenger that acts over a wide temporal and spatial range in nearly all cell types. In this universal sense, calcium signalling activates NFAT proteins in different types of cells.

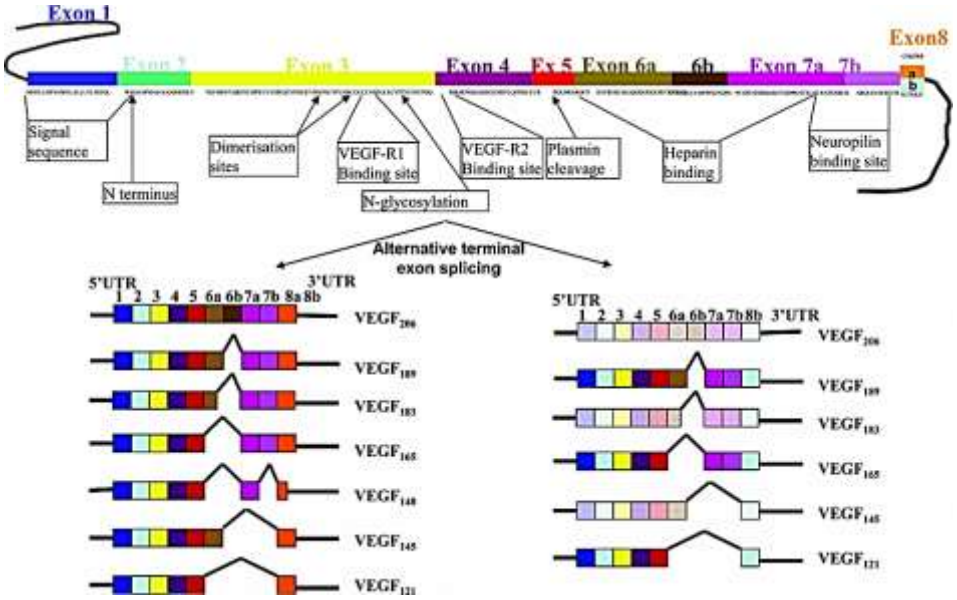



**Figure 10: Alternative splicing of human VEGF pre-mRNA resulting in multiple isoforms.**

While being best known for its pro-angiogenic role, VEGF signalling is also the main implicated in the process of valve formatiom[18] (Figure 7). 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 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[14]. Collectively, tight regulation of VEGF expression in terms of space and time is indispensable for normal EMT.

An array of developmental cardiac abnormalities was associated with disrupted VEGF signalling. The heterozygous expression of VEGF allele was shown to be embryonic lethal due to cardiovascular defects established by compromised endothelial proliferation[32,33]. Comparable lethality events owed to severe cardiac abnormalities of myocardial and septal defects was seen in mice embryos overexpressing VEGF-A[33]. Collectively, those facts reinforce the idea that dose - dependent regulation of VEGF expression is a requirement for normal cardiac development. Various molecular effectors tend to cooperate with VEGF in order to fulfill the requirements for normal cardiac development, some of which are: the *NFATC1* gene belonging to the NFAT family of transcription factors and the calcineurin a calcium-dependent phosphatase. Lately, it has been demonstrated that VEGF plays an essential role in regulating NFATC1 during valve leaflet elongation[16]. Studies have shown that CyclosporinA (CsA), a calcineurin inhibitor, or even the absence of NFATC1 terminates the VEGF induced proliferation of the human pulmonary valve endothelial cells[34] (Figure11[35]).

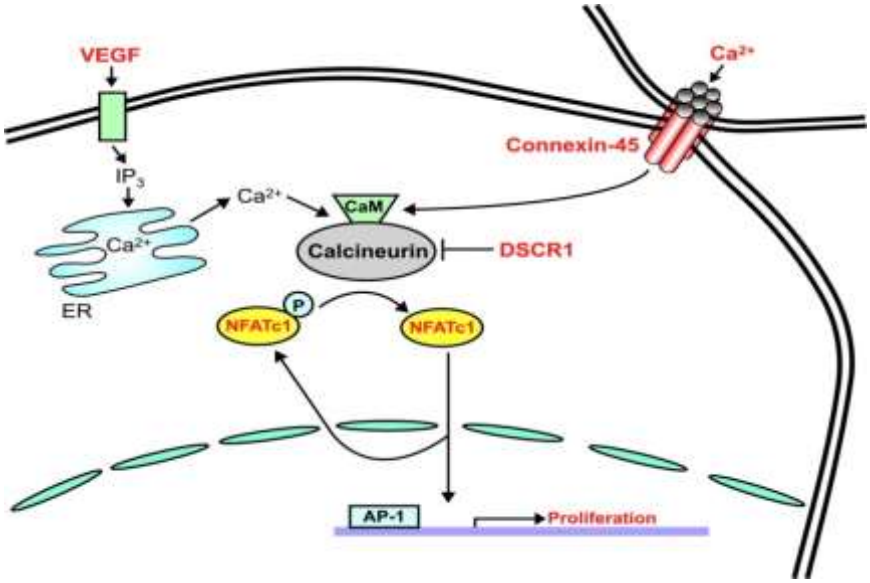



**Figure 11: A Model for VEGF-NFATC1 mediated regulation of valve endothelial cells Proliferation.**

VEGF has thus a distinct spatio-temporal role during heart valve development. Being a potent inhibitor of EMT, does not imply that VEGF has a negative role during valve maturation. Precise expression of VEGF initiates EMT from one side and maintains the endothelial integrity from the other. To date, a well-defined frame describing VEGF contribution to valve development is still missing.

### *2. The NFAT Family of Transcription Factors*

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. This family of transcription factors encircles five NFAT proteins closely related to the Rel/NFKB family. Through only

five members: NFAT1-4 and NFAT5/TonEBP, the NFAT family possesses a wide pattern of expression and fulfils distinct cell-specific developmental jobs inside the human body (Table 2[36]). NFAT1-4 or (NFATc1-4) respond to intracellular fluxes of calcium ions and regulate accordingly the transcription of downstream target genes in a calcineurin dependent- manner[37]. Conversely, NFAT5 is the only calcineurin – independent member, known to be responsive to hypertonic stress[37,38]. NFAT5 is the pristine member among other NFATs that emerged as early as *Drosophila Melanogaster*, being the only NFAT-related protein in this specie[37].

| 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 |

NFAT, nuclear factor of activated T cells; TonEBP, tonicity-responsive enhancer-binding protein.

**Table 2: The NFAT family of transcription factors and their respective inductive signals.**

In the context of evolution, the genes of NFAT family are known to be conserved among different species[37]. Members of the NFAT family are characterized by distinct chromosomal localization and amino acid lengths. The central regions of NFAT genes convey homology of 80% for exons 5-7 and 50% for the NFAT gene as a whole. The 5’ and 3’ ends are the less conserved regions. As a result of alternative splicing and

usage of alternative 5’ and 3’ exons, several transcripts of the NFAT gene were generated in mouse and human[39] (Figure 12[39]). Driven by distinct expression profiles, the resulting protein isoforms fulfill various regulatory functions in the cardiovascular, immune, musculoskeletal and nervous system.

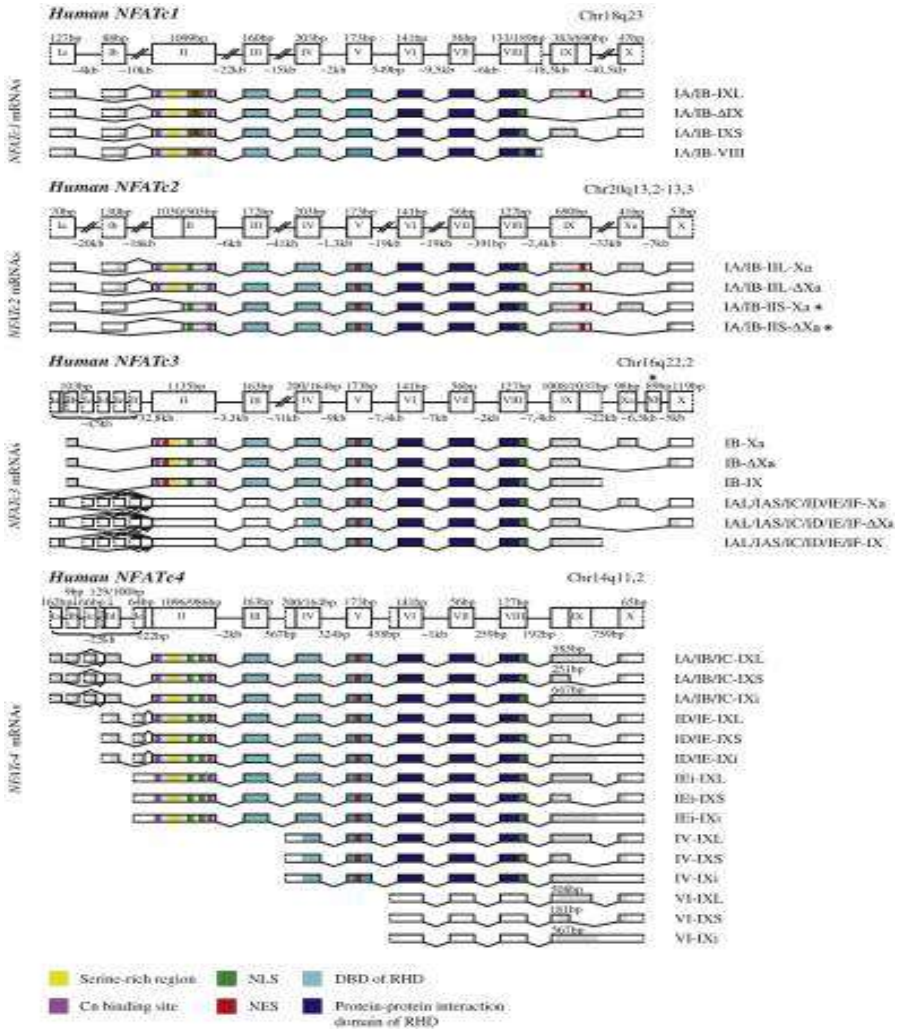



**Figure 12: Human NFATCc1/2/3/4 alternative splicing.**

### *3. NFAT Family: Regulatory Domains and DNA Binding*

The NFAT family of transcription factors is characterized by four major regulatory domains: an amino-terminal transactivation domain (TAD), a moderately conserved region (NFAT homology region, NHR), a highly conserved DNA-binding domain (Rel homology domain, RHD) and a less conserved C-terminal domain. NFAT proteins harbor a Rel-resembling DNA-binding domain and an AP-1 interaction region made up of two independent immunoglobulins known as RHR-C (C terminal subdomain) and RHR-N (N terminal subdomain)[40]. 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.

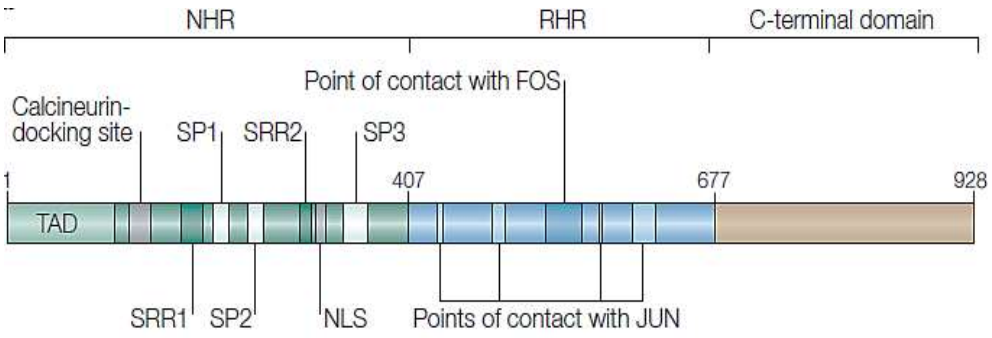



**Figure 13: A schematic representation of the different regulatory domains of NFATC1.** The transactivation domain (TAD), regulatory motifs (NHR), DNA binding and carboxy (C) - terminal domain are shown here.

The NFATn group includes: GATA, AP-1, Mef2 and cMAF. NFAT/NFATn complexes recognize the consensus DNA sequence (A/T) GGAAA and subsequently regulate the transcription of specific target genes[40]. Serine-rich residues, SPXX repeats (SP), nuclear localization signal (NLS) and calcineurin docking sites span the NFAT homology region.

