Chapter 2

SCIENTIFIC BACKGROUND
Epstein-Barr virus (EBV), discovered in 1964 [2] by examining electron-micrographs of cells cultured from Burkitt’s lymphoma was the first identified human tumor virus to be associated with various malignancies. The virus has potent transforming activity in-vitro and is found in several human epithelial and lymphoid cancers. However, EBV establishes a lifelong asymptomatic infection in over 90% of the immunocompetent human adult population. Using PCR detection system, it is found that nearly 1 in $10^5$ resting mature B cells carries the viral genome. Virus and host have co-evolved over millions of years with a fine dynamic equilibrium between viral persistence and immune control. Under normal condition EBV initiates, establishes and maintains persistent infection by subtly using virtually every aspect of normal B cell development. Ultimately, this allows the virus to persist lifelong within memory B cells in a nonpathogenic form [50, 51]. However, EBV is ubiquitous and associated with Burkitt’s lymphoma (BL), Hodgkin’s Disease (HD) and Nasopharyngeal Carcinoma (NPC) to a major extent therefore it has raised the serious investigation about the oncogenic potential of the virus [52]. From an innocent bystander now EBV has been classified as group I carcinogen by the International Agency for Research on Cancer (IARC monograph, 1997).

2.1: **Epstein-Barr Virus (EBV)**

The characteristic features of EBV structure are: (1) an icosahedral capsid, approximately 100-110 nm in diameter and containing 162 capsomers, (2) a core containing a linear, dsDNA molecule, (3) an amorphous, sometimes asymmetric material that surrounds the capsid, designated as the tegument and (4) an envelope containing viral glycoprotein spikes on its surface. The double stranded DNA genome of EBV is 172 kb long and encodes more than 85 genes (figure 2.1.1).
The viral genome contains a series of 0.5 kb terminal repeats (TR) at the both ends and four internal repeat (IR) sequences. There are two origin of replication within the viral genome for latent and lytic phase of life cycle i.e. oriP and oriLyt respectively. Since, EBV genome was sequenced from a Bam HI fragment cloned library, ORFs or genes are referenced to specific Bam HI fragments. The major glycoprotein of EBV, gp350 interacts with CD21 molecule on B cell during viral infection [1]. In case of epithelial cells infection (figure 2.1.2), the virus uses
different form of glycoprotein complexes and close cell to cell contact with virus producing B cells is required for efficient infection [53].

**Life cycle of Epstein Barr Virus:** After infection, the viral genome enters into the nucleus and forms a circular episome using the TR sequence. It is considered that individual infection events lead to episome formation that differs in the terminal repeat number. Thus analysis of the TR region by Southern blot hybridization can provide information about the clonality of the virus infected cell population [54]. In EBV-infected cells, virus lytic replication with production of infectious virion is a rare event. Typically EBV establishes a latent infection, which is
characterized by a restricted set of 10 latent genes including EBER1 and 2, EBNA1, 2, 3A, 3B, 3C and leader protein and LMP1, 2A and 2B. A combination of virus isolation and sero-epidemiological studies suggest that all EBV isolates can be classified as EBV1 (type A) or EBV2 (type B) on the basis of sequence variation in the genes encoding EBNA proteins, particularly EBNA2. The in vitro studies suggest that type A virus is more potent than type B in B cell transformation process. In addition to variation within the C terminus sequence of LMP1 gene, a 30 bp deletion is also observed which might be important for the transformation effects of that protein. However, the extent to which that variation is directly related to the pathogenesis remains to be established. Investigations on different cell lines and virus associated malignancies have identified three different types of latency [55]. Gene expression pattern in all three forms of latency is depicted in figure 2.1.3.

During latency I only EBERs and EBNA1 are expressed. This kind of latency is observed in BL [56]. In normal individuals EBV-associated B cell subpopulation also exhibits identical latency programme with additional expression of LMP2A in few cases [57]. Similar type of latency programme with EBERs, EBNA1 and LMP2A expression also has been demonstrated in EBVaGC cases [58]. In non-B cell malignancies such as HD, T cell lymphoma and NPC the virus expresses EBERs, EBNA1 and both the LMPs (i.e. LMP1 and LMP2). This is termed as latency II [59, 60]. In addition a transcript containing the BamHI A rightward open reading frame (BARF0) has been detected at very low level in all situations [61].
Coordinate expression of all the above latent genes leads to a dramatic change in cellular phenotype, including growth transformation. It is termed as latency III. In real life this kind of latency is observed in immunosuppression associated lymphoproliferative disorder and in infectious mononucleosis [62]. Differences between these latency programmes are detectable at the level of mRNA transcript of EBNA1 gene. In latency I and II, the EBNA1 mRNA is initiated from Qp promoter whereas in latency III form all six EBNAs are initiated from Cp/Wp promoter [63]. The switch from latency to lytic cycle is mediated by early lytic proteins, BZLF1 and BRLF1 expression followed by coordinated expression of about 80 lytic genes [64]. During productive cycle, replication is originated from oriLyt and transcription is initiated from Qp promoter. The switch over in usage between the different promoters is tightly regulated by the hypermethylation of the CpG islands of the promoter regions [65].

