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Altered regulation of cell migration in IRF6-mutated orofacial cleft patients-derived primary cells reveals a novel role of RhoGTPases in cleft/lip palate development

By
Mounir Al Saneh

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School of Arts and Sciences
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Roy Khalaf

[redacted]

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Altered regulation of cell migration in IRF6-mutated orofacial cleft patients-derived primary cells reveals a novel role of RhoGTPases in cleft/lip palate development

Abstract

Cleft lip and/or palate (CL/P) affects on average 1 in 700 live births and is considered to be the most common congenital craniofacial birth defect (Setó-Salvia and Stanier 2014). It results from a failure in the lip buds and/or palatal shelves to properly position, proliferate, migrate, or fuse (Bush and Jiang 2012). In order to unravel the causes leading to the occurrence of the cleft, both genetics and molecular factors are being studied. The genetic approach consisted of collecting DNA samples from different families with CL/P and conducting targeted sequencing on all of them. Analysis of the generated reads consisted of aligning, annotating and calling the variants. This was followed by prioritization of candidate variants through several filtering steps. In the molecular part, we isolated two cellular populations from two patients: a control and a patient with a CL/P phenotype typical of van der Woude syndrome (VWS). We obtained primary cell cultures, specifically fibroblasts, derived from the anterior ossified region of the palate. The aim is to look for significant differences in the behaviour of the CL/P patient-derived cells *in vitro* when compared to control cells from the healthy donor through the study of different mechanisms, including cell proliferation, cell migration and adhesion. *IRF6* targeted sequencing revealed mutations in two distinct families; one of the two mutations (p.Ala2Valfs*53), has not been reported in the literature previously. In the second family, a well-known mutation, p.Arg250Gln, was detected in an *a priori* isolated sporadic cleft. Our results showed that there is no difference in viability of the VWS patient cells compared to healthy control cells. However, a significant decrease in the migratory and adhesion ability of the VWS patient cells was detected compared to the control, which could account for the phenotype. The primary fibroblast cells of the affected individual with VWS showed a loss of directionality as was detected by the motility assays. Additionally, Rac1 activity was upregulated in the patient cells, and reflected by the accumulation of WAVE2 and Arp2 at the cell periphery resulting in a

loss of productive migration, as opposed to their localization to a defined leading edge in motile healthy cells. The *IRF6* mutation holds diagnostic value and provides better risk estimations. The results showed an increased understanding of the molecular mechanisms of VWS and expand the knowledge of its occurrence. They provide a strong molecular evidence that CLP/VWS phenotype could be caused by a defect in the migratory ability of the cell.

Keywords: CL/P, defect, VWS, proliferation, migration, differentiation, fibroblast.

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LIST OF ABBREVIATIONS

CL/P: cleft lip and/or palate

NSCLP: non-syndromic cleft lip palate

TGFA: transforming growth factor alpha

SHH: sonic hedgehog family

TGfβ: transforming growth factor β

FGF: Fibroblast growth factor

CPO: cleft palate

Fgf10: fibroblast growth factor 10

Fgfr2b: fibroblast growth factor receptor 2b

IRF6: interferon regulatory factor 6

VWS: van der soude syndrome

FAK: focal adhesion kinase

MAPK: mitogen activated protein kinase

ECM: extra cellular matrix

NME: nucleoside diphosphate kinase

WAVE: WASP-Family Verprolin-Homologous

Rho A: Ras homologous member A

PCR: polymerase chain reaction

DNA: Deoxyribonucleic acid

Rho: Ras homologous

GTPase: Guanosine triphosphatase

Arp2/3: Actin-related protein 2/3

Rac1: Ras-related C3 botulinum toxin substrate 1

GTP: Guanosine triphosphate

GDP: Guanosine diphosphate

GEF: Guanine Nucleotide Exchange Factor

GAP: GTPase Activating Protein

GDI: Guanine Nucleotide Dissociation Inhibitor

PSA: Penicillin Streptomycin Amphotericin

FBS: Fetal Bovine Serum

DMEM: Dulbecco's Modified Eagle Medium

SRR: serine rich region

TIAM: T-lymphoma invasion and metastasis-inducing protein 1

TCF: tissues from craniofacial cases

ECL: Enhanced Chemiluminescence

PBS: Phosphate Buffered Saline

HEPES: hydroxyethyl piperazin-1-ethanesulfonic acid

BSA: bovine serum albumin

PFA: paraformaldehyde

SDS: sodium dodecyl sulfate

Chapter 1

Literature Review

1.1 Facial Development:

Facial development is a complex mechanism that involves a precise coordination of cell signaling pathways (Welf and Haugh 2011). It is a process that includes all three germ layers: endoderm, ectoderm and mesoderm (Chen et al. 2017). It occurs at an early stage of development during embryogenesis, at around the third week of gestation where the oropharyngeal membrane starts to form and can be seen at the area between the heart and the brain (Ansari and Bordoni 2020). At this early stage of fetal development, the oropharyngeal membrane that is made up of both an inner and outer endodermal layer would separate the primitive oral cavity from the developing pharynx. The oropharyngeal membrane soon breaks down to establish continuity between the ectoderm-lined oral cavity and the endoderm-lined pharynx (Master Dentistry Volume 3 Oral Biology Oral Anatomy, Academia.edu n.d.). The oropharyngeal membrane is surrounded by elements that will eventually form the face that are the tissues of the first mandibular pharyngeal arch and the frontonasal process. The frontonasal process would rise from neural crest cells and it will give two medial and two lateral nasal processes. The intermaxillary process would form at the midline after fusion occurs between the medial nasal processes. After ten weeks of gestation, the philtrum of the upper lip and nasal bridge would be formed. On the other hand, the mandibular pharyngeal arch that originates from the neural crest and mesoderm would give rise to two mandibular and maxillary processes that will form a pair of palatal processes at later

stages (Ansari and Bordoni 2020). The nasal placodes would then sink into the mesenchyme in order to form the primitive nasal cavities. The nasal pits would continue to deepen until they eventually approach the roof of the primitive oral cavity. By the end of the fifth week, the membranes would rupture to form communications between the developing nasal and oral cavities (Themes 2015).

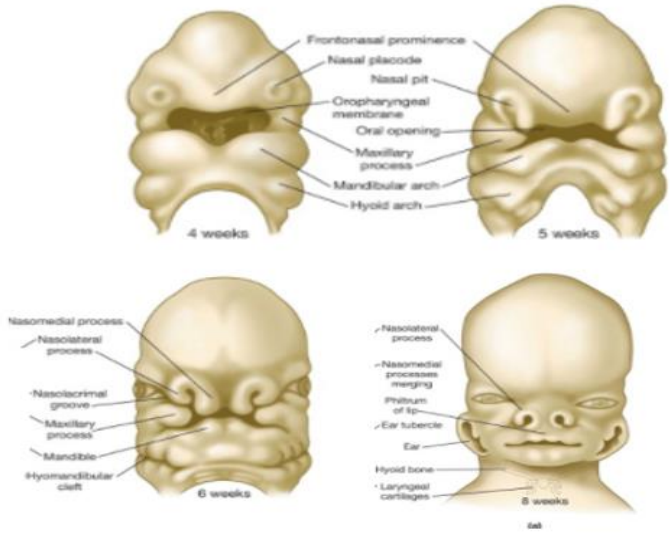


Figure 1: Facial development, from week 4 till week 8. Adopted and modified from Ultrasound Evaluation of the Fetal Face and Neck.

The palate is an anatomical component that separates the nasal from the oral cavity and forms the roof of the mouth (Helwany and Rathee 2020). The primary palate's core would be formed by the fusion of the maxillary, nasal and medial prominences during the first six weeks of gestation (Jiang, Bush, and Lidral 2006). Anteriorly, the nasal and oral epithelium will touch and form the oro-nasal membrane (Ansari and Bordoni 2020).

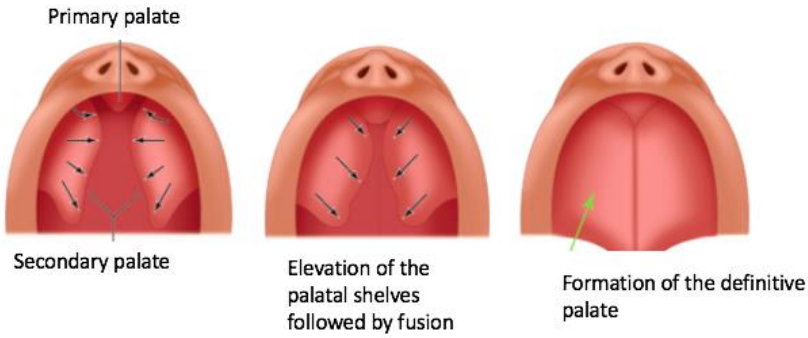


Figure 2: Palate formation steps. Taken and modified from Development of Cleft Lip and Palate

The formation of the hard and soft palates left is done by the secondary palate during the seventh to eighth week of gestation. The secondary palate is formed from the two palatal shelves that would grow parallel to the tongue, and by the end of this stage the palatal processes would fuse along with the primary palate to eventually form the definitive palate (Secondary Palate - an overview | ScienceDirect Topics n.d.).