### *4. NFAT Family: Regulation of Expression*

$Ca^{2+}$ mobilization and $Ca^{2+}$/calmodulin- dependent calcineurin activity regulate NFAT activity. Being phosphorylated, NFAT are set inactive, residing in the cytoplasm. Elevated levels of $Ca^{2+}$ tend to activate the serine/threonine phosphatase calcineurin B that will in turns dephosphorylate the serine/proline residues in the regulatory domain of NFAT (NFATc1-c4)[38]. NFAT will then expose its nuclear localization signal (NLS) and get imported into the nucleus. Once in the nucleus, NFAT in conjunction with Fos and Jun heterodimers of the AP-1 transcription factor, are recruited to regulate different biological and developmental processes[38,40] (Figure 11[35]). This regulatory process can be abolished by the action of cyclosporine A and FK506, which are specific suppressants of calcineurin[38,41]. Nuclear kinases will then phosphorylate the serine/proline residues thus exposing the NES (Nuclear Export Signal) and exporting NFAT back to the cytoplasm[41].

### *5. NFAT Family and Calcineurin: The Signaling Twin*

Calcineurin (Cn) or PP2B is $Ca^{2+}$ calmodulin-dependent serine/threonine phosphatase, composed of calcineurin A (Cn A) catalytic and calcineurin B (Cn B) regulatory subunits, and the calcium- binding protein calmodulin. Cn A engages three regulatory domains: a Cn B binding unit, a CAM interacting region and an auto inhibitory domain. Cn B subunit bears four EF calcium binding motifs. At low levels of calcium, the two subunits Cn A and Cn B are tightly bound together, in a way that the Cn A active site is masked by the auto inhibitory subunit. However, elevated levels of calcium induce a conformational change in the regulatory domain of CAM, thus exposing the calmodulin binding site. CAM will bind Cn A and activate the catalytic subunit Cn B.

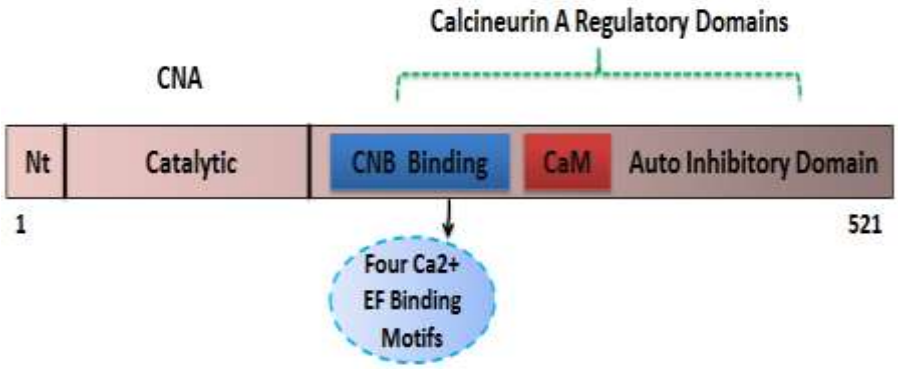



**Figure 14: Calcineurin A Domain Structure.** It is made up of a catalytic and regulatory domain. The regulatory domain is comprised of Cn B binding domain, calmodulin binding domain, and auto inhibitory domain. Cn B has four calcium EF binding motifs.

Other than vertebrates, genetic studies have shown that calcineurin subunit genes are highly conserved among invertebrates such as: *Drosophila Melanogaster and Caenorhabditis elegans* and that it contributes to the development of flies and worms[42].

Calcineurin signalling has been implicated in a wide range of developmental processes. The heart, eyes, blood vessels, and muscles are few of many organs, where calcineurin is a key developmental candidate. Members of the NFAT family of transcription factors except for NFAT5 are substrates of the phosphatase calcineurin. Calcineurin signalling through NFAT family was first described in the immune system[42]. Studies have shown that both calcineurin and NFATs are essential elements in the differentiation of muscles, skin, bone and cartilage[42]. Besides, much of the pathological cases affecting the skin, heart, skeletal muscles and the nervous system were associated with NFAT/calcineurin regulatory pathway[42]. No distortion in the heart

tube formation or cardiac differentiation was detected in mice embryos with mutated calcineurin or NFAT[42]. Besides, studies in transgenic mice have shown that NFAT/calcineurin mediated activation is by itself indispensable and enough for regulating hypertrophic responses of adult cardiomyocytes[42]. Calcineurin B (Cn B) is expressed in both the endocardium and myocardium of the developing heart. Interestingly, calcineurin B and NFATC1 mutant mice possess similar phenotype of undeveloped cardiac cushions and immature valve leaflets. Adding to that, VEGF activation of NFATC1[42] and NFATC1 translocation in to the nucleus was blocked[43] under the action of FK506 or CsA treatment. Collectively, accumulating evidence hint for a possible role for VEGF signalling and NFAT activation through calcineurin in the development of valvuloseptal structures. Though, the exact mechanism that integrates NFATC1, VEGF, and calcineurin in terms of regulatory function at the level of the valves, is still poorly understood.

### *6. NFATC1: A Potential Regulatory Target*

Nuclear Factor of Activated T-cells cytoplasmic 1 also known as NFAT2, is a member of the NFAT family of transcription factors, first purified from bovine thymus and associated with osteoclast differentiation. The human NFATC1 is located on the long arm of chromosome 18 (q23) spanning approximately 135kbp. Out of the ten exons that make up the gene, only eight exons are coding ones. NFATC1 protein follows the same description for its regulatory domains as other NFAT members. Exons 6, 7 and 8 are most important since they encode the DNA-binding domain, while exons 2 and 3 encode the calcineurin docking sites. Ten potential spliced mRNAs are the

result of alternative splicing of the human NFATc1 gene. Driven by calcineurin, NFATc1 is turned on to regulate a broad spectrum of developmental events in the human body.

Beside its role in the immune system, NFATC1 is emerging as a pivotal regulator of heart valve development[10,42] (Figure 15[44]). NFATC1 is exclusively expressed in the endocardial cushions; the early precursors of heart valves. Predominant expression of NFATC1 protein is also detected in endocardial cells lining the endocardial cushions, valve and septal primordial, and the bulbus cordis. Studies done so far on murine embryos, have demonstrated the NFATC1 expression profile during the different stages of heart development. It showed a uniform broad expression of NFATC1 in the embryonic heart between day E7.5 and E11.5, a spatial restricted expression pattern at the site of emerging valvular structures, and a complete loss of expression at day E13.5.

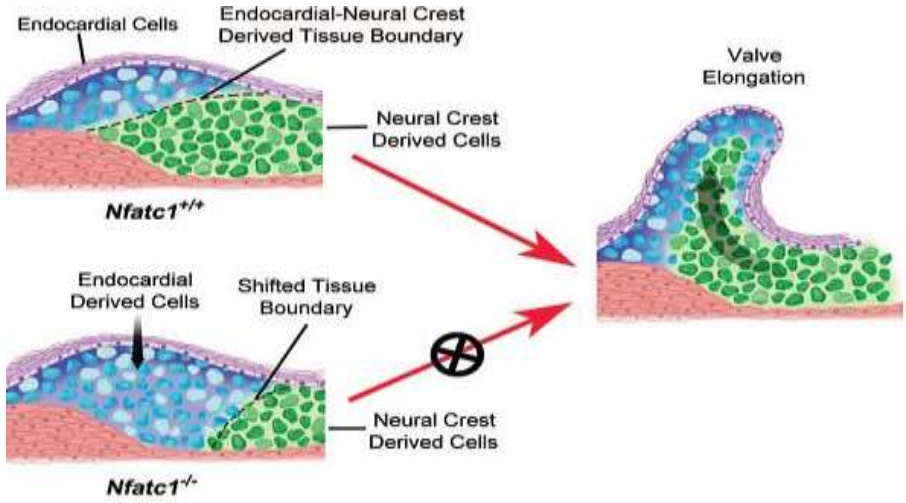



**Figure 15: A hypothetical model depicting a promising role for NFATC1 in semilunar valve development.**

"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[42,43]. Relevant studies have described a defective hypoplastic phenotype of semilunar and atrioventricular valves that failed to remodel in *Nfatc1*$^{-/-}$ mice[45,46]. Calcineurin is expressed in both myocardium and endocardium at day E9.5 and normal progress of EMT in AVC explants was detected in mutated Nfatc1 and Cn B mutants[14]. This indicates that NFAT/calcineurin signalling is not important for EMT initiation, though it is indispensable for subsequent stages of valve remodelling. The role of both NFATC1 and calcineurin in endocardial cushion formation and proliferation was highlighted in *Nfatc1*$^{-/-}$ and *Ppp3cb*$^{-/-}$ embryos, who suffered severe defects in semilunar and atrioventricular valves[47]. In the context of proliferation, it has been shown that NFATC1-VEGF signalling enhances the proliferation of the pulmonary valve endothelial cells[42]. Driven by VEGF signals, calcineurin B – NFATC1 activity was shown to be crucial for proper valve maturation after EMT and for endocardial cell proliferation[14]. Despite all mentioned evidences, it is essential to recapitulate answers explaining the up regulation of VEGF in the overlying endothelium as well as the mechanism by which endocardial NFATC1 is activated during valve remodelling.

In order to decipher the complex signalling 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.

### *7. T-Box Family of Transcription Factors*

The T-box family of transcription factors is a large family of 18 members, named after its founding member, the T protein, known as Brachyury. T-box proteins are characterized by a highly conserved T-box domain involved in DNA binding and dimerization[48]. The T-box domain comprises around 180 amino acid residues of the entire T-box protein and recognizes the palindromic DNA sequence TCACACCTAGGTGTGA. T-box factors in conjunction with the NKX2.5 homeodomain, the zinc finger GATA and the MEF2 MADS transcription factors, appear to guide cardiac specification, cell fate determination and differentiation form *Drosophila* to man[7].

Most of the T-box genes act within the progenitor fields of the developing embryo, to shape the formation of the early mesoderm, the heart and limbs[7]. A differential expression of at least seven T-box factors including *Tbx1-5*, *Tbx18* and *Tbx20*, was detected in the heart of mammalians and different vertebrate models[7] (Figure 16[49]). Heart precursor lineages, the myocardium, the endocardium and valves in addition to the conduction system and epicardium, all show an overlapping and dose-dependent expression of T-box factors. So far, clinical and basic research studies have linked many cases of embryonic lethality, syndromic events, and CHDs of valves, septa and conduction system to T-box factor mutations and happloinsufficiency[48].

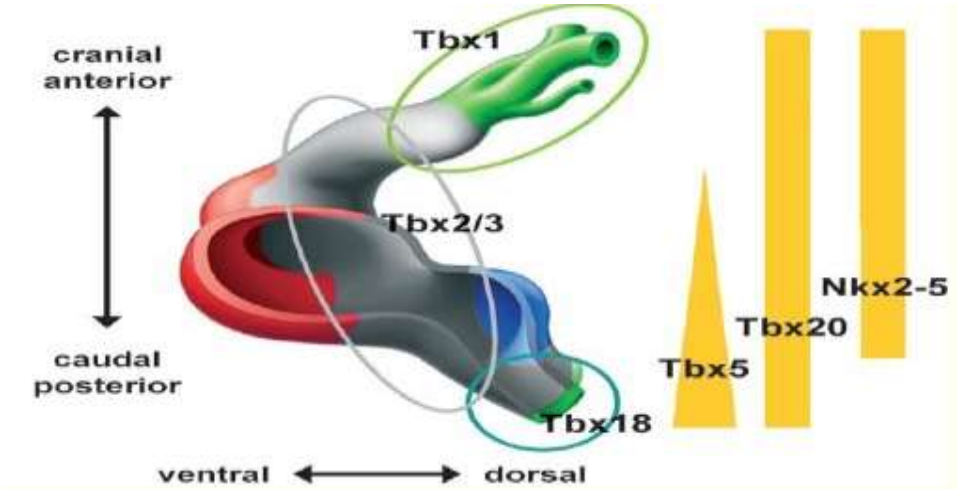



**Figure 16: Role of T-box transcription factors in heart development.** Tbx5 is required for anterior - posterior patterning, and in collaboration with Tbx20 and NKx2.5 for chamber differentiation.

In this part, we will review the role of Tbx5 gene in cardiac development and try to investigate a function for Tbx5 in valvuloseptal development.

### *8. Tbx5 Gene: Insight into Heart Development*

Tbx5 is a member of the T-box family of transcription factors; implicated in cardiac specification, chamber morphogenesis and limb development. It is widely expressed in a subset of cells in the developing heart, eyes and limbs. It consists of nine exons and spans over 54.4Kb on chromosome number 12. The expression profile of Tbx5 regulates its transcriptional activity and can be described as being dose-sensitive, temporal and spatially specific. Alternative splicing of Tbx5 gene generates different spliced isoforms, each with unique activity and distribution. Tbx5a and Tbx5b are two isoforms of this gene (Figure 15[50]).Tbx5b is a C-terminal truncated transcript described by Georges et.al in 2008[50]. This short isoform can bind DNA successfully and can be found in both the nucleus and cytoplasm. The loss of the C-terminal region disrupted its

physical interaction with other collaborators such as: GATA4 and NKx2-5[50]. Tbx5a is the longer isoform described as a nuclear component; implicated chiefly in proliferation events[50]. Tbx5a comprises 518 amino acid residues with two potent activation domains, two NLSs at both the C- and N- terminus, and NES in its DNA binding domain.