Fig. 2.1.3: EBV gene transcription in the three forms of latency. The top panel shows the position of the exons on a linear map of the genome. The lower panels show the direction of transcription from each promoter (arrows) and the splicing structure between the exons. Coding exons are shown in black and non-coding exons in white. (Adapted from: Young et al., 2000)
2.2: EBV and Cancer

Since its discovery as the first human virus, EBV has been implicated in the development of a wide range of cancers as depicted in the figure 2.2.1. A brief description of EBV association with various cancers is given below:

(1) **Burkitt’s lymphoma** - Since B-lymphocytes are the natural host of EBV infection; the earliest evidence about the association of EBV with malignancy was established from endemic BL restricted within equatorial Africa. Endemic BL is a childhood disorder and occurs mostly in areas where malaria is holoendemic. All patients affected with BL characteristically carry a chromosomal translocation t(8;14), t(8;2) or t(8;22), placing the c-myc oncogene under the control of an immunoglobulin gene that results in over expression of c-myc oncogene [66]. C-myc is acting as a chromatin remodeling and nuclear matrix attachment factor, which may help EBNA1 to keep the EBV genome in the nucleus of the BL cell. It may explain the high prevalence of the viral genome in BL cases [67]. In EBV-associated BL, the viral genome is present as a monoclonal episome form and only EBERs and EBNA1 genes are expressed. As there is a difference in growth phenotype between EBV-associated BL compared to negative cases. Therefore it is suggested that potent growth transforming ability of EBV and high c-myc gene expression can cooperate to alter B cell growth phenotype [68].

(2) **Hodgkin’s Disease** - HD is characterized by an expansion of Reed-Sternberg cells, which are now postulated to be of B cell lineage. In western population, EBV is detectable in 20-50% of HD cases while in developing countries up to 100% of cases may be associated with the virus. In all EBV-associated HD, viral gene expression follows the latency II pattern with EBNA1, LMP1,
LMP2A and EBERs. Interestingly, although LMP1 and LMP2A are expressed there seems to be no mounted CTL response to the affected cells in vivo [69].

(3) **T cell lymphoma** - EBV is known primarily for its association with B cell, later it was found that EBV could infect the T cell. Nasal T/ NK-cell lymphoma is an aggressive subtype of non-Hodgkin lymphoma (NHL) that is closely associated with EBV and the virus is localized in most of the tumor cells. Lymphoma cell population exhibits different phenotypic and genotypic features including an absence of T cells antigens, expression of NK cell marker CD56 and absence of T cell receptor gene rearrangement [70]. The virus is consistently associated with this lymphoma regardless of geographical location and the gene expression is restricted within EBERs, EBNA1, LMP1 and LMP2 like latency II [71].