Any defect in facial development during gestation can lead to clefts of the lip or palate. For patients diagnosed with cleft lip, the lip forms between the fourth and the seventh week of gestation (Smarius et al. 2017). During the sixth week, most of the maxilla, lateral upper lip and the secondary palate would form. In order for this formation to occur, the maxillary processes that are found on each side of the mouth would grow and merge in with the medial nasal processes (Jiang, Bush, and Lidral 2006). Any failure in the fusion process would lead to an opening in the upper lip, either unilateral or bilateral (Mossey et al. 2009). In a group of NSCLP patients, *TGFA* was sequenced and five mutations were found that could be a cause for cleft lip (Machida et al. 1999). The combined effect of the *TGFA* TaqI C2 mutation and

maternal smoking increases the risk of CL/P by two if multivitamins were not taken in the first trimester of pregnancy (Kohli and Kohli 2012).

For cleft palate patients, any disturbance in the development of the palate might lead to a cleft. For example if there was a problem in the palatal shelf growth, a failure in palate elevation or a fusion block during the process would cause a cleft palate (Li, Lan, and Jiang 2017). Events such as cellular growth, proliferation and palatal shelf formation operate through several molecular networks between the palatal shelf epithelium and mesenchyme (Yu et al. 2009). The tight spatio-temporal control especially in developing organs is important because too little or too much control could be detrimental. Growth factors and signaling molecules such as the sonic hedgehog family (Shh), transforming growth factor β (TGfβ) super family, fibroblast growth factors (Fgfs) with their receptors and targets are all included in these molecular networks (Greene and Pisano 2010). For instance, the role of fibroblast growth factor (Fgf) is crucial during palatogenesis in Shox2 mutant mice and it is subject to the tight spatio-temporal regulation. Mice that have a mutation in the Shox2 gene grow a type of CPO detected in humans also. This type of CPO is characterized with cleft of the hard palate along with an intact soft palate. It is due to an abnormal proliferation and apoptosis since even if a number of protagonists are expressed normally. The site and time of signalling play a crucial role for development to occur normally, for example: in Shox2 mutant mice, Fgf10 and Fgfr2b expression was at ectopic sites in the mesenchyme of the mice. This shows that timing and site of signaling are crucial for normal development (Yu et al. 2009b).

1.2 Orofacial clefts

1.2.1 Definition

Orofacial clefts are one of the most common congenital birth defects (Schutte and Murray 1999). They have a global incidence of 1/700 live births (Gowans et al. 2016). Cleft lip may result from perturbations in proliferation, migration and survival of the neural crest cells. Also, they may rise due to a problem in the fusion between the medial nasal and maxillary processes. A problem however in the growth of the palatal shelf, such as its fusion or elevation may result in cleft palate (Bush and Jiang 2012). Thus, the process of facial development requires a precise coordination of a series of events since it is a very sensitive process; events that involve cell migration, migration, growth and differentiation. (Setó-Salvia and Stanier 2014).

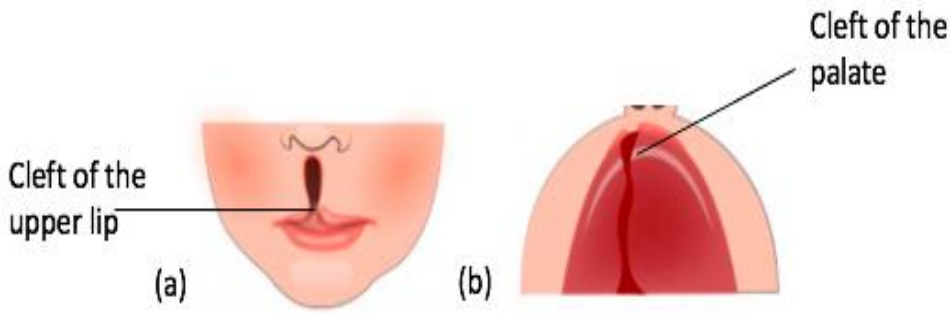


Figure 3: Illustration of the cleft lip and cleft palate. Adopted and modified from Cleft Palate and Cleft Lip

1.2.2 Statistics and Epidemiology

The prevalence of orofacial clefts can vary depending on ethnicity, sex, socioeconomic status, and geographical location. Cleft of the lip and/or the clefts of the palate lip are more common than palate clefts only. It is reported that the highest prevalence rates are known to be North American Indians and Asians (1/500), Caucasian populations are intermediate (1/1000) and Africans have the lowest

incidence (1/2500) (Murthy and Bhaskar 2009). The complexity of this orofacial malformation that is characterized by disruptions of the normal facial structure is considered to be a major psychosocial and economic burden for families and societies (Bueno et al. 2011). CL/P may not be a major cause of mortality, however it does cause morbidity to children (Dixon et al. 2011a).

1.2.3 Forms

There are two forms of CL/P: syndromic and non-syndromic. Several genes such as genes which code for growth and transcription factors, and receptors that are important in wound repair have been reported to be involved in both syndromic and non-syndromic CL/P (Beyeler et al. 2014). 70% of CL/P patients are non-syndromic. Such patients suffer from no physical or developmental problems. Syndromic CL/P can be caused by chromosomal aberrations or monogenic diseases (Pala and Sonvanshi 2016).

CL/P represents a heterogeneous group of disorders affecting the lips and the oral cavity. Due to the phenotypic diversity, classification was made difficult. A cleft lip can be unilateral which means on one side, or bilateral which is on both sides of the upper lip. Cleft lip can occur alone or can occur with a cleft palate. Similarly, a cleft palate may occur in isolation, without any deformity of the upper lip. The cleft lip can be a combination of either complete or incomplete unilateral or bilateral form, and the cleft palate can be of complete, incomplete, or submucous form (Allori et al. 2017).

Reports show that the most common type of CL/P is unilateral, occurring more frequently on the left side (Jensen et al. 1988). According to the International Perinatal Database of Typical Oral Clefts, 30.2% of the CL/P group had bilateral cleft and 69.8% had unilateral cleft (IPDTOC Working Group 2011). Ratios were

41.1% on the right and 58.9% on the left side (Yılmaz, Özbilen, and Üstün 2019). CL/P was seen more often in males than females however CPO was more frequent in females (Derijcke, Eerens, and Carels 1996).

1.2.4 Complications

Individuals with CL/P may face several problems. The most common problems are with communication skills such as speaking and hearing. The patient's appearance is also affected, he might face problems with feeding that cause long lasting outcomes on both health and social integration (Mossey et al. 2009). Care accessibility has increased in the past few years, but its quality varies substantially. These problems can be corrected through several ways and up to several degrees such as surgery, dental treatment and speech therapy (Dixon et al. 2011b). Psychosocial issues in CL/P patients are noted. It is important to provide care for the patient as he they might suffer of lowered self-esteem and interaction difficulties (Sousa, Devare, and Ghanshani 2009).

1.2.5 Causes

In most of the cases, the cause of the isolated cleft is unknown because both environmental and genetic factors and the interactions between them have been implied in the etiology of this malformation. In general, sporadic cases that have no family history may be more related to environmental risks coupled to genetic predisposition factors, while genetic factors including aberrant gene variants inherited from the mother or father may be directly responsible for the development of familial CL/P that is frequently present along with other signs or symptoms (Setó-Salvia and Stanier 2014). In families with no history of clefts, the risk of cleft in the siblings is not important. During pregnancy, there is an increase in the risk of orofacial clefts as high

as 20% if the mother smokes. Dietary intakes, nutrition, alcohol and medications mothers take during pregnancy such as anti-seizure/anticonvulsant drugs, acutane containing drugs, drugs used to treat cancer, arthritis and psoriasis all play a role in the cleft pathogenesis (WERLER et al. 2011). The core of cleft research is through the identification of candidate genes, which relied on linkage and association studies, gene expression and developmental analysis performed in model organisms such as mice. Candidate gene analysis is used either to identify a causative gene or to confirm an association (Dixon et al. 2011). Gene deletions in certain metabolic pathways such as in GSTT1 (glutathione S-transferase theta) also account during maternal smoking for five-times risk increase to have a child with an oral cleft compared to wild type genotype; due to a deficiency in detoxification pathways (van Rooij et al. 2001). Another example is a defect in the *IRF6* gene on chromosome one. Mutations in *IRF6* cause VWS, known to be the most common form of syndromic clefting, accounting for approximately 2% of all CL/P cases (Leslie et al. 2016).

1.2.6 Diagnosis, management and treatment

Although postnatal diagnosis of CL/P is clinically easy, recent advances in prenatal imaging have made the diagnosis of CL/P and associated deformities and anomalies possible during the fetal period (Abramson et al. 2015). Antenatal scans carried out at the 20th week of gestation provided up to 45% of the diagnosis of cleft lip and/or palate (Taib et al. 2015). Of the children who were not diagnosed antenatally, 72% are diagnosed at birth and the others are diagnosed later since isolated cleft palate is more difficult to diagnose during newborn examination when using the torch and tongue depressor, instead of relying on the digital palpation of the palate (Habel et al. 2006). Ultrasonographic examination is widely used in order to screen for abnormalities (D. W. Kim et al. 2015). Antenatal diagnosis may prepare the parents

and provide them information of treatment protocols for the child post-delivery, however it may negatively impact the rest of the pregnancy if taken as a burden for the family (Blakeley et al. 2019). This shows the importance of an appropriate and timely support for the family. The management of a patient with cleft lip and/or palate represents the commitment of a dedicated team of specialists that provide care at the time of diagnosis until adulthood: a feeding specialist who helps manage the special feeding needs, a geneticist who diagnoses associated syndromes and provides counseling regarding genetic risks, and a specialty nurse that aids the cooperation between the patient, his family and the craniofacial team (Nahai et al. 2005). The purpose of undergoing a surgery is to regain the normal functioning of the orofacial area (Conway et al. 2015). The number of surgeries can vary according to the type of cleft the patient is diagnosed with. (Guerrero 2012). They might reach up to four surgeries depending whether the cleft is unilateral or bilateral (Taib et al. 2015). Speech therapy is considered to be a post-surgery requirement that helps the patient establish the correct placement of articulators and correct air flow in order to minimize speech problems and enhance communication (Nagarajan, Savitha, and Subramaniyan 2009).