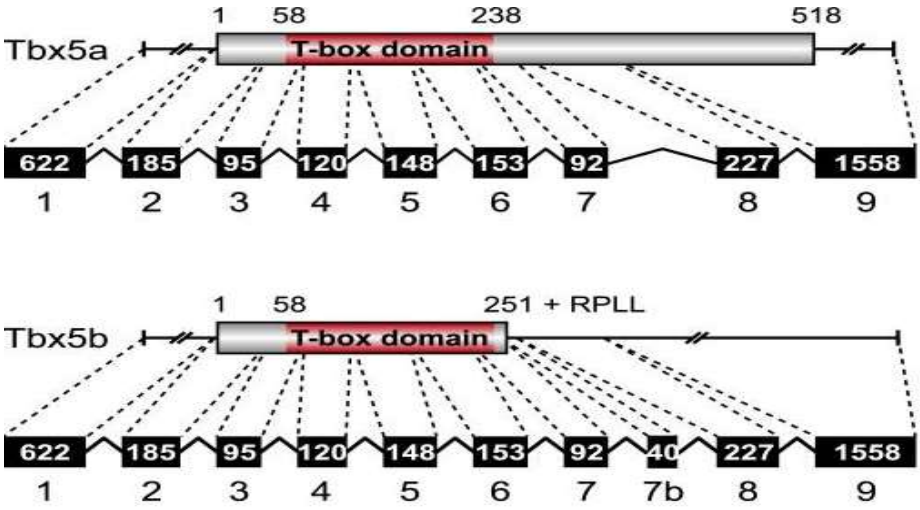



**Figure 17: Scheme showing the Tbx5 isoforms resulting from alternative splicing.**

Tbx5 is expressed in a posterior-to-anterior gradient during heart development, with high levels in the inflow tract, atrium and left ventricle and low levels in the right ventricle. The loss of Tbx5 has been associated with the human CHD Holt Oram syndrome (HOS). HOS is a rare autosomal-dominant syndrome characterized by forelimb and cardiac congenital abnormalities. The latter include atrial and ventricular septal defects, and conduction anomalies. Comparable defective phenotypes of atrial septal defects and conduction problems were also seen in Tbx5-hetrozygous mice[51,52] .Tbx5 expression has been also detected on the atrial roof of the atrioventricular valves in mouse and chick embryos as has been also reported in humans[48]. Interestingly, Dr. Georges Nemer's lab has correlated a heterozygous mutation in the Tbx5 gene to a

defective valvular and septal phenotype including aortic stenosis and atrial septal defect (Unpublished work). This additional feature of expression suggests strongly that Tbx5 may have a prominent regulatory role in valvulogenesis and septation.

## E. Eye Development: Where Complexity Meets Structure

### *1. Embryonic Eye Development: From Late Gastrula to Optic Cup*

The process of embryonic eye development is a complex event of self-assembly of many biological structures. It is built up by three major tissues, the cornea, the lens and the retina. During gastrulation, the anterior neural plate reorganizes into a single eye field at the centre of the forebrain. This single eye field will split into two lateral parts referred as optic pits. During neurulation, the two optic pits become evident and start to enlarge forming the optic vesicles. By day 28 of gestation in humans, the overlying ectodermal surface will invaginate forming a thickened ectodermal layer known as lens placode. At this stage, the lens placode will come into a close proximity to touch the underlying optic vesicles. This cell-to-cell contact will activate the neuroectoderm to fold inwardly, thus giving rise to the optic cup.

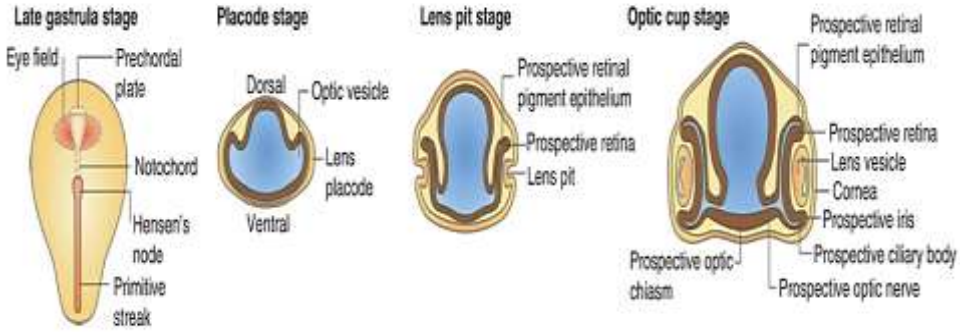



**Figure 18: A scheme depicting the developing eye: from late gastrula stage to optic cup stage.**

Reaching the optic cup stage, the major ocular structures will appear as follows: the inner layer of the optic cup will give rise to the retina, the lens will appear as an invagination product from the lens placode, and the cornea will become visible as the lens detaches from the surface epithelium (Figure 18[53]). In summary, different embryonic origins collaborate to shape major ocular structures during the early stages of eye development. Neural and ectodermal surfaces are the building blocks of the retina, the lens, and part of the cornea. However, the major part of the cornea has neural crest origins.

### *2. Eye field Induction: A Molecular Web*

The eye is the ideal model for studying mechanisms of embryonic induction, proliferation, migration and gene regulation. Much of our knowledge of the molecular genetics of the eye is extracted from *Drosophila* developing eye models, which helped uncover a number of highly conservative regulatory genes in the genome of vertebrates[54]. A group of eye field transcription factors (EFTFs) are dynamically expressed in the anterior neural plate, where they act as developmental markers for eye field induction. This group comprises master regulatory genes such as: *Pax6, Rx1, ET* (also known as transcription repression factor Tbx3), *Lhx2* (Lim homeobox protein 2), the orphan nuclear receptor *Tll, Optx2*, and *Six3*[55,53]. EFTFs are not expressed until neurulation proceeds and their expression is not restricted to the eye, where *Pax6* and *Rx1* signals penetrate regions of the brain and neurons upon development[56,54]. They possess a unique overlapping pattern of expression in the presumptive eye field, thus making it difficult to assign independent roles for each factor[56]. Moreover, it is important to consider the interaction among EFTFs and with other transcription factors

and downstream targets at multiple regulatory levels and through various signalling systems (Figure 19[53]).

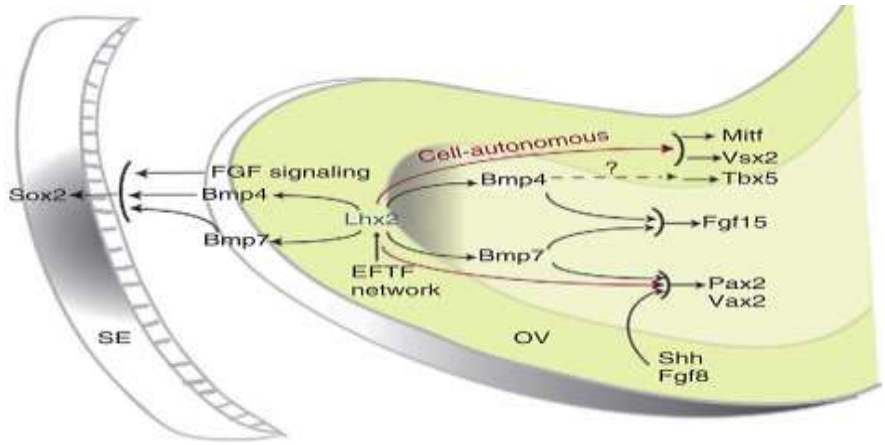



**Figure 19: A hypothetical model recapitulating overlapping expression of different transcription factors during the early stages of eye development.** Lhx2 is acting as a central regulatory point in collaboration with EFTF network and BMP signalling play a crucial role in linking the early stages of lens specification to optic vesicle patterning. The dashed arrow indicates unresolved regulatory signals controlling Tbx5 other than Bmp4. OV, optic vesicle; EFTF, eye field transcription factors.

Genetic studies have strongly suggested that EFTFs are not only necessary for proper eye morphogenesis; however it is by itself sufficient for eye tissues induction. Overexpression studies of *Pax6, Six3, Optx2* and *Rx,* have demonstrated events of eye tissue induction and expansion in the vertebrate nervous system[55]. Conversely, targeted or spontaneous mutations of *Pax6, Six3, Six6, Rx,* and *Lhx2* in mice, resulted in abnormal morphogenesis of eyes or even no eyes[55]. Likewise, homozygous *Pax6* knockout mice, failed to develop their lens and eye cup and heterozygous ones showed retarded development of lens placode[54]. At a higher level of regulation, NOTCH and EGFR signalling pathways were proposed to regulate the early stages of eye development. Mice with homozygous NOTCH2 mutations suffered bilateral Microphthalmia[55].

### *3. Optic Cup Formation:*

The patterning of the optic vesicle into an optic cup is a crucial transition stage by which major ocular structures are sculpted. The formation of the optic cup initiates the process by which the neural retina, retinal pigment epithelium, parts of the ciliary body, and a portion of the iris are formed[57]. This transition step can be summarized by two major events; one including a regional and axial patterning of the optic neuroepithelium and the other corresponds to lens induction in the surface ectoderm. The sonic hedgehog (Shh), FGFs, TGFβ, and BMPs signals from tissues of distinct embryonic origins in conjunction with ETFTs orchestrate those patterning steps[58]. One important outcome of this multilevel regulatory pattern is the establishment of defined expression domains for a panel of transcription factors such as: *Pax2, Vsx2, Tbx5, Vax2, Mitf,* and *Sox2*[58]. Recent studies have integrated ETFT network and complex signalling pathways into a uniform model, depicting the process of optic cup formation. In this model, *Lhx2* gene product is central and indispensable. Under the umbrella of ETFT expression, *Lhx2* links lens specification to optic vesicle modelling through the regulation of BMP (BMP4 and BMP7) signalling. Briefly, it acts to induce and maintain the expression of patterning genes (*Mitf, Vsx2, Pax2),* establish the dorsoventral polarity of optic cup (*Tbx5* and *Vax2*), and maintain retinal progenitor cell properties (*Fgf15*) and lens specification (*Sox2*)[57,58].

To date, hundreds of genes are registered as eye disease candidates; however less than 20 have well- defined molecular basis. The malformations that affect the optic cup as well as other induced ocular structures such as: the retina and the iris have been linked either to single causative genes or to multiple overlapping pathways of

expression. Little is known about the exact mechanism by which the optic cup is formed and oriented. Lhx2$^{-/-}$ mouse embryos showed arrested optic cup formation in both optic neuroepithelium and lens ectoderm[58]. It has been reported that *Pax6*$^{-/-}$ mouse mutant small eye (Sey), have no expression of *Tbx5* and extended expression of *Vax1/Vax2* over the entire optic vesicle[57]. Likewise, *Pax6* together with *Tbx5*, *cVax* , and *Bmp4* patterns the dorsoventral polarity of retinoptical axis in chick optic cup[57]. Relevant studies have also linked the phenotype of eye dorsalizarion to distorted expression of *Tbx5* and *Bmp4*[57].

### *4. Tbx5 in the Eye*

Tbx5 was first detected in the retina of chick embryos mainly in the dorsal retina[59]. Later in development, Tbx5 expression becomes confined to the dorsal optic cup, retinal pigment epithelium and neural retina. The contribution of Tbx5 to the formation of the optic cup was sufficiently dissected above. The circuitry comprising Pax6, Tbx5, Bmp4, and cVax has been extensively studied in term of dorsoventral polarity of the optic cup and in turns the retinal axis.