(4) **Nasopharyngeal Carcinoma** - Although lymphocytes are the most potent infection sites there is now evidence that suggests the normal epithelial cells in nasopharynx express a distinct EBV receptor that can facilitate the in vivo infection [72]. NPC is endemic in South East Asia and Northern America but rare in most other parts of the world. The association of EBV with undifferentiated NPC has been documented in as early as 1966; later it was substantiated by means of ISH against EBERs, that every undifferentiated NPC cases are EBV positive regardless of their geographical origin [73]. Southern blot experiment has revealed monoclonality of the resident viral genome and gene expression follows the type II latency pattern. In addition to latent gene expression with LMP1 and BARF1, early lytic gene BZLF1 has been detected in some cases [74]. Moreover elevated IgA antibody titer to the viral structural proteins like VCA, EA and MA complexes can be detected several years before the disease prognosis [75]. In several studies EBV-association in majority of NPC cases has been strongly correlated with high incidence of cellular migration *in-vitro* and metastasis *in-vivo* [48, 76].
(5) **Gastric cancer** - In recent years, EBV has been reported to link with the development of GC throughout the world. However, there are some regional differences in the incidence of EBVaGC with highest incidence in USA and Germany and lowest in China [77]. Generally the frequency of EBVaGC is higher in men and also more prevalent in young age group. As observed in other malignancies, EBV is present as a clonal form with the fact that every malignant cell is EBV-positive. It suggests that infection of GC by the EBV occurs early in tumorigenesis process [78]. Although EBERs, EBNA1, BARF0 and BARF1 genes are expressed as similar with type I latency in BL, but LMP2A gene is also expressed as in NPC [58]. Recent results suggest that BARF1 expression in gastric cancer cells may provide a protective role against apoptosis through an increased Bcl-2 to Bax ratio, thus promoting cancer cell survival [79]. There were reports demonstrating that the early lytic gene BZLF1 was expressed in few cases of EBV associated gastric cancer identified so far [80, 81]. Surprisingly, although EBVaGC cell population is very much immunogenic and viral latent gene restricted CTL response is present, but it could not eliminate the virus infected malignant cells. In addition EBVaGC is often accompanied with strong lymphocytes infiltration predominantly CD8+ CTLs but that could not improve any prognosis over the virus non-associated malignancies [5]. Very recently, the invasive behavior of EBVaGC has been reported [82].

(6) **Other cancers** - EBV infection is associated with a few of other epithelial cancers. The most surprising observation was the absence of detectable EBERs expression indicating a new type of latency [83] in Hepatocellular cancer. Carcinomas showing morphological features similar to undifferentiated NPCs, termed as Lymphoepithelial Carcinoma can occur at different sites such as thymus, tonsils, lungs, stomach and uterine cervix. These types of cancer are EBV-positive with a wide variation. Lymphoepithelial carcinoma of the stomach is EBV associated in nearly
all cases regardless of the geographical location [84]. EBV has been detected in thymic epithelial tumors from Chinese patients but not in western population, although several case reports have demonstrated the absence of EBV from cervical and skin cancers of this type [85]. Recently using sensitive detection techniques, the presence of EBERs has been found in EBV-associated invasive Breast cancer [22, 23, 86-88].

![Diagram of EBV expression and contributing factors in distinct malignancies](image.jpg)

**Fig 2.2.1: EBV expression and contributing factors in distinct malignancies** (Adapted from Raab Traub N; 2012).

### 2.3: Gastric cancer and EBV

EBV-association in gastric cancer is characterized by expression of Latency I genes EBERs and LMP2A. EBV EBERs are common to all of the EBV-associated malignancies and therefore considered as the gold standard to detect EBV-association. EBERs are the most abundant viral transcripts in latently EBV-infected cells and contribute significantly in the maintenance of malignant phenotypes. EBER1 and EBER2 genes are separated by 161 base pairs and are transcribed from left to right on the EBV map. The primary sequence similarity is only 54% between EBER1 and EBER2, but both shows striking similarity in their secondary structures.
These secondary structures are similar to the adenovirus-encoded small RNAs, VA1 and VA2 [6]. The primary sequences of the EBERs are strongly conserved among a number of EBV strains. Within the 1 kb EBER region, ten single base changes have been identified. The EBER1 sequence is completely conserved, and two base substitutions are within the EBER2 sequence, while eight are outside the coding regions [89]. The overall high conservation of the sequence suggests that EBERs are important for the life cycle of the virus. However, studies with mutant EBV lacking EBER genes have indicated that EBERs have no role in viral infection, replication and virus-induced transformation of primary lymphocytes [90].