1.3 Van der Woude Syndrome

VWS is a rare autosomal dominant condition that has high penetrance and variable expression as first described by Demarquay in 1845. This syndrome includes bilateral lower lip pits, cleft lip and palate along with hypodontia (Gurpal-Chhabda and Singh-Chhabda 2018). These lip pits occur on the paramedian portion of the vermillion border of the lip and represent the most common clinical problem occurring in 80% of the patients (Deshmukh et al. 2014). They result due to notching of the lips at an early stage of development with fixation of tissues at the base of the

notch or may result due to the failure of complete union of embryonic lateral sulci of lip (Hersh and Verdi 1992).

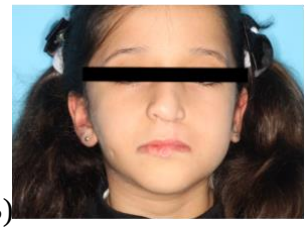
(A)  B) 

Figure 4(A): Patient with a submucous cleft, bifid uvula, two bilateral symmetrical lip pits and no hypodontia. 4(B): Patient with a unilateral CL/P, one left lip pit and no hypodontia.

1.4 Cell motility and migration

Cell migration is a fundamental biological process to establish and maintain a proper organization of multicellular organisms. It is a key process that is utilized by many eukaryotic cells for survival such as the case of single-cell eukaryotes, or in order to accomplish a variety of essential processes for a proper immune response, wound repair and tissue homeostasis (Trepat, Chen, and Jacobson 2012). Cells might migrate either through swimming, which is achieved by the flagella, or through crawling with the aid of actin rich structures that undergo cycles of assembly and disassembly (Fritz-Laylin, Lord, and Mullins 2017). It is a complex phenomenon that is primarily driven by the actin network beneath the cell membrane, and each of these steps is driven by physical forces generated by specific segments of the cytoskeleton. Cell migration can be divided into three general steps or components that are: protrusion of the leading edge, adhesion of the leading edge, deadhesion at the cell body and its rear, and contraction of the cytoskeleton to be able to pull the cell forward (Trepat, Chen, and Jacobson 2012b). Adhesions between the cells or cells with the extracellular matrix share several attributes including cytoskeletal

linkages, signaling molecules and proteins that allow multiple cellular functions. The knowledge of these proteins has grown rapidly in recent years (Weber, Bjerke, and DeSimone 2011). Focal adhesions are protein structures important in the adhesion, deadhesion and migration of cells (Focal Adhesion n.d.). They are found at the end of stress fibers and have a lifetime between minutes and hours. Integrins are transmembrane proteins that recognize the extracellular matrix proteins and they are made of two distinct subunits (α and β), and each combination binds a distinct ligand (Alberts et al. 2002). Focal adhesions undergo cycles of assembly and disassembly. Its role in assembly is activated with the initiation of downstream signaling once a growth factor binds the integrin β-subunit domain (M. Kim, Carman, and Springer 2003). After their activation, integrins bind the extracellular matrix that would then recruit signaling proteins and initiates downstream signaling that includes FAK phosphorylation and MAPK activation along with several other proteins that influence cell adhesion, cell migration, migration speed, number of focal adhesions that all play along and affect the migration process in total (Laukaitis et al. 2001). Focal adhesions disassembly is a process that is not well clear because several factors contribute to it. It has been reported that the degradation of the extracellular matrix is in part responsible for the adhesion disassembly, thus the degradation of the extracellular matrix by the matrix metalloproteinases could be one of the factors resulting in an increase in cell motility and invasion (Degraded Collagen Fragments Promote Rapid Disassembly of Smooth Muscle Focal Adhesions That Correlates with Cleavage of Pp125FAK, Paxillin, and Talin n.d.). Cells therefore would move in response to a signal such as an inflammatory response factors for macrophages (Inflammatory responses and inflammation-associated diseases in organs n.d.). The signal is detected by the cell membrane receptors that transmit it to the interior of the

cell where the direction of motion is decided. The signal would be then transduced in a series of pathways that would thus lead to actin cytoskeleton remodeling at the leading edge and form protrusions from the cytoplasm called lamellipodia and filopodia, then the actin polymers are stabilized with the aid of focal adhesions at the front end of the cell while the rear end is retracted by the detaching of focal adhesions from the ECM (Redox regulation of the actin cytoskeleton and its role in the vascular system n.d.)

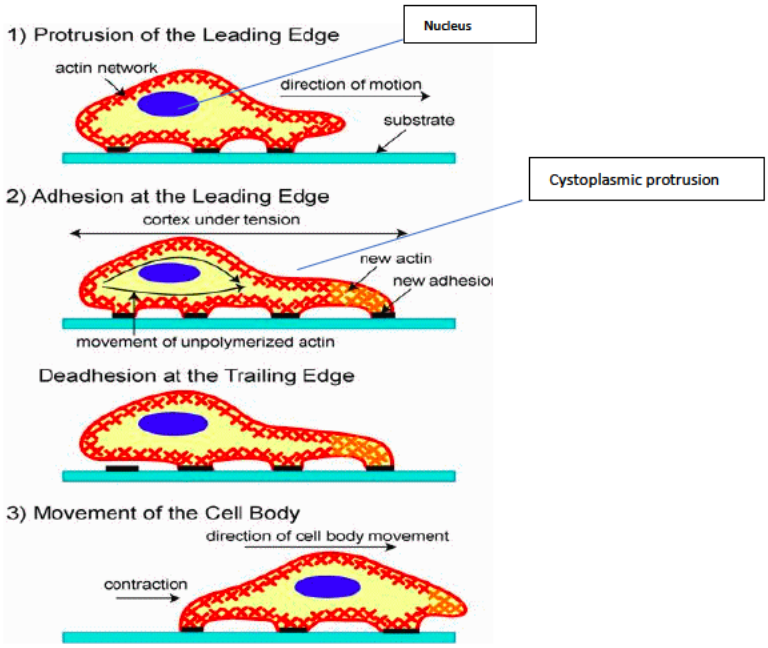


Figure 5: Cell motility cycle upon the stimulation of cell migration. The cell would extend its cytoplasmic protrusions in the direction of the signal then adheres to the extracellular matrix, then detachment of the structure in the tail ends and the cell would retract. From Introduction to cell motility. Taken and modified from Leibzig University.

1.5 Family of Rho GTPases

1.5.1 Definition and roles

The family of Rho GTPases is a part of the Ras superfamily, and they are well known for their role in regulating cell migration (Haga and Ridley 2016). They consist of over 20 genes and are the main actin cytoskeleton dynamic regulators.

They contribute to a variety of cellular processes such as cell cycle progression, cell polarity, cell motility, and vesicular trafficking (Kumawat, Chakrabarty, and Kulkarni 2017). This family of proteins has various and tight roles in vital and critical aspects of the cell, so any dysregulation would lead to major consequences.

1.5.2 Rho GTPase alternations

Rho GTPases usually alternate between two forms, an active GTP-bound form and an inactive GDP-bound form (Kumawat, Chakrabarty, and Kulkarni 2017). This tight cycle is basically achieved with the aid of a group of upstream regulators that are the Rho: GEFs, GAPs and GDIs. The GEFs or guanosine exchange factors catalyze the exchange of GDP with GTP in order to activate the Rho GTPase, the GAPs (GTPase-activating proteins) enhance the intrinsic GTPase activity of the Rho GTPase that would therefore lead to its inactivation through the hydrolysis of the GTP. The guanine nucleotide dissociation inhibitors (GDIs) provide an additional regulation through GDP sequestering to maintain an adequate pool of inactive Rho GTPases in the cytosol (Garcia-Mata, Boulter, and E