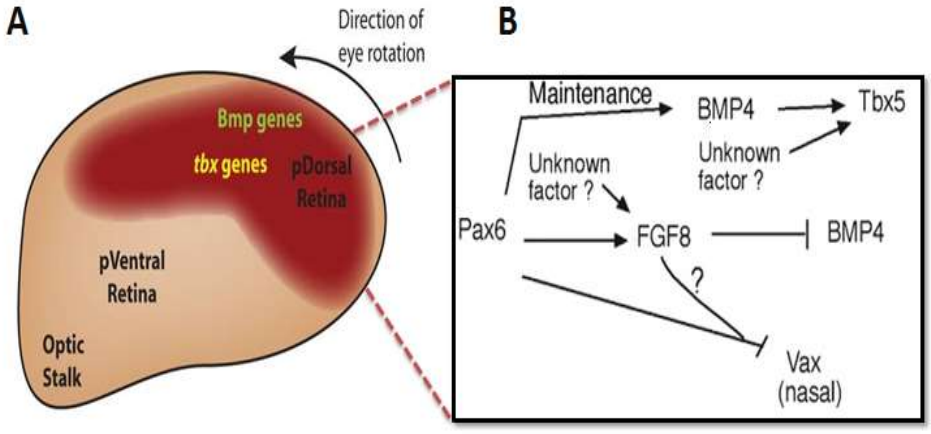


**Figure 20: Expression profile regulating optic cup and retinal axis orientation during early stages of eye development. A.** Retinal diagram showing expression domains of BMP and Tbx genes. **B.** Major role for PAX6 in keeping balance between dorsal (Tbx5) and ventral (Vax) markers, through BMP and other unrevealed signalling pathways during optic cup formation.

Studies have shown that the inversion of dorsoventral polarity blocks optic vesicle development, arrests optic cup formation, and disturbs the patterning and differentiation of neural retina and pigment epithelium in chick embryos[60]. Multiple studies have shown that BMP signalling plays a crucial role in establishing the dorsal polarity and the neural retina in the optic neuroepithelium. Besides, BMP signalling in the dorsal optic vesicle is an upstream regulator for *Tbx5, Vsx2,* and *Fgf15* and distorted expression of BMP in the ventral optic cup have led to ectopic expression of *Tbx5* in chicks[58]. Interestingly, the reintroduction of Bmp4 signalling in culture media in Lhx2-/- chick embryos, failed to restore the expression of *Tbx5*[58]. This unresolved issue raises doubt of whether *Tbx5* expression is a potential target for other players and signalling pathways implicated during early stages of eye development.

### *5. VEGF and Cn/NFATc1 in the eye*

VEGF - mediated Cn/NFATc1 signalling circuit fits in more than one developmental scheme. The cardiac scheme was the most studied and well- described among other developmental events. Likewise, this evolutionary conserved developmental 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 signalling pathway[61].

VEGF and calcineurin-dependent NFATC1 signalling plays a key role in regulating angiogenic cell behaviours. VEGF is produced and secreted by at least five different types of retinal cells including: pigmented retinal epithelium, astrocytes, Müller cells, ganglion cells, and vascular endothelium. Among all the growth factors implicated in retinal angiogenesis, VEGF is suggested to be the potent regulator and calcineurin-dependent *NFATC1*is one signalling pathway known to act downstream of VEGF. Studies have demonstrated that *NFATC1* signalling 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[61].

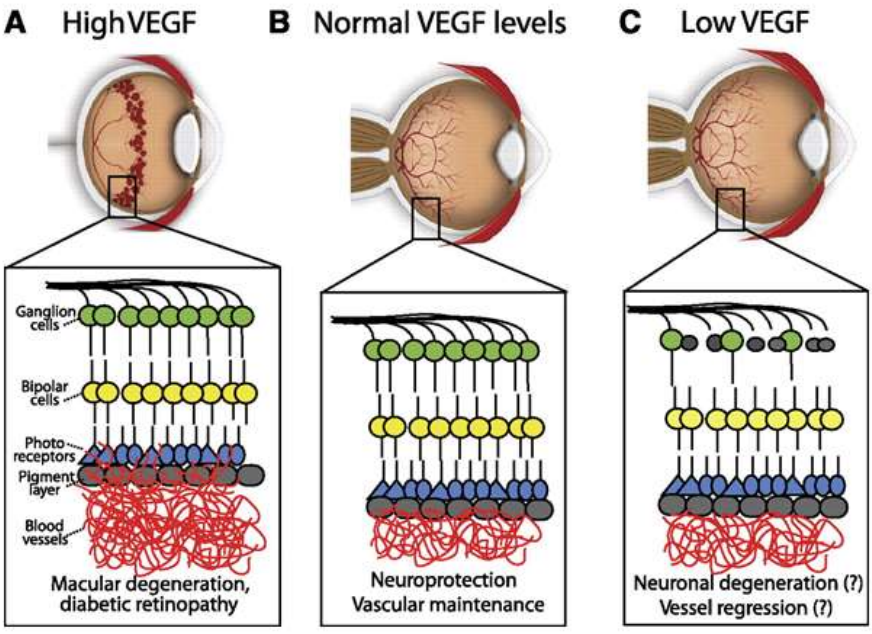



**Figure 21: Effect of variable levels of VEGF on retinal integrity**

The phosphatase calcineurin has also been linked 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 apoptotic events. 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[62].

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. The downstream targets of VEGF-mediated Cn/NFATC1 signalling were beyond the scope of most of the studies done to define the molecular genetics of the early stages of eye development.

### *6. Congenital Eye Defects*

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. Worldwide, 20 million children under the age of 16 suffer from cataracts and 1.4 million are blind[63]. Approximately, 50% of the patients with congenital eye anomalies develop glaucoma in their lifetime[64]. Congenital eye malformations include severe phenotypes of Aniridia, anterior segment dysgenesis, and glaucoma. 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. Despite this fact, a network of candidate

causative genes including: *PITX2, PITX3, PAX6, MAF, FOXC1, LMX1B, and CYP1B1* have been shown to be related to congenital eye defects[64].

### *7. Congenital Aniridia*

Aniridia is a developmental eye disorder associated with iris hypoplasia, corneal opacities, cataracts, and optic nerve hypoplasia. It is inherited in an autosomal-dominant fashion and can exist in both sporadic and familial forms[65]. 50% to 70% of Aniridia patients develop glaucoma as they reach adulthood. This panocular disease is associated with mutations in the paired box gene 6 (*PAX6*) gene, a homeobox master gene implicated in the early stages of eye development.

### *8. Congenital Secondary Glaucoma*

Congenital glaucoma is a group of hereditary optic neuropathies characterized by gradual and irreversible visual field loss and optic nerve degeneration. It is the most common cause of irreversible blindness worldwide, resulting from damage to the aqueous outflow system with elevated intraocular pressure (IOP). Early - onset forms of glaucoma are inherited in an autosomal dominant and autosomal recessive pattern. Congenital secondary glaucoma incorporates severe defective eye phenotypes of Aniridia, Anterior Segment Dysgenesis (ASD), and lens defects. Genetic heterogeneity is the hallmark of congenital glaucomas and little is known about the genetic backgrounds of congenital glaucoma.

### *9. Anterior Segment Dysgenesis (ASD)*

The anterior segment of the eye includes the cornea, iris, lens, and chamber angle. ASD disorders comprise a wide spectrum of defective developmental conditions affecting the above listed structures. It has been associated with the Axenfeld- Rieger syndrome (ARS) and mutations in the transcription factors *PITX2* and *FOXC1*[66,67]. The major clinical concern is again demonstrated in the threat of developing glaucoma, the case of 50% of the patients with ASDs.

## F. Aims of the Study

NFATC1 gene has been extensively studied in our lab. A recent study has linked a defective valvular phenotype of tricuspid atresia and aortic stenosis to NFATc1 mutations. Unpublished data have also correlated a phenotype of valvular aortic stenosis with a heterozygous mutation in Tbx5 gene. Our preliminary data shows that both Tbx5/calcineurin and Tbx5/NFATC1induce transcriptional synergy upstream of VEGF. In this report, we will describe and characterize a previously reported mutation (V210M) in the NFATC1 gene and we will show its association with valvuloseptal defects and severe eye malformations.

Given the prominent role of *NFATC1* in valve development, we aim to investigate a role for *NFATC1* in the hallmark of valve remodelling and embryonic eye development. The main target is to fit this gene and its collaborators of transcription factors into a uniform regulatory model implicated in valvuloseptal and eye development.

We hypothesize that the V210M mutation in the NFATc1 gene is disease causing and CHD contributing. We suggest that the mutation will distort the expression of VEGF in both the valves and eyes and disrupt the physical interaction of NFATc1 protein with other collaborators of transcription factors some of which are: calcineurin, Tbx5, and GATA5.

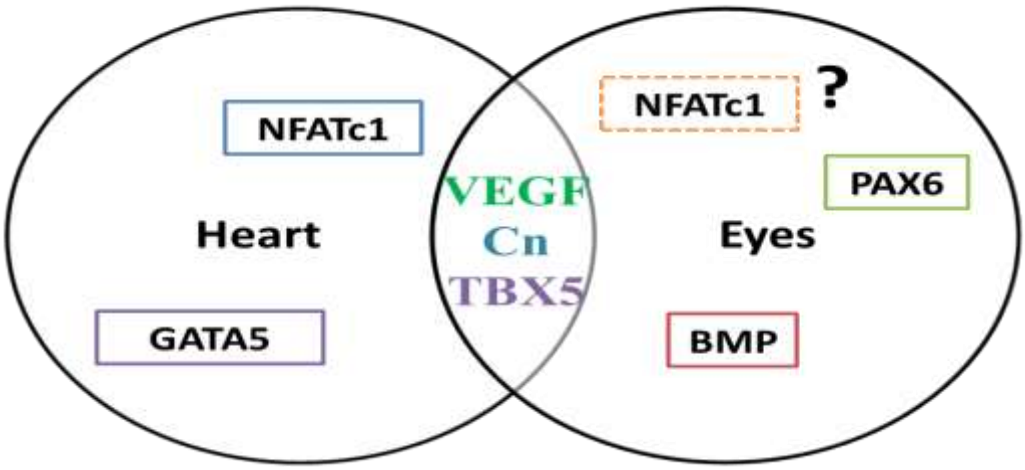



**Figure22: A scheme depicting the stated hypothesis of the study.**

# CHAPTER II

# MATERIALS AND METHODS

## A. Site –Directed Mutagenesis

After identifying each mutant gene sequence, oligonucleotides encoding for the desired mutation were annealed to one strand of the human NFATC1 gene cloned in the pCEP4 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 phosphorylated and PCR was performed using Site-Directed Mutagenesis kit from FINNZYMES (product code: F-541).

| Mutant | Primer Orientation | Primer Sequence |
|---|---|---|
| **3'V210M** | **Forward** | **5'GGCAGTCTCCCTGCATGTCTCCCAAGACCA3'** |
| | **Reverse** | **5'ATGGCGACGTCTGGGGGGAC3'** |

**Table 3: Primers used for site-directed mutagenesis.** The table represents the desired mutation, the primer orientation and sequence.

## B. Transformation and cloning of constructs in bacteria

The previously obtained constructs are then transformed into *E.coli, XL1 blue* strain bacteria initially stored at -80 $^{o}$C. Over 70 μL of bacteria, 1μg of the plasmids containing our DNA constructs is added. The mixture is placed for 2 minutes on ice, 5 minutes at 37$^{O}$C (in the water bath), then 2 minutes on ice. The transformed bacteria are placed and streaked on agar plate, and then incubated at 37$^{O}$C overnight. Note that this transformation process is performed in aseptic conditions i.e. close to the flame of a Bunsen burner. Exclusively, bacterial Colonies incorporating the desired plasmid will grow on the agar ampicillin selective medium. Bacterial colonies are then removed with pipette tips, and are then transferred into 15-mL falcon tubes containing 3 mL liquid broth. The tubes are then incubated overnight in the shaker, at 37$^{o}$C, at 200 rpm. Minipreps and maxipreps are performed using illustraTM plasmidPrep Midi Flow Kit by GE Healthcare according to the enclosed manufacturer's protocol.

## C. PCR and Electrophoresis

Blood was extracted from registered patients at the Children's Cardiac Registry Center at the American University of Beirut Medical Center (AUB-MC) after signing a consent form approved by the IRB. EDTA tubes were used for blood collection and DNA extraction was done via blood extraction kit (QIAGEN) as per protocol. The obtained DNA is then quantified at 260 nm via a Nanodrop. DNA were amplified in a 20 μl reaction mixture using Phusion polymerase kit (Finnzymes) under the following

conditions: 98ºC for 30 seconds as an initial denaturing step; 98ºC for 45 second, 68ºC for 45 seconds, 72ºC for 2 minutes for 35 cycles; a final extension step at 72ºC for 4 minutes followed by a hold temperature at 4ºC.DNA samples are run on 1.5% agarose gels with pSK ladder and separated by size. Desired bands are visualized and cut under UV lights and purified using the Gel Extraction kit following the manufacturer's protocol (peqGOLD Gel extraction kit).

**D. Cell Lines**

HEK 293T 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 at 37 ºC.

**E. 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, Tbx5, activated calcineurin and GATA5). Transfection methods are used for a range of applications, including regulation of gene expression, gene function studies, production of recombinant proteins and mutational analysis.

**F. Polyethyleneimine (PEI) transfection**

Hela 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.