EBERs have been located in the cell nucleus by in situ hybridization (ISH) analysis [91]. However, they have also been detected in cytoplasm as well as in nuclei of interphase cells by high resolution ISH using confocal laser scanning microscopy [92]. The staining pattern indicates a close association with the rough endoplasmic reticulum and Golgi apparatus. Several intracellular proteins, La antigen, EBER-associated protein (EAP)/L22, double-stranded (ds) RNA-dependent protein kinase (PKR) and Retinoic acid-inducible gene I (RIG-I) were shown to bind EBERs. La antigen stably binds the oligouridylate region at the 30 termini of EBERs [93]. A second cellular EBER-binding protein, EAP was originally identified in La antigen-containing RNP complexes, and it was proven to be L22 protein, which had been identified as a component of the ribosome subunit [16]. L22 binds a stem-loop structure (stem-loop 3) of EBER1. Although the domains on EBERs that interact with these molecules were identified, the significance remains unknown. PKR, an interferon (IFN)-inducible serine/threonine kinase, is the key mediator of antiviral activities of IFN [94]. Besides its antiviral effects, IFN has anti-proliferative and anti-tumor effects. IFN has been used in the treatment of several malignancies. The interaction between EBERs and PKR was demonstrated in a cell-free system. It was clarified that
EBERs compete with dsRNA for binding to PKR, suppress the PKR activity and block phosphorylation of eIF-2α, thus resulting in the maintenance of protein synthesis [95, 96]. These results suggested that EBERs antagonize the IFN-induced antiviral activity by blockage of the PKR pathway and contribute to the protection of EBV from the effect of IFN during the establishment of latent infections. RIG-I is a cytosolic protein that detects viral double-stranded RNA (dsRNA) inside the cell and initiates signaling pathways leading to the induction of protective cellular genes, including type I IFNs and inflammatory cytokines. RIG-I contains an N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H-box RNA helicase domain [97]. The helicase domain is responsible for dsRNA recognition, and the CARD domain activates downstream signals, resulting in the activation of transcription factors, NF-kB and IFN regulatory factor3 (IRF-3). RIG-I functions independently of toll-like receptor 3 (TLR3), which is involved in the recognition of viral dsRNA on the cell surface and induction of type I IFN responses. EBERs are observed to activate the expression of IFN and IL-10 though RIG-1 mediated signaling pathway [10, 18].

![Secondary structures of EBERs](Adapted from Nanbo et al., 2001).
Another EBV oncogene LMP2A was reported to contribute in malignant transformation by intervening in signaling pathways at multiple points, namely the B-cell receptor blockade mechanism, the ubiquitin-mediated (Notch and Wnt) pathways, the MAPK, PI3-K/Akt and NK-κB pathways, which can provide us with important insights into the roles of LMP2A in the EBV-associated latency state in various malignancies. The EBV LMP2 gene encodes 2 isoforms of the hydrophobic integral membrane protein, namely LMP2A and LMP2B [98]. LMP2A and LMP2B are transcribed from two different promoters separated by 3 kb through the fused terminal repeat of the EBV episome. The LMP2A promoter is upstream of the LMP2 gene, while LMP2B shares a bidirectional promoter with LMP1 [99]. The LMP2A and LMP2B transcripts are respectively 2.0 kb and 1.7 kb in length. These transcripts share eight common 3´ exons, but have unique 5´ exons. The eight common exons shared by both LMP2A and LMP2B encode a hydrophobic stretch encompassing 12 membrane-spanning domains and a 27-amino acid cytoplasmic C-terminal domain (figure 2.3.2). The 5´ exon of LMP2A encodes a hydrophilic N-terminal 119-amino acid cytoplasmic-signaling domain (NTD), whereas the 5´ exon of LMP2B is non-coding [98]. LMP2A is composed of a variety of highly conserved motifs with functional significance (figure 2.3.3). LMP2A tyrosine residues Y74 and Y85 (Y74/85) with conserved sequences of paired tyrosine and leucine (YXXL) constitute the Immunoreceptor Tyrosine-based Activation Motif (ITAM) participating in signal transduction events [100]. Tyrosine 112 (Y112) within the YEEA motif of LMP2A demonstrates sequence homology to the PTK SH2 binding motif (YEEI) of the Src family and interacts with the tandem SH2 domains of Syk [101].
The sequence homology of five other tyrosine residues (Y23, Y31, Y60, Y64 and Y101) of the LMP2A N-terminal to ITAM might imply their molecular interaction and functional significance. The conserved N-terminal proline-rich motifs contain a consensus sequence that is required to interact with SH3 and WW domains, which in turn yield various biological effects [28]. The cysteine-containing motifs in the C-terminal and transmembrane domains of LMP2A are thought to be the protein interaction motifs involved in molecular clustering, which is required for LMP2A function [102]. However, the precise mechanism by which LMP2A alters these pathways remains elusive.