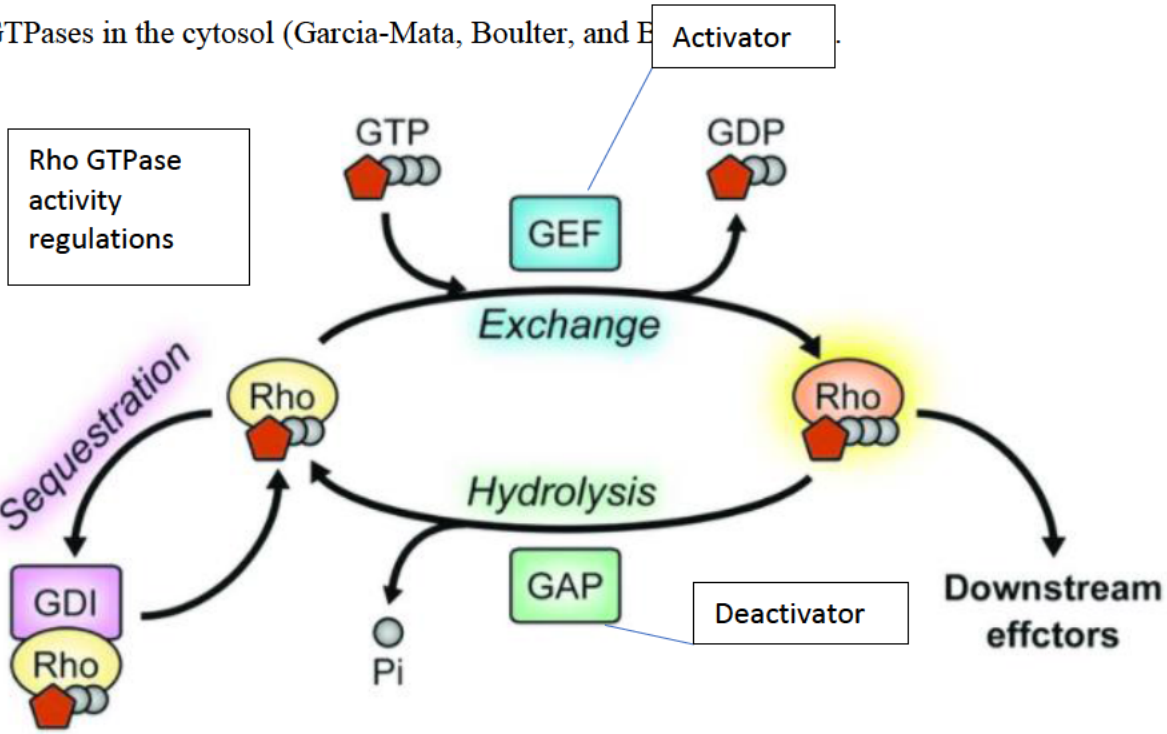


Figure 6: Rho GTPase activity regulation. Activated by GEFs, inactivated by GAPs, GDIs sequester inactive RhoGTPases. Adopted and modified from Emerging roles of Ephexins in Physiology and Disease.

It is important to note that some Rho GTPase proteins are continuously activated because they have a greater GTP hydrolyzing capability with minimal GTP/GDP cycling. Thus, these proteins are considered to be atypical, because they are not regulated by nucleotide cycling but through an epigenetic or post expression modulation by degradation (Spiering and Hodgson 2011).

1.6 IRF6

1.6.1 Definition

IRF6 belongs to a family of nine transcription factors. Most IRF6 proteins regulate the expression of interferons alpha and beta after viral infections, but the function of IRF6 is unknown. The gene that encodes IRF6 is located on chromosome 1 (Kondo et al. 2002). Cytoplasmic IRF6 regulates the complex formed of NME1 and NME2, two principal well-known regulators of Rho GTPases. Thus, a mutation in *IRF6* would affect the dynamic behavior of epithelia during lip/palate development (Whale et al. 2006).

1.6.2 IRF6 and Rho GTPases and their role in motility

It has been shown recently that there are correlations between IRF6 and Rho GTPases when it comes to cellular motility. NME1 and NME2 are two well-known regulators of the Rho GTPases and epithelial junctional remodeling, which therefore regulates the dynamic behavior of the epithelia during the lip and palate development (Disrupted IRF6-NME1/2 Complexes as a Cause of Cleft Lip/Palate n.d.). NME1 and NME2 reside on chromosome 17q11.33 (Hsu 2011). Also, it has been reported that there is an elevation in the levels of Rac1 and RhoA respectively in cultured keratinocytes (Interferon regulatory factor 6 regulates keratinocyte migration -

PubMed n.d.) and in ameloblasts *in vivo* when IRF6 is ablated (Full Spectrum of Postnatal Tooth Phenotypes in a Novel Irf6 Cleft Lip Model n.d.).

Cells enter migrating mode in response to the presence of a chemoattractant. The cell moves in an amoeboid-like manner by determining the direction of the migration according to the place where the chemoattractant is present (Chemotactic signaling in mesenchymal cells compared to amoeboid cells n.d.). Moreover, the direction of motion depends on the localized nucleation of actin and the polymerization at the edge that is leading in order to create membrane protrusions (Alblazi and Siar 2015). When it comes to cytoskeletal rearrangement, the Rho family of GTPases have been proven to have a very high contribution (Karnoub and Der 2013). Rac1 is responsible for the actin polymerization regulation in membrane ruffles and protrusions (Machacek et al. 2009). Furthermore, other studies indicate that Rac1 also mediates the protrusion at the cell's leading edge (Ridley 2015).

Other models suggest that the polymerization of actin at the protruding edge of a cell that is moving depends on the local activation of Rac, which in turn engages WAVE members of the N-WASP family through a complex of proteins that would lead to the Arp2/3-mediated actin filament nucleation and branching (Swaney and Li 2016). All these processes would therefore result in the application of a force that is protrusive to the cell membrane and the extension of the lamellipodia (Al-Koussa et al. 2020).

NME1, a protein that encodes nucleoside diphosphate kinases, interacts with several numerous cellular proteins including the Rho-GEF TIAM1 (T-lymphoma invasion and metastasis-inducing protein 1) that is responsible for activating Rac1 (Hsu 2011). IRF6 through its SMAD-like domain interacts with NME1. This interaction is achieved through the phosphorylation of the SRR (serine rich region)

of the IRF6 SMAD domain. When the SRR is removed, the binding of IRF6 and NME1 is disrupted. IRF6 and NME1 binding would allow NME1 to bind TIAM1, inhibit its activity, thus inhibiting Rac1 activation (Parada-Sanchez et al. 2017).

1.7 Aim of this study

In order to unravel the causes leading to the occurrence of the cleft, both genetics and molecular factors are being investigated. We investigate the genetic basis in DNA samples derived from blood, and molecular mechanisms in primary cells derived from a VWS patient. *IRF6* screening was conducted in a cohort of 200 participants, the results were analysed and the cosegregation of the mutation was verified in all multi-generational families. Primary fibroblastic cells derived from the upper right gingiva and palatal regions were isolated, and two cellular populations from two participants were obtained: a control from a healthy 5-year old boy with no cleft phenotype and the patient from family 2 with a cleft phenotype typical of VWS. The participants are not related. The aim is to look for significant differences in both the gene expression and behaviour of the patient-derived cells *in vitro* when compared to control cells from the healthy donor through the study of different mechanisms, including cell proliferation, cell migration and adhesion**.**

Chapter 2

Materials and Methods

2.1 Patient Selection

As part of the craniomaxillofacial research project at the Lebanese American University, we have collected over 200 individuals from 18 different families presenting orofacial dysmorphic features. Participants provided written informed consent prior to filling out the questionnaire. Parents of the minor participants provided a written declaration of consent as they were legally authorized representatives. Research and data collection were carried out in compliance with the Helsinki Declaration, with the approval of the LAU Institutional Review Board and the Committee on Human Subjects in Research (CHSR). De-identified data about the neurological, muscular, epithelial, and connective tissues related development was collected and all medical conditions were obtained from medical charts.

2.2 Study subjects and screening

Blood samples were obtained by a blood draw and fresh alveolar tissue sample were obtained during surgical repair. For adults, we obtained a blood sample of two collection tubes (4-6 mL/tube). For children, the amount was adapted to their weight. Blood was drawn in EDTA tubes. Genomic DNA was extracted from peripheral blood of the participants using the standard phenol-chloroform extraction procedure.

IRF6 targeted sequencing was conducted on the genomic DNA of the proband from every recruited family. Exons 2- 9 of *IRF6* were amplified by PCR (primers available upon request). PCR experiments were performed in a 10µl total volume mixture containing 20 ng of genomic DNA, 0.5µM each primer, 200µM dNTPs, 0.2

unit Taq polymerase, and 1X PCR buffer supplied by the manufacturer. PCR conditions are as follows: initial denaturation 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 57°C for 30 sec, elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. The amplified products were cleaned and sequenced using Big Dye sequencing kit as recommended. Sequence samples were purified with magnetic beads and run on an automated sequencer model ABI Prism 3700. DNA sequences were aligned and analyzed using UGENE (Okonechnikov, Golosova, and Fursov 2012), a free open-source cross-platform. DNA sequence variants were confirmed by sequencing the opposite strand in the proband and, if possible, by co-segregation of the mutation in all affected individuals.

2.3 Primary cell culture

Primary cells were extracted from the orofacial region of the family 2 proband during palatal surgical closure. In parallel, a tissue biopsy was obtained from the upper right gingiva of a healthy 5 years-old boy. The excess gingival tissue was removed from the crown for dental reasons. Tissues obtained from healthy and CL/P patients were washed three times with PBS (Lonza) supplemented with 10% Penicillin Streptomycin Amphotericin (PSA) (Lonza). They were then cut into small pieces and plated into 6-well plates containing Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma) and 1% PS (Sigma) at 37˚C in the CO2 incubator. After reaching confluence, fibroblasts were passaged subsequently into T25 cm^2 and T75 cm^2 flasks and the medium was replaced every 3 days. At passage 3, cells were cryopreserved in liquid nitrogen. For the assays, fibroblasts were thawed, cultured, and expanded in T75cm^2 flasks containing DMEM (Sigma) supplemented with 10% FBS (Sigma) and 1% PS (Sigma) at 37˚C in the CO2 incubator. All experiments were performed using passages 6-8. The cells were

thereafter annotated TCF (tissues from craniofacial cases) and in this study TCF2 cells were derived from a healthy tissue and TCF4 from a VWS patient.