## G. Luciferase assay

In order to investigate the transcriptional regulation of VEGF promoter by NFATc1 mutant, Hela cells were transfected with VEGF /Luc as well as NFATc1 protein (Wt, V210M) and/or calcineurin (Cn) (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.

## H. Immunofluorescence

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). The primary antibodies were diluted (1:500) 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 anti-fading 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 60% 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 HEK293T cells were obtained according to the following protocol. The cells were first washed with 1X PBS. Then 2mL of 1X Phosphate buffered 41 saline (PBS) and 20-30µL of Ethylenediaminetetraacetic acid (EDTA) (chelating agent) were added to the petri dishes to detach the cells. The petri dishes were then placed on the shaker for 20 minutes, to allow the detachment of the cells, and then the cells are harvested in eppendorf tubes and centrifuged for 90 seconds at 11000rpm (fixed-angle rotor). The supernatant is discarded, and the pellet is resuspended in 800µL of buffer A (0.5 M extraction buffer) (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 tubes are placed on ice for 15 minutes. 50µL of NP40 10% is added for each tube, the tubes are vortexed for 10 seconds, and then centrifuged for 90 seconds at maximum speed. The supernatant is carefully discarded,

and the transparent pellet is resuspended in 150µL 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) and the tubes are placed on the shaker in a cold room (4 ºC) for 20 minutes. After that, the tubes are centrifuged for 90 minutes at maximum speed, the pellet is discarded, and 50µL aliquots of the obtained supernatant are prepared, to be stored at -80 ºC 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 protein (20µg protein) were resuspended in 5x lamelli buffer (1mL glycerol, 0.5mL β-mercapto ethanol, 3mL 10% SDS, 1.25mL 1M Tris pH6.7 and 2mg bromophenol blue). The samples were boiled for 3min and were resolved under reducing conditions, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (12%). The proteins were transferred to a Polyvinylidenedifluoride membrane (PVDF) membrane (Amersham), blocked with 5% non-fat dry milk (Nido) solution in Tris-buffered saline (TBS) 1X for 45 minutes with shaking at room temperature. They are 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 TBS-T (TBS, 0.05% Tween 20), the membranes were incubated with the corresponding secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase-conjugated

antibodies) at a dilution of 1:40000 for 2 hours at room temperature. Bound antibodies were detected with the ECL 43 Western blotting analysis kit or Western Lightening Chemiluminescence Kit (Perkin Elmer, Cat # NEL 103) and the protein bands were visualized by autoradiography and ChemiDoc (Biorad).

## N. Co-immunoprecipitation assays

Co-immunoprecipitation (Co-IP) of HA-(calcineurin, Tbx5 and GATA5) and endogenous Flag-NFATC1 (Wt and mutant) were performed using nuclear extracts of HEK293 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 rabbit anti-HA (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 mouse anti- Flag (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. Electrophoretic Mobility Shift Assays (EMSA)

In order to assess the binding affinity of NFATc1 (Wt and mutant) on its consensus region, gel retardation assays were done. The cells were harvested and protein extracts were prepared as previously described. For probe synthesis, two pairs of primers were designed one corresponding to NFAT consensus region. The complementary strands were first annealed, then phosphorylated with T4 polynucleotide kinase and γ-32P-ATP at the 5'-end. It is then allowed to migrate on a non-denaturing 12% Bis-Acrylamide gel (Acrylamide: Bis (38:2), 1.6% APS, TEMED, water and 1x TBE) for 45 minutes at 125 volts. Radioactive double stranded are extracted from the gel and purified using Costar Spin-X columns according to the manufacturer's protocol. The binding reaction was performed by incubating 10µg of proteins with 1µl of the labeled probe, 4µl binding buffer (20mM Tris pH 7.9, 120mM KCl, 2mM EDTA, 25mM MgCl2 and 25% glycerol) and 1µl poly dI/dC (Amersham 1 unit/µl) in a final volume of 20µl completed with water for 20 minutes at 25°C. The samples are run on a 6% non-denaturing polyacrylamide gel (Acrylamide: Bis (29:1), 1.6% APS, TEMED, water and 0.25X TBE) in 0.25 X TBE buffer at 200 volts. The gel is then dried using the BioRad gel dryer (Model 583) for 2 hours at 80ºC; and once dried, is exposed to a phosphor imager screen. The screen is then scanned using the STORM (Molecular Dynamics) scanner in the Molecular Core Facility at AUB.

## P. 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 *NFAC1* 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 mutant is designated as V210M. The activated form of calcineurin will be designated as Cn and NFATC1 double mutant will be designated as 5-55.

## 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. Sanger sequencing was conducted to sequence all the coding exons (exons 1 to 8) of NFATc1 gene in all family members. We found a previously reported SNP mutation (V210M) in exon 2 of the *NFATC1* gen in two patients with an atrial septal defect (ASD), tricuspid valve regurgitation and eye malformations including Aniridia, secondary glaucoma, and anterior segment dysgenesis (ASD). This SNP was inherited from their father in a heterozygous pattern, leading to the substitution of valine with methionine at position 210. The mother was phenotypically normal. None of the 100 control individuals enrolled in this study showed this genetic alteration, suggesting that the SNP could be the mutation responsible for the phenotype.

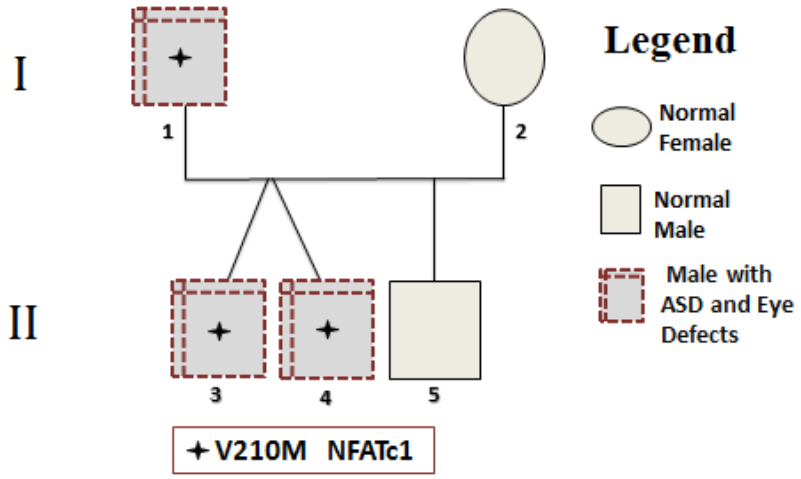



**Figure 23: Genotype-phenotype correlation showing the Mendelian inheritance of the V210M missense mutation.** ASD; atrial septal defect.

To further confirm the V210M mutation, whole exome sequencing was done for this family at the Seidman Lab at Harvard Medical School Department of Genetics by Dr. Akl Fahed. The results reconfirmed those obtained by Sanger sequencing and showed no mutation in any other candidate causative gene such as: Tbx5, PAX6, and BMP4.

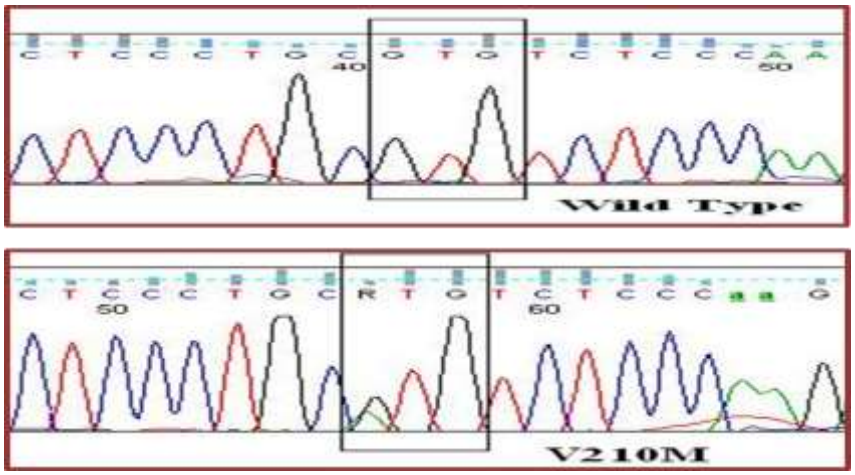



**Figure 24: Sequencing result for the mother (Wild Type) and the affected individuals with V210M mutation (patients and their father).** The boxed region indicates the position of the polymorphism in the patient as compared to normal

sequence. The representative chromatogram shows a heterozygous mutation by which valine was substituted by a methionine at position 210, through a nucleotide change G to A.

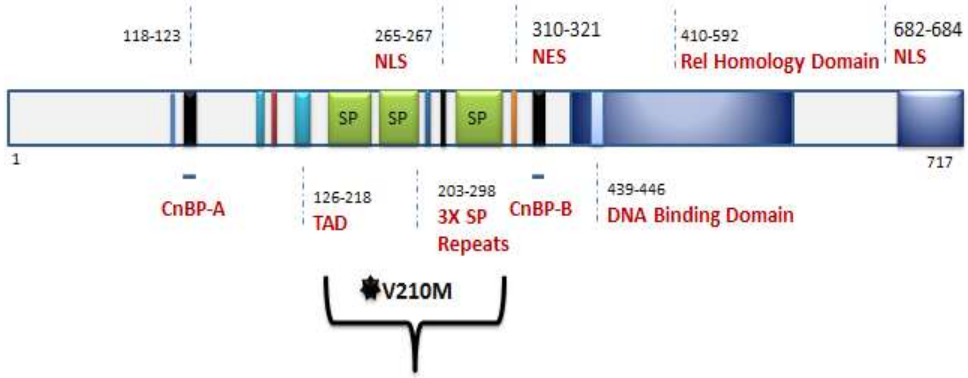



**Figure 25: NFATc1 Regulatory Domains.** The missense SNP leads to V210M substitution in the transactivation domain (TAD) and Serine/Proline repeats (SP Repeats). The scheme above represents isoform A, the most abundant NFATC1 protein with 717 amino acids, a transactivation domain (TAD) at the N-terminus a DNA-binding domain at the C-terminus. (NLS= nuclear localization signal, NES= nuclear export signal, SP= serine- proline).

## B. SNPs Prediction Tools

Prediction tools were used to predict the effect of this missense SNP on the protein function. The *in silico* predictions done by the Polyphen-2 software used by the Exome Variant Server (EVS) database, showed that the V210M mutation is probably damaging to the protein function with a score of 1. SIFT Blink prediction tool predicted that this missense mutation will be deleterious to the function of the protein with a tolerance probability (0.00) less than 0.05. However, PROVEAN protein prediction tool considered the mutation as being neutral with a PROVEAN score of -1.768 (more than -2.5).

Consequently, one may hypothesize that the valvular and ocular defective phenotype seen in patients harboring the V210M mutation, stems from disturbances affecting the regulatory network of transcription factors controlling the two

developmental processes. In order to validate this hypothesis, functional assays will be conducted to assess the effect of the mutation on the cellular localization of NFATC1 protein, its transcriptional activity, DNA-binding affinity, and protein-protein interaction.

## C. Cellular Localization of the NFATc1 Protein

In order to investigate the effect of the mutation on the cellular localization of NFATc1 protein, immunofluorescence assays were done. HeLa cells were transfected with generated plasmids of NFATC1 Wt and mutant, with and without calcineurin.