Therefore, identifying the LMP2A signaling molecules that are involved in the pathogenesis of EBV-associated malignancies is important for the development of potential therapeutic targets. Potent small molecule inhibitors that impair the LMP2A oncogenic signaling pathways could possibly become novel therapeutic modalities.
EBV infection is implicated in the etiology of several different lymphoid and epithelial malignancies. Studies by various research groups have pointed out that there is a correlation between EBV-association and state of host cell differentiation. More than 80% of lymphoepithelioma-like poorly differentiated gastric carcinoma is infected with EBV [103]. On the other hand, ordinary-type GC, comprising 5–10% of all cases of GC, shows features of moderately or poorly differentiated adenocarcinomas with various degrees of lymphocytic infiltration. Within a solid tumor cancer cells have the unique ability to initiate tumor growth and sustain self-renewal as well. This in turn may lead to cell motility & invasiveness i.e. metastatic potential of the cells. EBV Latency I genes especially LMP2A have been shown to modulate some very important self renewal pathways such as the Notch, the Sonic Hedgehog and the
Wnt/β catenin in B cells and epithelial cells which aid in maintenance and progression of cancers. Notch receptors are activated by transmembrane ligands of the delta and jagged families expressed on neighboring cells (figure 2.4.1) [104]. This initiates a cascade of proteolytic cleavages of the receptor close to and within the transmembrane domain. Ultimately, the intramembrane γ-secretase protease liberates NICD (Notch intracellular domain), which translocates to the nucleus and engages the transcription factor CSL {CBF1/RBPjk in vertebrates, suppressor of hairless [Su(H)] in Drosophila, and Lag-1 in C. elegans}, thereby activating transcription. Some of the best-characterized Notch target genes encode members of the hairy-enhancer of split (Hes) family of transcriptional repressors, which are nuclear basic helix-loop-helix (bHLH) proteins. These HES family members regulate developmental decisions. Wnt signaling is initiated when ligands engage a complex consisting of a serpentine receptor of the frizzled family (figure 2.4.1). The key molecule in the cascade is a cytoplasmic protein termed β-catenin, whose stability is regulated by the so-called destruction complex. When Wnt receptors are not engaged, two scaffolding proteins in the destruction complex, the tumor suppressors Adenomatous Polyposis Coli (APC) and axin, bind newly synthesized β-catenin. CKI and GSK3, two kinases residing in the destruction complex, then sequentially phosphorylate a set of conserved Ser and Thr residues in the N-terminus of β-catenin. The resulting phosphorylated footprint recruits ubiquitin ligase, which targets β-catenin for proteasomal degradation. When Wnts bind the receptor complex, the activity of the destruction complex is inhibited. As a consequence, β-catenin accumulates and binds to nuclear DNA binding proteins of the Tcf/Lef family [105]. In sum, the canonical pathway translates a Wnt signal into transient transcription of a Tcf/Lef target gene program. In the absence of Hedgehog signaling, Patched family receptors (PTCH1/PTCH and PTCH2) inhibit the Smoothened (SMO) signal transducer [106]. SMO inactivation leads to
formation of the cytoplasmic Gli degradation complex, in which Gli family members (Gli1, Gli2 and Gli3) are phosphorylated by casein kinase I· (CKI·), glycogen synthase kinase-3β (GSK3β) and protein kinase A (PKA). Phosphorylated GLI is recognized for ubiquitination and ubiquitinated Gli is partially degraded to release its intact N-terminal half functioning as transcriptional repressor. Hedgehog-binding to Patched family receptors releases the SMO signal transducer from Patched-dependent suppression (figure 2.4.1). SMO then activates STK36 serine/threonine kinase to inhibit the assembly of Gli degradation complex for the stabilization of full-length Gli. Hedgehog signaling activation leads to Gli-dependent transcriptional activation of target genes, e.g. Gli1, Ptch1, CCND2, FOXL1 and JAG2. These signaling cascades are the critical controllers of self-renewal ability of cancer stem cells within a solid tumor [107] and key regulators of cell growth, metastatic potential and drug resistance [108, 109].

Fig 2.4.1: Outline of the Notch, Wnt and Sonic Hedgehog pathway

2.5: Mitochondria and Mitochondrial Dynamics

Mitochondria, organelles found in virtually every eukaryotic cell, are involved in a number of cellular functions and are essential for both life and death. Mitochondria, via oxidative
phosphorylation ensues the conversion of the dietary intake into ATP, the energy currency required for most of the biological reactions. In addition, they are the site of many important reactions such as urea cycle, lipid metabolism, steroid hormone and porphyrin synthesis and interconversion of amino acids. Moreover, mitochondria play a central role in complex physiological processes including cellular proliferation, differentiation, and apoptosis and as well as in cellular processes like glucose sensing and insulin regulation, cellular Ca2+ and ROS (reactive oxygen species) homeostasis. This wide array of functions performed by the mitochondria takes advantage of the specialized morphology of the organelle: it consists of two membranes, an outer one that separates them from the cytosol, and an inner one that constitutes the active site of the oxidative phosphorylation, where the four complexes of the respiratory chain (complex I, II, III and IV) as well as the ATPase (complex V) reside. The two membranes enclose two aqueous spaces, the matrix, where Krebs’ cycle is located, and the intermembrane space, where many proteins are stored, including cytochrome C. This mitochondrial ultrastructure is modulated in response to various physiological and pathological signals [110, 111].