2.4 Viability Assay

Cells were seeded in a 96-well plate for 24h, 48h, and 72h (Corning Inc. Corning, NY, USA). Cell viability was determined using water-soluble cell proliferation reagent WST-1 (Roche, Mannheim, Germany). After the addition of WST-1 for 2 hours in a humidified incubator at 37˚C and 5% CO2, the color change caused by the cleavage of the tetrazolium salt into formazan by mitochondrial dehydrogenases was measured using a Varioskan Flash plate reader at 450mm (Thermo Fisher Scientific,Waltham, MA, USA).

2.5 Pull-down Assays and Western Blots

Cell lysates were collected from cells treated with the various conditions for the indicated time points. The RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs, Sand Diego, CA, USA) was used for pull-down assay following the manufacturer's instructions. Briefly, cell lysates were incubated with GST-CRIB or GST-RBD for 1 hour at 4 ℃ with gentle shaking. Then, the samples were centrifuged, and the pellet washed for several times. GTP-Rac1 was detected by western blotting using anti-Rac. For the Western blot, protein samples were separated by SDS-PAGE, transferred to PVDF membranes and the proteins of interest detected after incubating the membrane with the corresponding primary antibodies. Secondary antibodies were visualized with chemiluminescent reagent ECL (GE Healthcare). The results were obtained using the Chemidoc imaging system. The levels of protein expression were compared by densitometry using ImageJ (National Institutes of Health, MA, USA).

2.6 Wound Healing

Cells were grown to confluence on culture plates. After 24 h, a wound was made in the monolayer with a sterile pipette tip. Cells were then washed twice with PBS to remove debris and a new medium was added. Phase-contrast images of the wounded area were captured at 0, 12, 24 and 48 h after wounding. Wound widths were measured at 13 different points for each wound, and the average rate of wound closure was calculated (in μm/h). The assay was done using infinity-corrected optics on a Zeiss Observer Z1 microscope supplemented with a computer-driven Roper cooled CCD camera and operated by Zen software (Zeiss).

2.7 Motility assay

Cells were plated to confluence on culture plates. Motility assay was performed 24 hours after seeding. For motility analysis, cells were imaged in *RPMI*-1640 AQ (10% FBS, 1% non-essential amino acid) media, buffered using HEPES, and overlaid with mineral oil on a 37°C stage. Images were collected every 60 seconds for 4 hours using a 20X objective lens on the Zeiss Observer Z1 microscope. The speed of cell movement was quantified using the ROI tracker plugin which was used to calculate the total distance traveled by individual cells. The speed is then calculated by dividing this distance by the time (120 minutes) and reported in μm/min. The speed of at least 10 cells for each condition was calculated. The net distance traveled by the cell was calculated by measuring the distance traveled between the first and the last frame.

2.8 Adhesion Assay

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37°C then washed with washing buffer (0.1% BSA in RPMI-1620 AQ media). The plates were then blocked with 0.5% BSA in RPMI-1620 AQ media at 37°C in a CO2 incubator for one hour. This was followed by washing the

plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to 4x105 cell/ml. 50 µl of cells were added in each well and incubated at 37°C in a CO2 incubator for 30 minutes. The plates were then shaken and washed 3 times. Cells were then fixed with 4% PFA at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining, the plates were washed extensively with water and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 nm using a Thermo scientific Varioskan Flash Multimode reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.9 Immunostaining and quantification of focal adhesions

Cells were plated on glass coverslips. They were fixed with 4% paraformaldehyde for 10 minutes at 37°C and permeabilized with 0.5% Triton-X 10 for 15 minutes on ice. To decrease background fluorescence, cells were rinsed with 0.1 M glycine then incubated with 0.1 M glycine for 10 minutes. For blocking, cells were incubated with 1% filtered BSA in PBS for 1 hour. Samples were then stained with primary antibodies anti-vinculin, anti-Arp2 and anti-WAVE overnight at 4°C and with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 63x objective lens on a Zeiss Observer Z1 microscope supplemented with a computer-driven Roper cooled CCD camera and operated by Zen software (Zeiss). For focal adhesions quantifications, two main plugins were used, CLAHE and Log3D. Briefly, CLAHE enhances the local contrast of the image and Log3D filters the image based on user predefined parameters which allows detection and analysis of focal adhesions.

2.10 Statistical Analysis

The results reported represent average values from three independent experiments. The error estimates are given as ± SEM. The *p*-values were calculated by one way ANOVA or t-test to check if the changes observed in the results were significant.

Chapter 3

Results

3.1 Subjects clinical phenotype and genetic transmissions

In family A in *figure 7*, the proband presented a unilateral CL/P and a unilateral lip pit. Clinical examination and family history revealed the presence of lip pits in seven individuals across three generations. Clinical examination of the affected father and sibling revealed the presence of additional submucous cleft. No presence of hypodontia was noted (***Figure 7A***). Sanger sequencing revealed a c.5_40delinsTGGGGGTCACA at exon 2 leading to a frameshift followed by a premature stop codon (p.Ala2Valfs*53). The mutation was co-segregating in all affected family members.

In family B in *figure 7*, the only affected member was the proband who was diagnosed with isolated sporadic unilateral cleft lip and palate (***Figure 7B***). No other signs and no family history were noted. Targeted *IRF6* sequencing revealed a missense mutation in exon 7, c.749G>A leading to a substitution, p.Arg250Gln, present *de novo* in the patient. This arginine substitution at position 250 has been previously reported to cause VWS syndrome (Little et al. 2009).

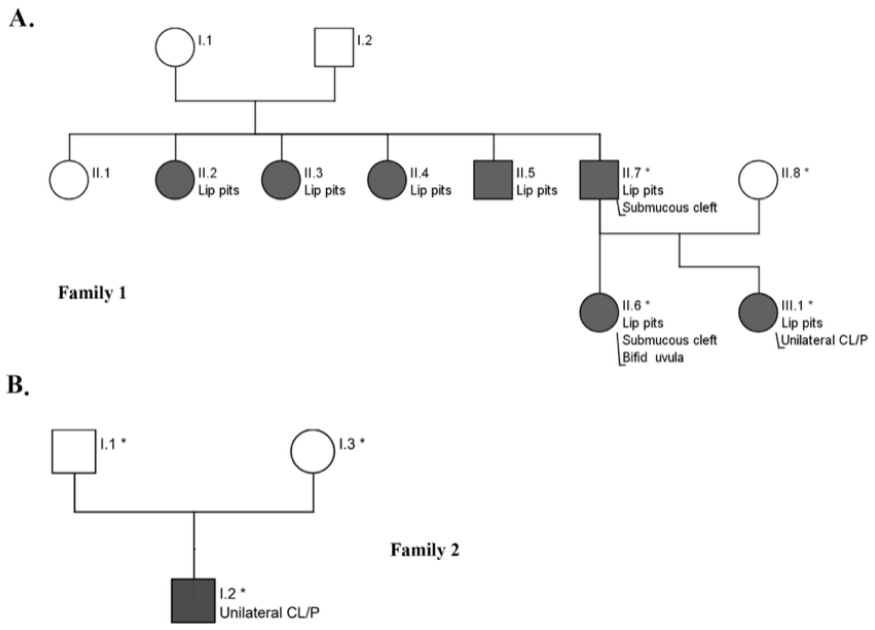


Figure 7: Pedigrees of the studied families. 7(A) Three-generational family with a p.Ala2Valfs*53 mutation. 7(B), Sporadic non-syndromic cleft patient with a p.Arg250Gln de novo mutation.

3.2 No difference in the proliferation rate between healthy donor cells and VWS patient cells

Orofacial clefting could be the result of several factors including insufficient proliferation (Jugessur and Murray 2005). Therefore, the proliferation rate was studied between the healthy donor cells TCF2 and VWS patient TCF4. The results of the WST-1 assay indicated that TCF4 patient cells did not show a significant change in proliferation compared to TCF2.