### *1. NFATC1 Wt and V210M*

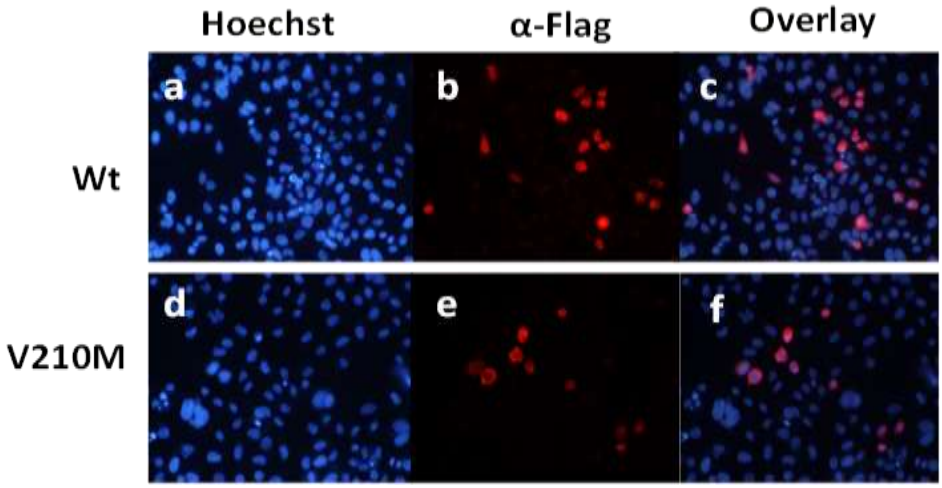



**Figure 26: Cellular localization of NFATc1 proteins in HeLa cells via immunofluorescence (NFATc1 alone)** Wt (a, b, c) and V210M (d, e, f) were found to be located in the cytoplasm as compared to the Hoechst stain (a, d). The localization of NFATc1 and V210M proteins was visualized using anti-Flag antibody (red color). Nuclei were visualized using the Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

*2. Calcineurin*

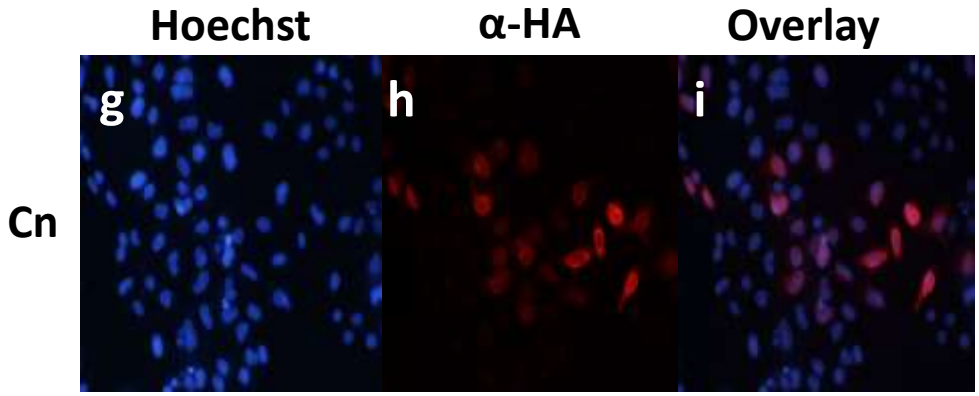



**Figure 27: Cellular localization of calcineurin proteins in HeLa cells via immunofluorescence.** Cn **(**Calcineurin) (h) was found to be located in the cytoplasm as compared to the Hoechst stain (g). The localization of calcineurin was visualized using anti-HA antibody (red color). Nuclei were visualized using the Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

*3. NFATc1 and Calcineurin*

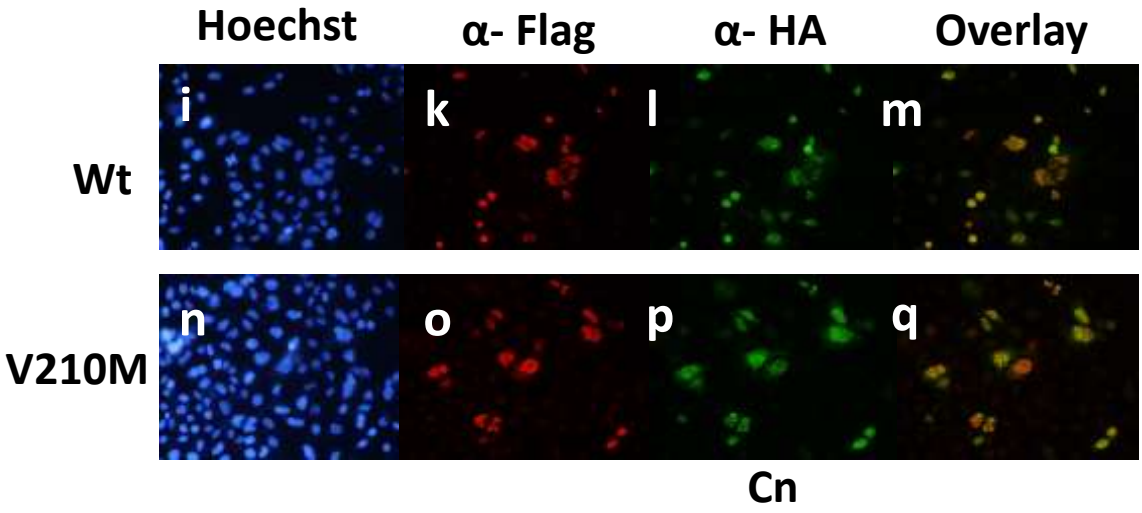



**Figure 28: Cellular localization of NFATc1 and calcineurin proteins in HeLa cells via immunofluorescence** Wt (I, k, l, m) and V210M (n, o, p, q) were found to be located in the nucleus as compared to the Hoechst stain (I, n) when cotransfected with PPP3CA. PPP3CA showed also a nuclear localization. The localization of NFATC1 was visualized using an anti-Flag antibody (red color) while PPP3CA was visualized using anti-HA antibody (green color). Nuclei of the cells were visualized using Hoechst dye (blue color). Images were taken by a fluorescence microscope and captured at x20.

In absence of calcineurin (PPP3CA), Immunostaining revealed that NFATC1 Wt and NFATC1 mutant are retained in the cytoplasm. As expected, calcineurin was localized inside the cytoplasm. When cotransfected with PPP3CA, HeLa cells showed a nuclear localization for NFATC1 Wt, V210M proteins, and calcineurin.

D. **DNA-Binding Affinity**

Gel shift assay was done in order to assess the effect of the V210M mutation on the binding affinity of NFATC1 protein to an NFATC consensus binding region. We performed electrophoretic mobility shift assay (EMSA) using NFATC probes and nuclear protein extracts form HEK293 cells. The latter were transfected with NFATC1 Wt, V210M and calcineurin. In order to retrieve the translocated nuclear NFATC1, nuclear extraction was done. Equal quantities of overexpressed proteins were verified by western blots and used for assessing the DNA-binding activity.

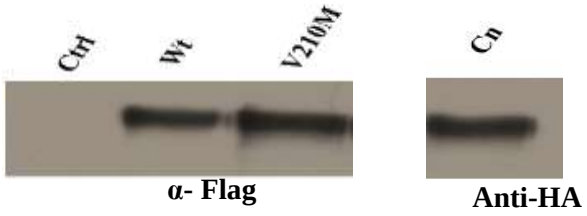



**Figure 29: Western Blot of NFATC1 and calcineurin extracts.** NFATC1 (Wt and mutant V210M) and calcineurin extracts from HEK293 cells were resolved on an SDS-PAGE prior to gel retardation assay. Western blot showed equal amounts of overexpressed proteins as visualized by anti- α-Flag and anti-HA antibodies. Nuclear extracts from mocked transfected HEK293 cells are referred to as Ctrl.

The binding patterns of NFATC1 Wt and V210M with activated form of calcineurin (PPP3CA) were investigated via gel shift assay. 5µg of each protein sample of equal concentration (Figure) was loaded into the gel.

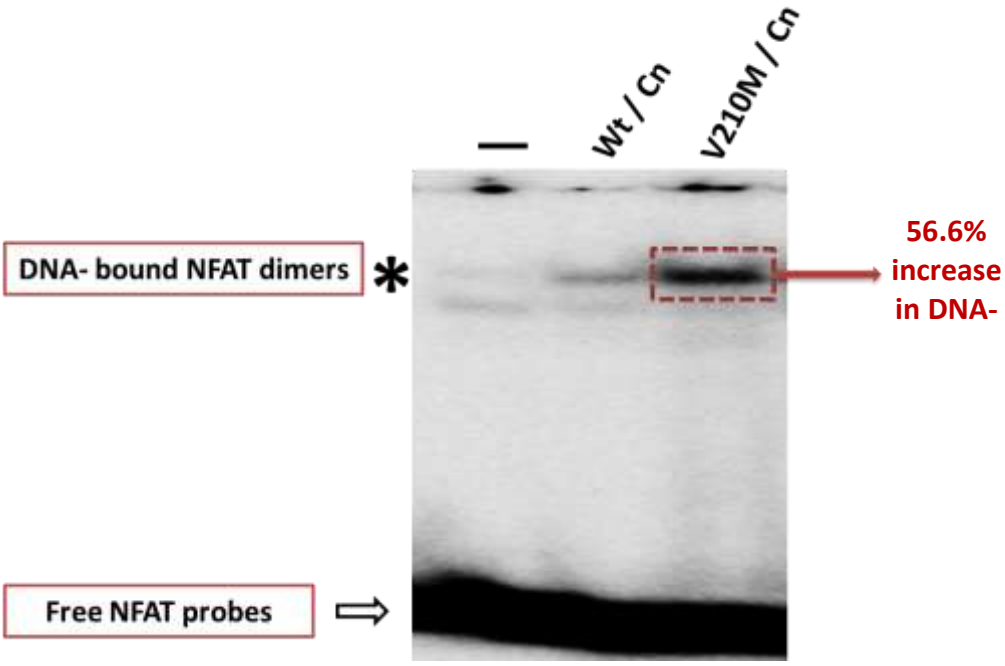



**Figure 30: DNA- binding pattern of NFATC1Wt and V210M mutant with calcineurin on NFATC- consensus binding site as a probe.** EMSA was done using equal amounts of nuclear extracts of NFATC1 Wt / Cn and V210M / Cn. _ sign corresponds to absence of nuclear extracts, * corresponds to NFATC1- DNA complexes, ⟹ corresponds to free $^{32}$P labelled probes.

Interestingly, the V210M mutant showed a stronger binding affinity to NFATC DNA probe as compared to NFATC1 Wt. NFATC1- DNA probe dimers were quantified using Image J software showing an increase of 56.6% in the DNA binding affinity of the V210M mutant as compared to NFATC1 Wt.

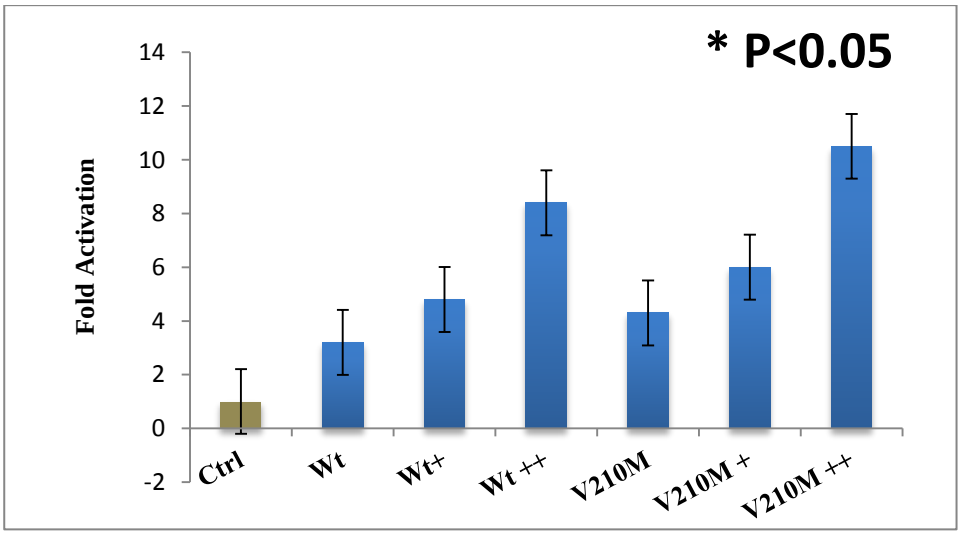
### E. Regulation of Gene Transcription

The vascular endothelial growth factor (VEGF) is a potent regulatory target for NFATC1. Bioinformatics tools were used to determine the potential NFATC1 binding

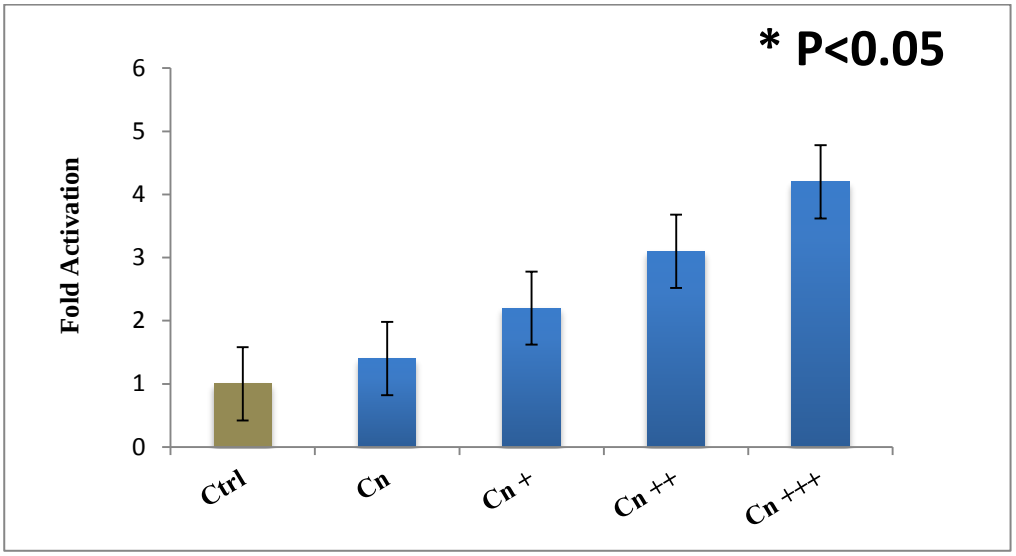
sites on the VEGF promoter. Conserved NFAT binding sites was found in the mouse 1.5 Kb VEGF promoters.

In order to assess the impact of the mutation on the regulatory function of NFATC1 protein, transactivation assays were done. HEK293 cells were transiently cotransfected with 0.8µg of the 1.5kb promoter (VEGF/luc) /well and increasing concentrations of NFATC1 Wt and V210M mutant with or without activated calcineurin (PPP3CA).

### *1. NFATC1*



## 2. *Calcineurin*




## 3. *NFATC1 and Calcineurin*

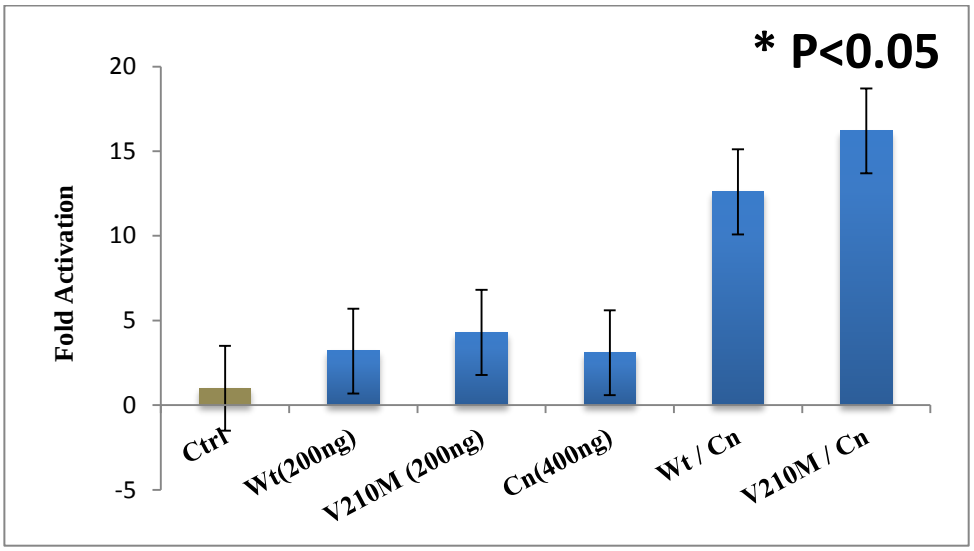



**Figure 31: Transcriptional Activity of NFATC1 Wt and V210M on VEGF promoter.** A- NFATC1 Wt and V210M mutant (200ng, 400ng, and 800ng) dose response. B- Calcineurin (100ng, 200ng, 400ng, and 500ng) dose response. C- Transcriptional activity of NFATC1Wt and V210M mutant (200ng) alone and with calcineurin (400ng). Relative luciferase activities are represented as fold activation. The

data are the means of 3 independent experiments done in duplicates ± standard error. Significance ($p<0.05$) was assessed using the Students T-test.

Luciferase assay was done to assess the effect of the V210M mutation on the transcriptional regulation of VEGF. Calcineurin alone induced a dose-dependent increase in transcriptional activity with a maximum of 4.2 folds. The results showed that NFATC1 Wt is a relatively weak activator of the VEGF promoter with a maximum fold increase of 2. Upon cotransfection with PPP3CA (400ng), the activation of VEGF promoter activity increased by 6 times reaching around 12.6 folds. Interestingly, V210M mutant showed a stronger transcriptional activity with a maximum fold increase of 3.1 and this activation was even stronger when cotransfected with PPP3CA reaching an increase of 16.2 folds.

### F. 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, calcineurin, GATA5, and Tbx5, 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.

### *1. NFATC1 and Calcineurin*

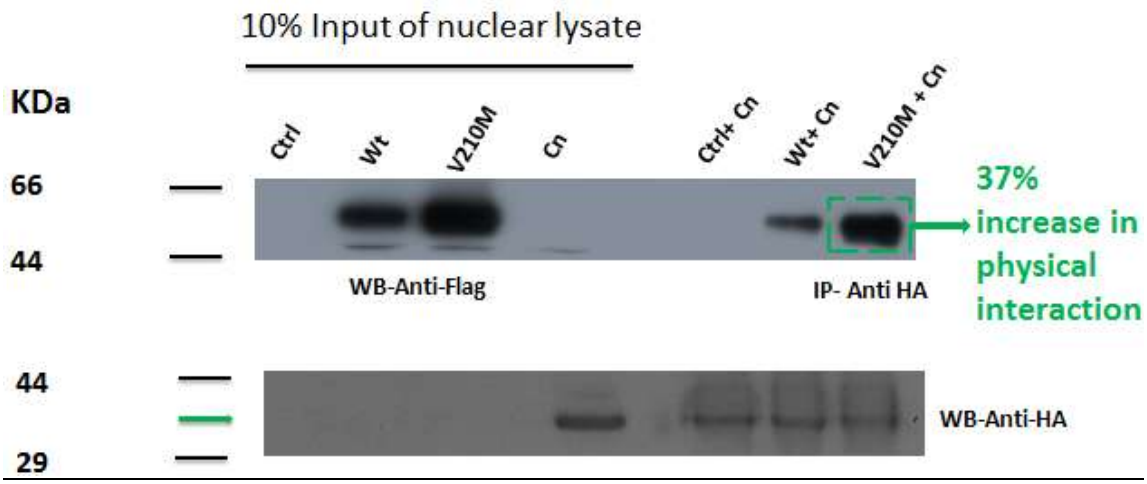



**Figure 32: Co-immunoprecipitation assay showing the physical interaction between NFATC1 and calcineurin.** Physical interaction between Flag-tagged NFATC1 (Wt and V210M mutant) and HA-tagged calcineurin is demonstrated. Ten times the quantity of proteins loaded for western blot was used for immunoprecipitation. 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. ⟶ was used to indicate the presence of calcineurin proteins. CoIP profiles of lower intensities were used for quantification. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD).

### *2. NFATC1 and Tbx5*

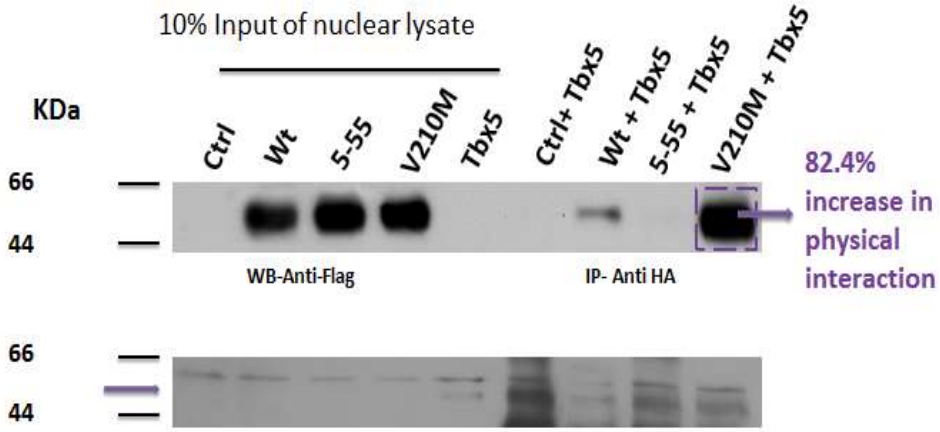



**Figure 33: Co-immunoprecipitation assay showing the physical interaction between NFATC1 and Tbx5.** Physical interaction between Flag-tagged NFATC1 (Wt, V210M mutant,

5-55; NFATC1 double mutant) and HA-tagged Tbx5 is demonstrated. Ten times the quantity of proteins loaded for western blot was used for immunoprecipitation. Nuclear lysates of NFATC1/Tbx5 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 identify Tbx5 proteins. ⟶ was used to indicate the presence of calcineurin protein. CoIP profiles of lower intensities were used for quantification. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD)

### *3. NFATC1 and GATA5*

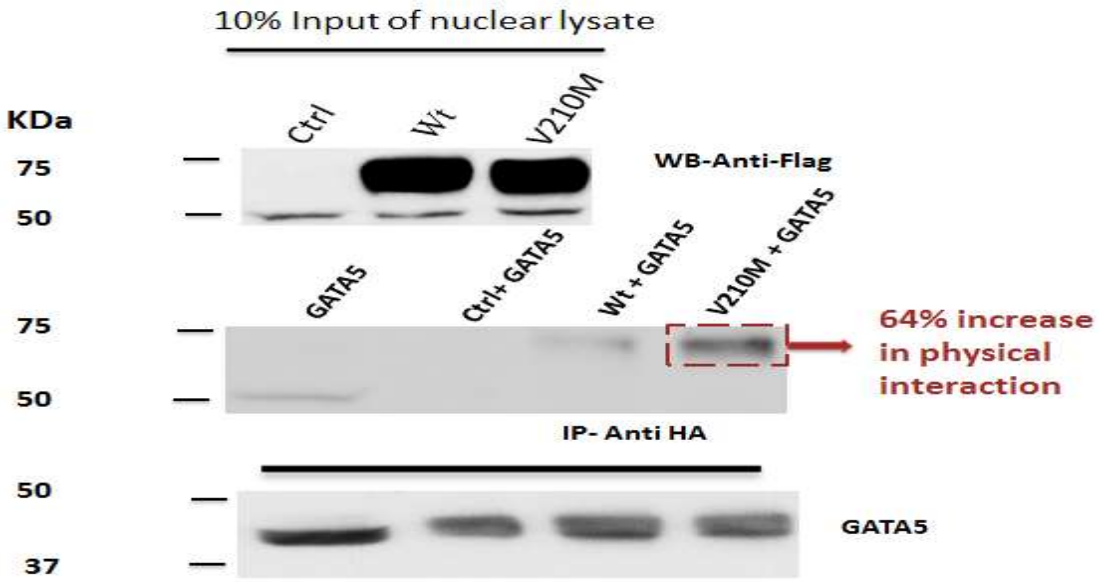



**Figure 34: Co-immunoprecipitation assay showing the physical interaction between NFATC1 and GATA5.** Physical interaction between Flag-tagged NFATC1 (Wt and V210M mutant) and HA-tagged GATA5 is demonstrated. Ten times the quantity of proteins loaded for western blot was used for immunoprecipitation. Nuclear lysates of NFATC1/GATA5 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 GATA5 proteins. CoIP profiles of lower intensities were used for quantification. Quantification was done using Image J software and Image Lab 5.0 software (BIORAD).

The physical interaction between NFATC1 (Wt and V210M protein) and activated form of calcineurin was assessed *in vitro* using CoIP. As expected, NFATC1 Wt interacts physically with calcineurin. Interestingly, the V210M protein showed an

increase in physical interaction with calcineurin of around 37% compared to NFATC1 Wt.

Immunoprecipitation assay showed a novel physical interaction between NFATC1 Wt and Tbx5. V210M mutant has even showed an increase in physical interaction of around 82.4% when immunoprecipitated with Tbx5 proteins. This increasing trend in physical interaction was perturbed as the NFATC1 double mutant (5-55) showed 8% decrease in physical interaction with Tbx5 as compared to NFATC1 Wt.

Given that GATA5 is a potent collaborator of NFATC1, it was used as a positive control and reference protein. The pattern of interaction between V210M mutant and GATA5 proteins, followed the same increasing trend seen with calcineurin and Tbx5. CoIP assay has revealed an increase of around 64% in interaction between GATA5 and V210M mutant.

# CHAPTER IV

# DISCUSSION

Congenital heart diseases account for the majority of congenital anomalies affecting newborns. Valvular and septal defects are among the most frequent forms of congenital heart diseases seen in infants and adults. Extensive research work has been dedicated to unravel and define the molecular mechanistics of valve and septa formation in many organisms. What is evident till now is that there exist a panel of transcription and growth factors taking the lead in such developmental processes. Cardiac-specific promoters are the main targets for candidate genes encoding transcription factors. Consequently, any mutation affecting the regulatory hierarchy implicated in valve morphogenesis will be reflected as a defective phenotype affecting any of the stages; starting from early inductive stages till late remodeling stages. *In vivo* mice models of knockout/ins and *in vitro* attempts of cloning and functional characterization of proteins, have weaved a general rule of genotype-phenotype correlation relating mutations in candidate cardiac genes to CHDs. This rule is validated in a dose-dependent manner, where the happloinsufficiency in some genes such as TBX5 and NKX2.5 proved to be disease causing. In a valvular context, NFATC1 is the prominent regulatory candidate gene, whose perturbations would be linked to valvular defects.