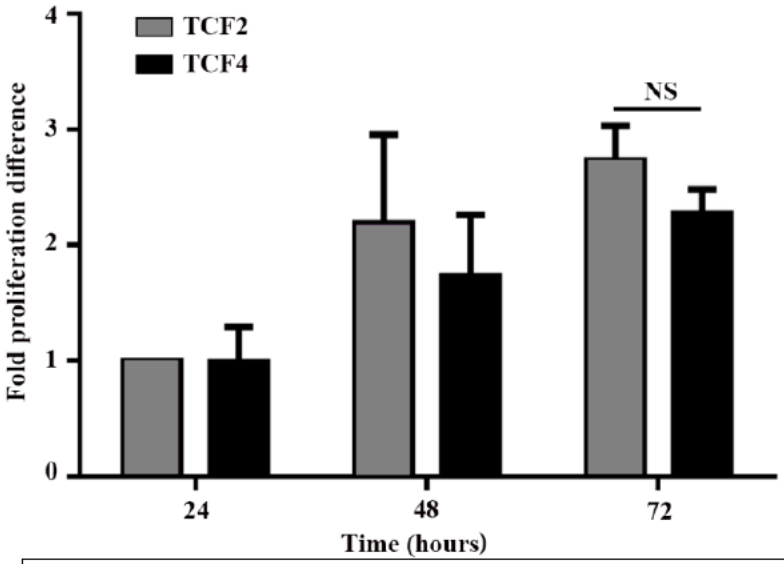
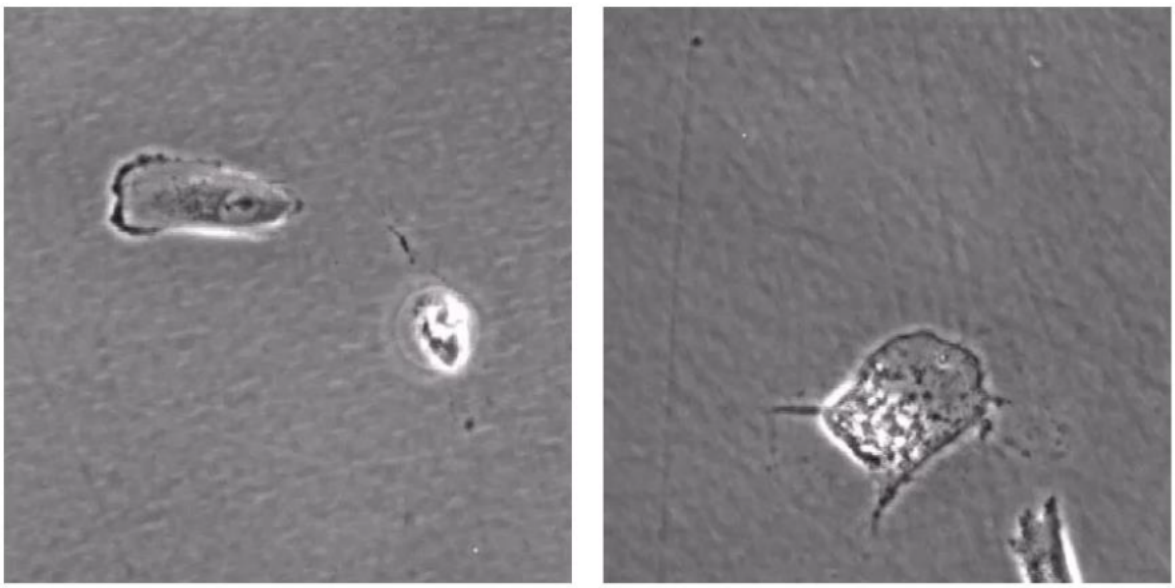


Figure 8: Proliferation rate and fold difference between TCF2 and TCF4

3.3 VWS patient cells' motility is lower than healthy donor cells'

We wanted to look at cell motility to understand the reason behind the gap in clefts, so we performed a wound healing assay for both lines. The results showed a decrease in cell migration in the patient's cells in compared with the TCF2 healthy cells. This effect was shown by a decrease in rate of wound closure at the 48th hour, as well as in random 2D migration in serum, there was a significant decrease in migration that may be a cause for the cleft defect phenotype.



Supplemental movies: S1. TCF2 cells undergoing random migration in serum. Time lapse in phase contrast (40X objective) with a frame every 2 min for a total of 2 hours.
Supplemental movies: S2. TCF4 cells undergoing random migration in serum. Time lapse in phase contrast (40X objective) with a frame every 2 min for a total of 2 hours.

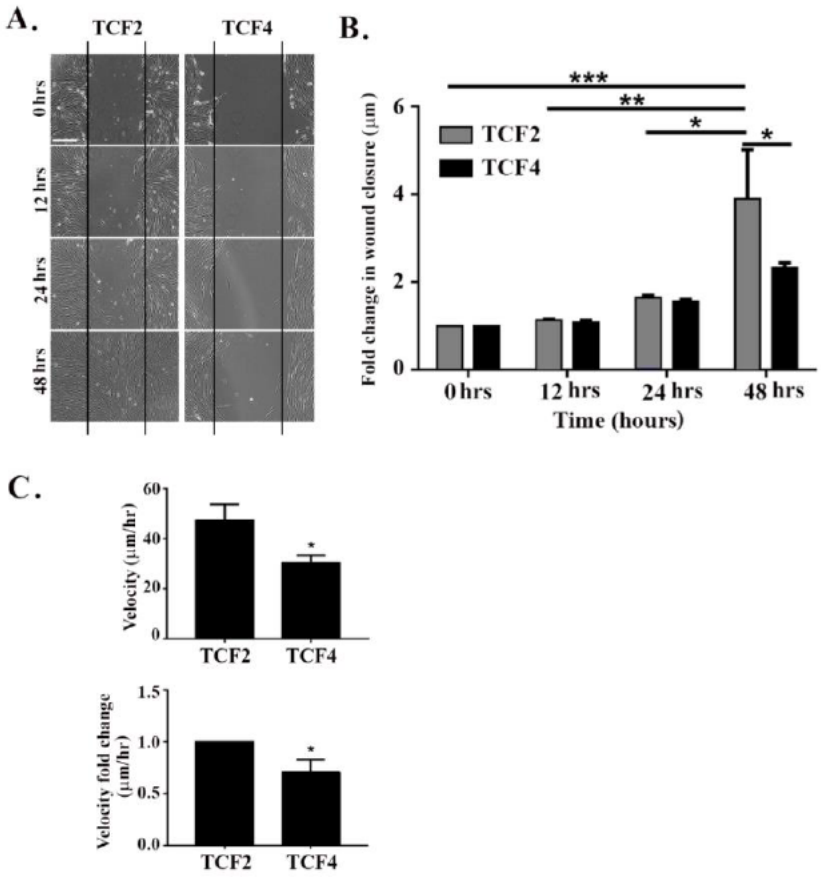


Figure 9(A) Representative wound closure images. 9(B) Rate of wound closure expressed as µm/h. 9(C) Random 2D migration quantitation

3.4 VWS patient cell adhesion to collagen is lower than healthy donors'

We plated the cells on collagen and an adhesion assay was performed in order to point out if there is a problem in adhesion as it is the second step in cell migration. According to figures *10A* and *10B*, TCF4 cells showed a significant decrease in cell adhesion ($p=0.0465$). In order to further look at the adhesion ability, TCF2 and TCF4 cell lines were immunostained for vinculin which is an essential component in adhesion structures. The figures showed that TCF4 cells are more round with less contractility ability, along with more point contacts and a decrease in focal adhesions.

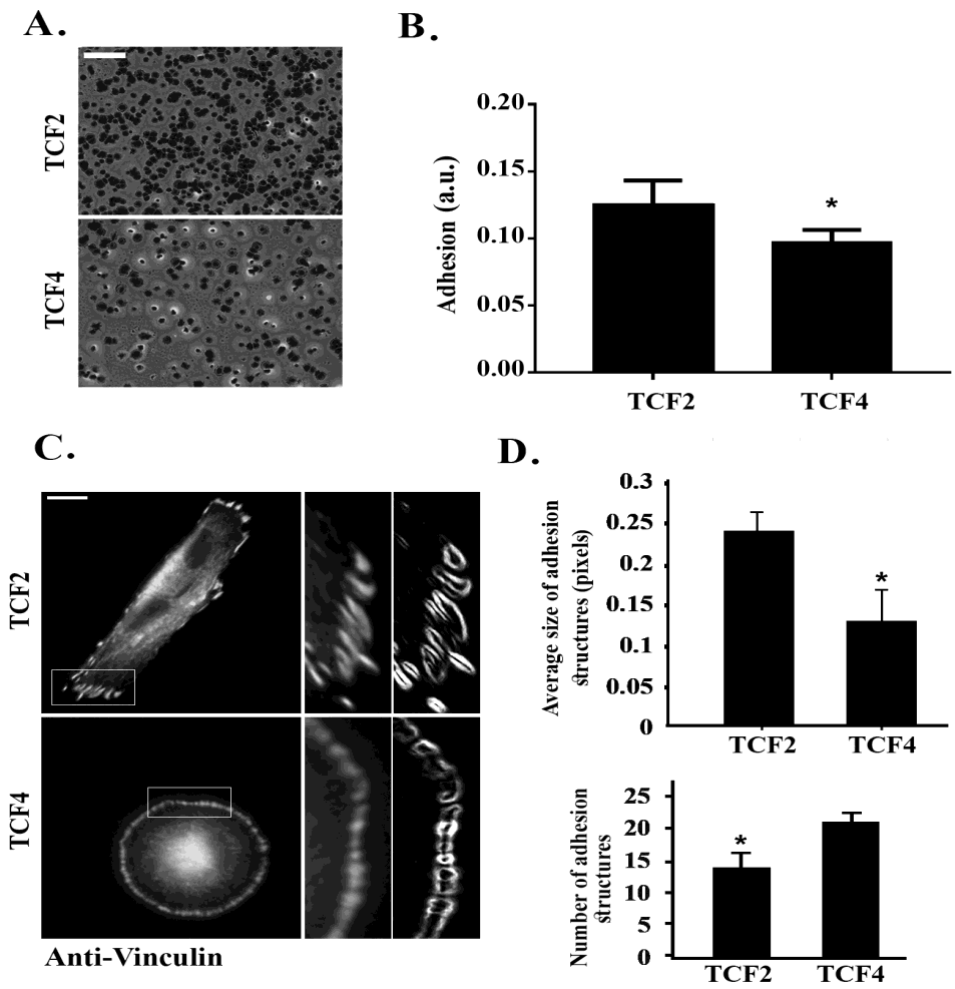


Figure 10(A) Representative micrographs of TCF2 and TCF4 cells plated, fixed and stained with crystal violet. (B) Quantitation of (A) measured at 550 nm using an ELISA plate reader. (C) Representative micrographs of TCF2 and TCF4 cells stained with anti-vinculin. Middle micrograph is 4X magnifications of the box shown on the cell in the left panel. The micrograph to the right shows a representation of the filter applied to the images to count adhesion structures. Scale is 10 mm. (D) Quantitation of focal adhesions.

3.5 VWS patient cell altered migration is due to dysregulation in the activation Rac1

According to the adhesion phenotype that was seen previously, the activation of Rac1 in TCF4 cells was investigated. According to the pull-down assay analysis showed more than 2-fold increase in Rac1 activation in TCF4 cells.

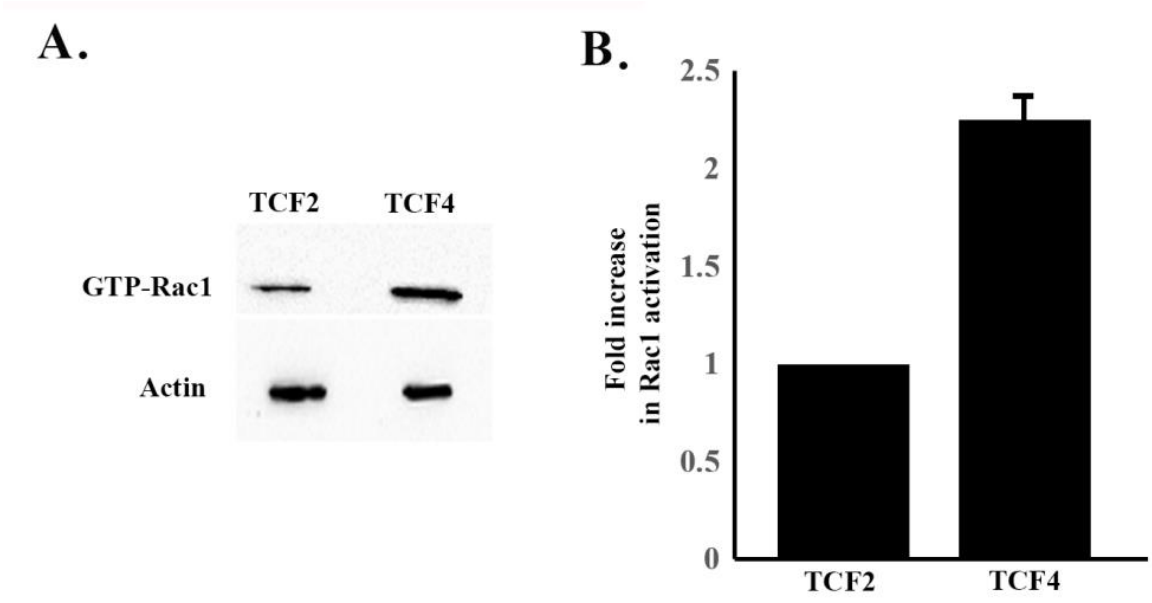


Figure 11(A) Increase in GTP-bound Rac1 in TCF4 compared to TCF2. (B) Graph showing the fold increase of activation of Rac1 in TCF4 compared to TCF2

3.6 Uncontrolled protrusions due to an increase in WAVE2 and Arp2 at the tail lead to unproductive migration of VWS patient cells

According to the immunostaining results, a diffuse staining of WAVE2 and Arp2 was shown all around the cells. Thus, this could affect the migration direction which is starting normally from the tail to the leading edge. The quantitation of WAVE2 and Arp2 show similar expressions of these proteins at the tail and leading edge of TCF4 patient cells, while there is a significant decrease in their expressions seen at the tail of TCF2 healthy donor cells.

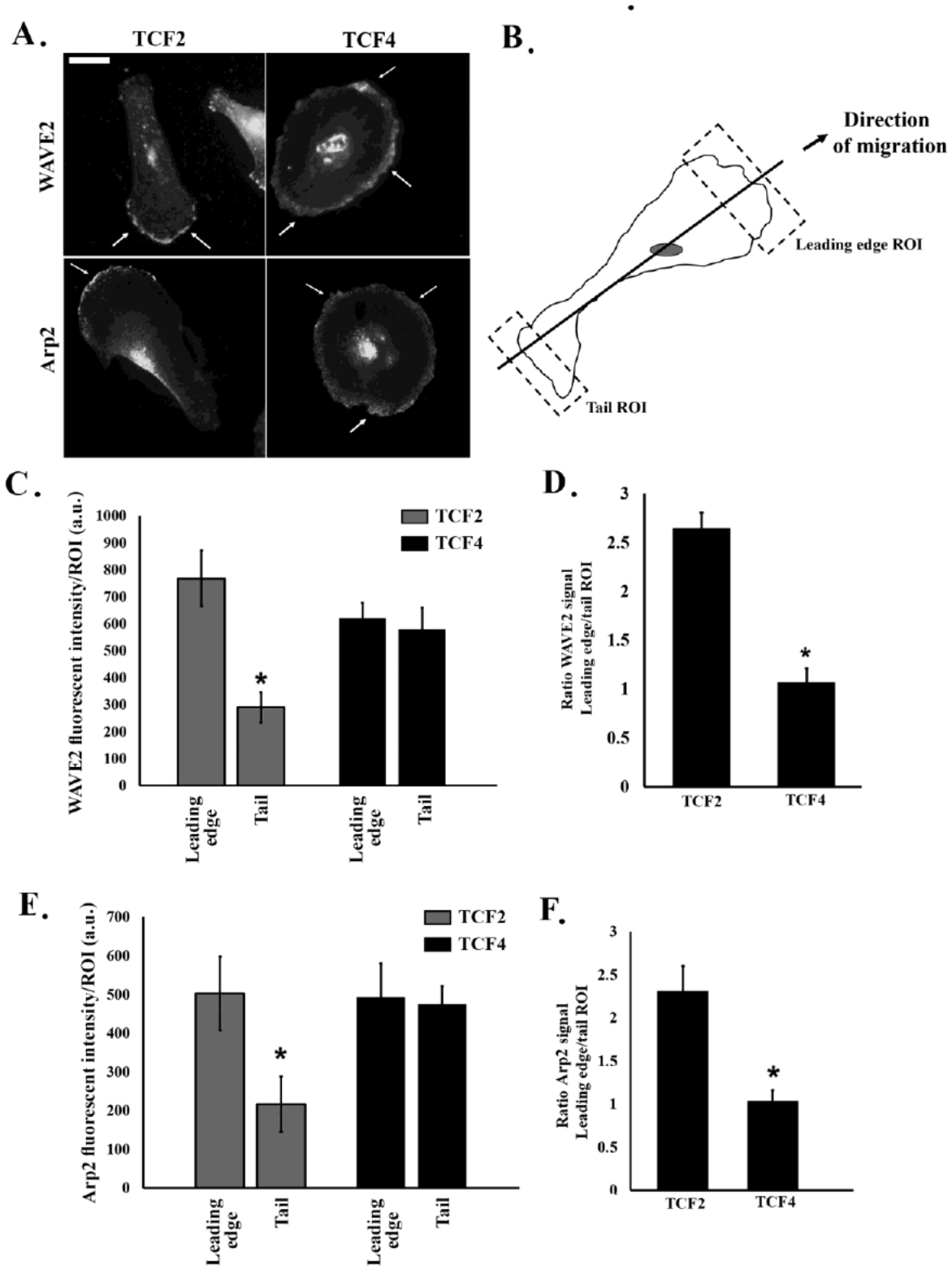


Figure 12: WAVE2 and Arp2 expressed at the tail of TCF2 and TCF4 cells. (A) Representative micrographs of TCF2 and TCF4 cells stained with anti-WAVE2 and anti-Arp2. Scale is 10 µm. (B) Schematic representation of the direction of migration of a normal cell. (C and E) Quantitation of (A). Data are the mean ± SEM. $p < 0.05$ (* $p < 0.05$) indicates a statistically significant difference. (D and F) Ratios of WAVE (D) and Arp2 (E) signals at the leading edge to tail.

Chapter 4

Discussion

IRF6 mutations are associated with both syndromic and non-syndromic CL/P (Parada-Sanchez et al. 2017). We decided in this study to pursue family 2, the individual that is diagnosed with VWS characterized with an IRF6 mutation. We present an overall view of the effects of a specific IRF6 mutation on the cellular physiological processes to increase our understanding of the molecular mechanisms in this VWS patient.