The process of eye development is as crucial as the process of valvuloseptal development. Heterogeneous and overlapping networks of transcription factors orchestrate the formation of ocular structures at early stages of embryology. Lhx2, PAX6, TBX5, BMP4, and BMP7 are few of many candidate transcription factors

implicated in eye development. Delineating the molecular pathways underlying such biological process is crucial in order to explain the genetic basis of many congenital eye defects such as: Aniridia, anterior segment dysgenesis and glaucoma. To date, *NFATC1* gene was not cited in the context of eye development.

Our preliminary results go along with that revealed by other genetic studies targeting the role of *NFATC1*gene in valvuloseptal development and pave the way for further investigation for a prominent role of *NFATC1* in eye development. We aim to fit *NFATC1* with other proteins such as: calcineurin, TBX5, and VEGF under a uniform genetic umbrella regulating the development of valvuloseptal and ocular structures.

### A. SNPs should be revisited: The NFATC1 V210M Story

We have detected a previously documented single nucleotide polymorphism (rs62096875) that has led to a mutation in the *NFATC1* gene in two patients within our cohort of patients with congenital heart and eye defects. Clinical approaches have diagnosed the patients with an atrial septal defect and severe ocular anomalies of Aniridia, secondary glaucoma, and anterior segment dysgenesis. The two patients have a paternal history of Ebstein's anomaly and glaucoma and a normal maternal history. Apparently, a simple heterozygous Mendelian pattern of inheritance is conveyed here. However, it is not well-translated at the phenotypic level as we have unresolved variable levels of severity in terms of cardiac and ocular defects between the two patients. This is not unfamiliar in genetic trait inheritance and is mainly due to a non-shared genetic background even among siblings of the same family. One such example is the variable spectrum of penetrance and expressivity of the *NKX 2-5* mutations,

which showed high and low penetrance patterns among different groups[68]. Comparable cases enduring such variability include also cases of retinoblastoma, Huntington's disease and breast cancer. What may account for such variability in expressivity is the intervention of many factors including: modifier genes, allelic variation, and environmental stress.

The V210M SNP was previously reported as a mere non-pathogenic polymorphism in other populations due to its high minor allele frequency (MAF). As for the Lebanese population, our screening results for more than 100 control cases and 200 patients with different forms of CHD were negative for this SNP suggesting that it could be related to the phenotype observed in the family where it was detected. Such SNPs with differential function in different populations are readily encountered, and one example is the Arg25Cys mutation; known to distort the transcriptional activity of NKX2-5 protein which has been extensively studied at the functional levels despite its high MAF in the studied populations[69]. While annotating our SNP as pathogenic, we took into consideration the genetic variation, susceptibility, and diversity among populations, where variants associated with Type 2 Diabetes, Crohn's disease, and pigmentation phenotypes exhibited large allele frequency differences among continental groups[70]. Trait-associated SNPs paradigms evaluate the SNPs significance based on their low MAF and appropriate segregation within paternal individuals and recent studies have revealed an error rate of 10% characterizing such associations[71]. The most cited examples of false discoveries are the two SNPs: V871M and R456H that have proved to be related to Type 2 Diabetes, though neither was statistically associated with Type 2 Diabetes due to intermediate allele frequencies[72]. Bioinformatics approaches

including SIFT and Polyphen-2 have predicted a deleterious effect for the V210M mutation on the protein function. Collectively, we refined the previous doubted association of the V210M as being non-pathogenic and resorted to functional studies as a tool to evaluate the functional significance of the SNP.

Whole Exome Sequencing has aided narrowing the area of genetic investigation in our study, by which none of other candidate genes such as: *TBX5, PAX6, BMP4,* and *PITX2* except *NFATC1* showed a disease causing polymorphism. Consequently, we postulate that this mutation contributes to the pathology of valvular and ocular lesions by disrupting the functional duties of the NFATc1 protein at the molecular level. Starting from here; our chief target is to characterize the effect of the V210M mutation at the molecular level. The Functional characterization of the *NFATC1* mutation will target four pivotal molecular levels: the level of subcellular localization, transcriptional regulation of downstream targets, integrity of DNA-binding affinity, and protein-protein physical interaction.

### B. V210M: Normal Translocation but Stronger DNA-Binding affinity

At the level of subcellular localization, our results reconfirmed the previously described role of calcineurin as an inductive factor for *NFATC1* nuclear translocation and gave additional evidence of the simultaneous translocation of both *NFATC1* and calcineurin proteins into the nucleus upon induction. The normal translocation of V210M mutated protein into the nucleus indicates that the calcineurin - based N-

terminus dephosphorylating events are probably kept undisturbed. However, a previously reported *NFATC1* double mutant harboring 2 mutations at its N- and C-terminus failed to show normal nuclear translocation[47]. As such, we can infer that any mutation –even not in the calcineurin docking sites- can alter the secondary and/ or tertiary structures of the protein. Consequently, it may disrupt calcineurin dephosphorylating functions and recruitment. This normal pattern of translocation raises additional intriguing questions, leading us to further investigate other molecular levels.

The mutant exhibited a stronger DNA-binding affinity visualized as a single band corresponding to DNA-bound *NFATC1* dimers. The characterized mutation does not affect the Rel Homology Domain (RHR) of NFATc1 protein comprising the RHR-N and RHR-C; the NFAT DNA-binding and dimerization sites respectively. Due to the fact that the locus of the mutation is too far from the DNA binding and dimerization domains, we can argue that the V210M mutant can form dimers more efficiently and in turns may exhibit prolonged periods of DNA binding and higher potential to recruit other collaborators of transcription factors. This conclusion is still unresolved and needs to be confirmed using bioinformatics predictive tools that may depict the changes in the mutant's secondary and tertiary structure. Hence, investigating other molecular levels is crucial to explain the abberative increased binding affinity and uncover the molecular aberrations that have probably led to valvular and ocular defects.

### C. V210M: A Gain of Interactive Function Mutation

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. *GATA5* and *NFATC1* are known to be strong developmental partners, best exemplified by their synergistic activation of endocardial transcription[73]. The inactivation of GATA5 in mice embryos has been linked to a severe phenotype of aortic stenosis, suggesting a role for *GATA5* in the proliferation of valve precursor cells and late remodeling events[73]. Our *in vitro* results showed that the interaction between GATA5 and NFATC1 is augmented by the mutation. Such interaction was shown to be relatively hampered by two mutations affecting the *NFATC1*gene in one patient with tricuspid atresia[47]. Taken together, it becomes evident that the *NFATC1-GATA5* pathway may be crucial at early stages of valvuloseptal development. In the context of human eye development, the expression of GATA5 was not previously reported.

We are the first to describe a novel *in vitro* physical interaction between *NFATC1* and *TBX5*. Our results revealed a drastic increase in the physical interaction between *NFATC1* mutant and *TBX5* in two patients with ASDs and severe ocular defects. To date, little is known about the role of *TBX5* gene during valvulogenesis. The expression of TBx5 is evident at later stages of heart development mainly in the AV cushions and emanating valve leaflets[48]. Moreover, mutations in the *TBX5* gene have been associated with the rare autosomal dominant disorder; the Holt - Oram syndrome (HOS) and cited as a causative gene for a circuit of cardiac defects including: atrial and ventricular septal defects, mitral valve prolapse, conduction problems, and patent ductus

arteriosus[48]. In the context of eye development, *TBX5* is expressed in the dorsal retina and its expression becomes confined to the optic cup, retinal pigment epithelium and neural retina at later stages, where it acts as a dorsal marker. Ophthalmic studies have shown that the expression of *TBX5* in the dorsal optic cup is not solely regulated by BMP4. Though, it is partially controlled by inhibitory BMP4 signals and stimulatory *PAX6* waves through an unknown modulator[74].

Our *in vitro* finding go in parallel with previous data from Dr. Nemer's Lab (Nancy Daouk Thesis 2012) associating a heterozygous mutation in the *TBX5* gene to a defective valvuloseptal phenotype of aortic stenosis and atrial septal defect. Other preliminary data (Theresa Farhat Thesis 2012) have supported this novel interaction at the transcriptional level as the couples *TBX5*-calcineurin and *TBX5*-*NFATC1* have been shown to synergistically activate VEGF promoter. Collectively, we could postulate that the interaction between *TBX5* and calcineurin-induced *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.

Our *in vitro* results have revealed an increasing physical interaction between *NFATC1* mutant and activated -form of calcineurin (PPP3CA). Calcineurin is widely expressed in the mammalian tissues including the retina, immune cells, and brain. The role of calcineurin as an *NFATC1*-activating phosphatase in the heart valves has been extensively studied. Activation of calcineurin has been reported to intervene in retinal

ganglion cells (RGC) apoptotic events in two rodents with ocular hypertension[62]. Relevant ophthalmic studies have also hypothesized that the activated form of calcineurin may contribute to RGC neurodegenration in glaucoma models even in the absence of ocular hypertension and elevated intraocular pressure (IOP)[62]. Such gain of interactive function between both players in conjunction with previous data describing the regulatory role of calcineurin in both eyes and valves, may partially explain the defective ocular and valvuloseptal phenotype. A calcineurin-*NFATC1* mediated pathway could be somehow convincing.

The aberrant increase in the interactive functions of the mutated protein can be correlated to the locus of the mutation that affected the transactivation domain (TAD). It is probable that the mutation has increased the efficiency of *NFATC1* to recruit other partners of transcription factors to different binding sites, thus stabilizing the transcriptional boosting complexes for longer periods of time upstream of target promoters implicated in valve and eye development. This conclusion may help explain the mutant's increasing DNA-binding affinity described above as well. In order to check for the validity of this scenario, we checked for the transcriptional regulation of target genes downstream of *NFATC1*; mainly the VEGF promoter.

*NFATC1* mutation has boosted the transcriptional activity of VEGF promoter and kept the classically described synergy with calcineurin unhampered. VEGF promoter is a potent downstream transcriptional target of *NFATC1* in the valves and probably in the eyes. Endocardial VEGF signaling was shown to be mediated by

*NFATC1*, whose nuclear translocation in endocardial cells is regulated by VEGF. Studies have also shown that VEGF expression is up regulated in the atrioventricular (AV) field of the heart tube after the onset of endocardial cushions. Besides, relevant studies have also revealed a crucial role for VEGF in mediating hypoxia- driven malformations in heart septation[75]. 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 yield mild valvular defects and severe septal ones. This may hint at an unknown regulatory pathway downstream of *NFATC1* contributing to septation. Ophthalmic evidence has suggested a chief role for VEGF in angiogenic ocular diseases, where it was found to promote angiogenesis[76]. Moreover, it has been demonstrated that *NFATC1* plays a crucial role in angiogenic cell behaviors in human retinal microvascular endothelial cells (HRMEC), through VEGF signaling[61]. Taken together and adding the accumulating preliminary data describing the existing synergy between *TBX5*-calcineurin and *TBX5-NFATC1* upstream of VEGF, has led us to integrate *NFATC1* as a central node with other transcription factors mainly *TBX5* and calcineurin in to a uniform regulatory scheme controlling the expression of VEGF in both valvuloseptal and ocular entities. This hypothesis does not eliminate the probable role for other transcription factors such as: Lhx2 and Sox9 and specific common cardiac and ocular promoters in regulating the two developmental processes.

## E. Conclusion

It is the first report describing and characterizing a potential role for *NFATC1* gene in the context of valve and eye development. The report depicts a mutant with super functional potentials at the levels of transcriptional regulation, physical

interaction and DNA-binding affinity. 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*, *TBX5*, and calcineurin at two levels; at the level of transcriptional synergy and combinatorial physical interaction. This model comprehends the unreported expression of *NFATC1* in the eyes through the novel *in vitro* finding of interaction between *NFATC1* and *TBX5* and hints for unregistered candidates implicated in the regulation of *TBX5* during the early stages of eye development other than BMPs. Looking deeply at the spatial and temporal expression of *NFATC1* during EMT in the valves and the evident severe atrial septal defect compared to milder valvular ones seen in both patients, writes down major remarks defining other downstream targets of *NFATC1* during valvuloseptal development.

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.

## F. Limitations and Drawbacks

- The expression system of HEK 293 and HeLa cells is quite far from valvular and ocular profiles.
- The *in vitro* system is not a perfect representative of the *in vivo* system, where multiple pathways can be forcedly down / up regulated upon overexpression and induction.
- *In vivo* studies done via knock in / outs mice models are essential to validate our hypothetical model.

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