The mutation that we are interested in is a singular mutation at a single gene locus that causes the VWS phenotype. It is a mutation in the IRF6 gene found on chromosome 1 at the q32 locus after targeted *IRF6* sequencing in a *a priori* isolated sporadic cleft in family 2. It is a well-known p.Arg250Gln missense mutation on exon 7 that leads to a change in amino acid from an arginine to a glutamine. This arginine substitution at this position has been previously reported to cause VWS. The position of the missense mutation gives more insight on the function of the IRF6 gene product. IRF6 has two domains, a DNA binding domain (amino acids 13-113) and a protein binding domain (amino acids 226-394) known to be the Smad-interferon regulatory factor-binding domain or SMAD (Kondo et al. 2002). The mutation detected is on the protein binding domain of IRF6, thus disrupting the ability to bind and interact with the NME proteins in the cytoplasm, eventually affecting the activation of Rho GTPases such as Rac 1. The interaction of NME proteins with IRF6 is enhanced by the phosphorylation of key serine residues in the IRF6 C-terminus (Parada-Sanchez et al. 2017). So, after extracting primary fibroblastic cells, TCF2 from a control and TCF4 from the individual with the IRF6

mutation we wanted to look at significant differences in the behaviour of the CL/P patient-derived cells with this specific IRF6 mutation *in vitro,* through the study of different mechanisms: including cell proliferation, cell migration and adhesion.

A WST-1 proliferation assay was performed, and there was no significance in the proliferation difference between TCF2 cells and TCF4 cells, meaning that the proliferation levels are similar, and proliferation does not play a role in the cleft defect phenotype.

The presence of gap in CL/P patients might be due to the inability of the cells to migrate properly. To learn the effect of the IRF6 mutation on the TCF4 cell motility,we looked at the wound healing assay and the 2-D migration assay. In both assays cell motility appeared to decrease, and cell directionality was lost in TCF4 cell. Therefore, it was important to look more at each mechanism of cellular motility individually and try to point out exactly what is going on.

As mentioned earlier, the second step in cell migration is the adhesion of the leading edge. This fact raised our interest in the effect of IRF6 mutation on focal adhesion proteins and the process of adhesion to a substrate. Therefore, an adhesion assay was performed, and it showed that the ability of TCF4 cells to adhere on collagen is significantly lower compared to the normal TCF2 cells. Also, when we stained for vinculin, a marker of focal adhesions, we noticed that the number of focal adhesions in the TCF4 cells was significantly higher than the TCF2 cells, however the size of these structures was smaller. It is not about the quantity of focal adhesions; it is more about the size. In TCF4, the point contacts or small adhesion structures were not able to grow and mature into actual focal adhesions that are needed and are what allow the second step in motility. We also noticed from the vinculin immunostaining that the TCF4 cells have a round phenotype and a complete

lack of directionality compared to TCF2 cells that have a clear directionality, and vinculin was more located at the leading edge of the cells in TCF2 whereas it was located all around the edges of the TCF4 cells. So, there is a clear problem in the distribution of the proteins that are going to allow the formation of these adhesions.

In order to further explain the motility ability, we decided to look at the Rac1 protein that mediates the formation of protrusions at the leading edge of the cell. And it was noted that there was an increase in the activation of Rac1. So, downstream of the IRF6 mutation, there is an increase in the activation of Rac1.

Rac1 plays a role in actin polymerization, and to look more into the effects of the IRF6 mutation we decided to look at the localization of WAVE2 and ARP2 protein. We noticed that in TCF4, the activation of Rac1 and the distribution of WAVE2 and ARP2 proteins were all around the edges of the IRF6 mutated cells as we can see in the suggested hypothetical model in figure 13A. This is what explained the loss of directionality. It is not that there are no protrusions, it is that we have too many protrusions all around the cells forming everywhere. We have the expression of WAVE2 and ARP2 in a diffuse form such that they are all around the cell, leading to the formation of many vectors of direction, the cell would try to move in a 360 direction, eventually they cancel each other out and the cell stays in place.

In order to have a productive migration, as we can see in figure 13B, we have to have a low T/N ratio. T is the total path that the cell takes, and N is the net path. So, a low ratio means the cell is not making unnecessary off-road motion, it is mostly staying on road, thus leading to a high productive migration. In TCF4 cells the ratio is a greater compared to TCF2 cells meaning that there is no productive migration. These results demonstrated molecular evidence that the IRF6 mutation causes defects in the normal physiological process of migration.

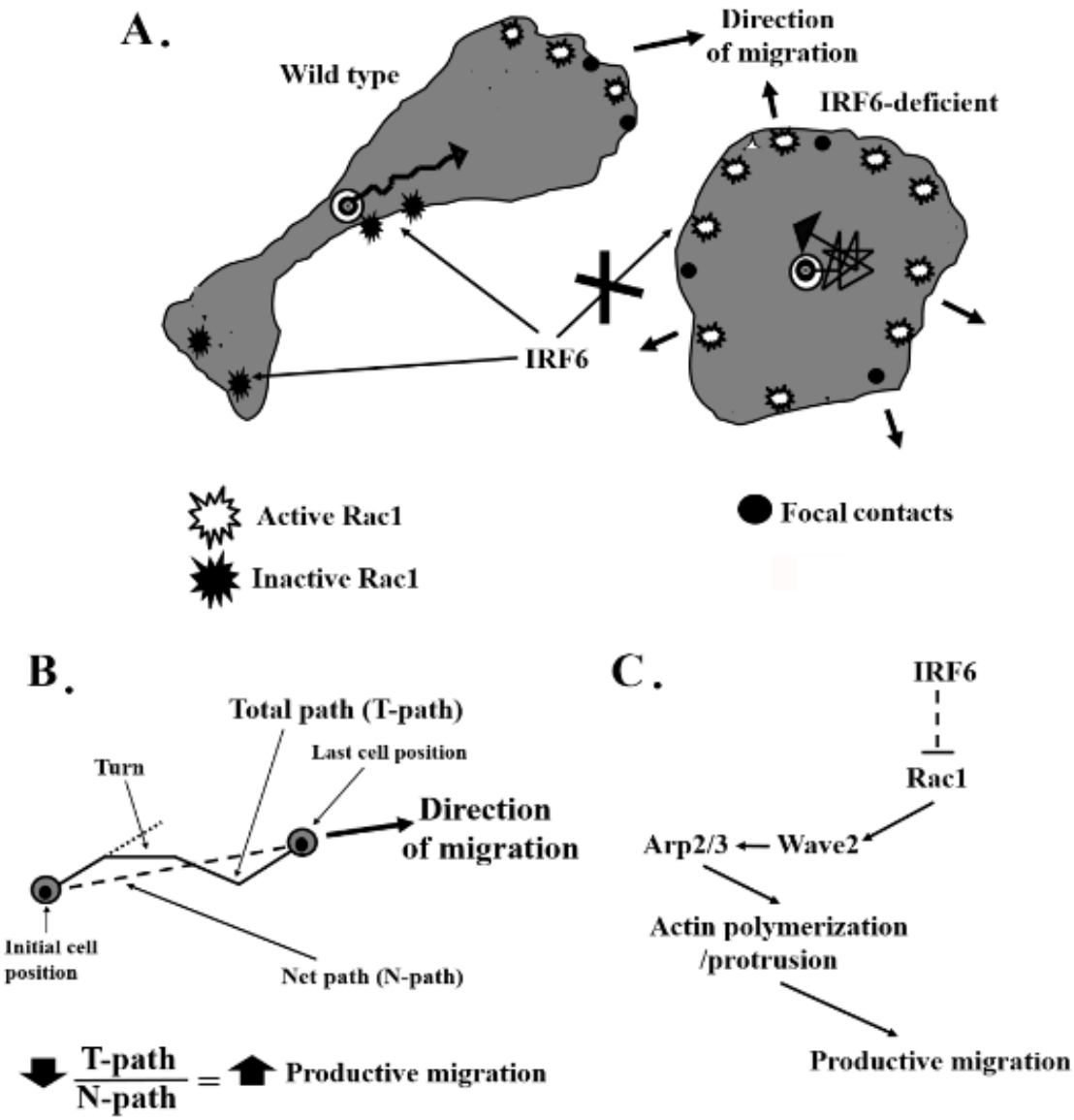


Figure 13: Schematic model of the disrupted multi-directional motility of IRF6-mutated cells. (A) Representation of the distribution and activity of Rac1 in IRF6 wild type cells and IRF6-deficient cells. (B) Drawing of the total and net paths of a wild type cell (C) Proposed model of the correlation existing between IRF6 and Rac1 implicated in the cellular migration control.

Chapter 5

Conclusion

Based on these data that we got, we propose that a mutation in *IRF6* is a loss of function mutation, *IRF6* would lose the ability to inhibit Rac1, thus Rac1 goes out of control, there is an increase in Rac1 expression as well as an increase in WAVE2 and Arp2 expression all over the edge of the cell. It is not just a matter of increased expression; it is a matter of the difuse localization and almost equal distribution around the edges. So, this would give us actin polymerization and prot everywhere causing nonproductive migration. This significantly contributes to morphogenetic cellular changes responsible for the cleft phenotype in VWS. Our findings give increased understanding of the molecular mechanisms of VWS by providing a strong molecular evidence that VWS phenotype is caused by a defect in normal physiological processes of cells orchestrated by *IRF6*. Identifying these mechanisms will bring to light the contribution of *IRF6* to CL/P occurrence.

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