Mitochondria are evolutionarily derived from the endosymbiotic relationship of prokaryotic bacteria within eukaryotic cells. Owing to this, mitochondria possess its own genome. Mammalian mitochondria have multi copies (approximately $10^3$ to $10^4$ copies/cell) of mtDNA, 16,569bp of circular DNA which is replicated and expressed within mitochondria. MtDNA is maternally inherited and shows non Mendelian inheritance patterns. In order to protect, maintain, and propagate the mitochondrial genome accurately, mtDNA is packaged into protein–DNA assemblies called nucleoids [112]. Mammalian mtDNA encodes 13 polypeptides, which are essential subunits of complexes I, III, IV and V of oxidative phosphorylation, 22 tRNAs and 2rRNAs which are necessary for the translation of these13 polypeptides. The remaining
mitochondrial proteins for oxidative phosphorylation, metabolic enzymes, DNA and RNA polymerases, and ribosomal proteins are all encoded by nuclear genome [113].

**Mitochondrial dynamics:** The term *mitochondrion* has been derived from the Greek words *mitos* and *chondros*, meaning thread and grain, respectively. Mitochondria can appear as a network of elongated and interconnected filaments that allow rapid exchange of mitochondrial contents, as a collection of rod like particles formed upon division, or as a mixture of both. At the beginning of the 20th century, owing to advances in brightfield microscopy and in cell culture, it was revealed that mitochondria show distinct morphologies, probably due to their dynamic nature [45]. A traditional concept derived from electron microscopy view of mitochondria as isolated kidney shaped organelles with inner structures of folded sheet-like cristae no longer represents the reality. Extended mitochondrial membranous networks are frequently found in metabolically active cells and serve distinct important functions. Thus isolated mitochondria may be considered as a fractionation artifact due to the inevitable presence of vesicles that have been cut and fused from the original mitochondrial network present within the cell [114, 115]. It was only in the 1990s, that the development of mitochondrial-targeted fluorescent dyes and proteins allowed the visualization of mitochondrial dynamics in several cellular models, from yeast to hepatocytes. In1997, the first gene (Fzo1) that participates in the fusion of mitochondria was discovered in *Drosophila melanogaster*. In the last decade, several genes that modulate mitochondrial fusion and fission have been identified, first in yeast and later in mammalian cells [46].

Mitochondrial dynamics implies that mitochondria can continuously fuse (*fusion*) and divide (*fission*) (figure 2.5.1). Mitochondrial fusion occurs when two mitochondria unite to form a single giant mitochondrion ensuring the exchange of lipid membrane and intra-mitochondrial
contents including mtDNA between the fusing mitochondria [46]. Mitochondrion fission is said to occur when a mitochondrion within a cell divides to form two or more separate mitochondrial compartments. When mitochondria divide, both the inner and outer membranes have to be cut and the mtDNA and other critical mitochondrial contents redistributed to the daughter mitochondria and mitochondria transform into isolated, small, round organelles. Owing to these events, mitochondria in a cell exist in different shapes (long filamentous or short grain like) and numbers, depending on their metabolic requirements, nutrient availability and type of cell. These fusion and fission events occur on an average every 2 minutes in several cell types [47]. Mitochondria undergo major morphological changes during the cell cycle, fragment massively in apoptotic cells, become highly elongated in senescent cells and elongate through hyperfusion upon selective stresses. The fusion and fission processes occur in a careful balance in order to maintain proper mitochondrial dynamics. An increase in fusion or a decrease in fission can lead to elongated, interconnected mitochondria, whereas a decrease in fusion or an increase in fission can lead to punctate, fragmented mitochondria.

Fig 2.5.1: Mitochondrial fission and fusion. Adapted from Tieu et al, 2014.)
**Key players of mitochondrial dynamics:**

Genetic and biochemical studies in several organisms including Drosophila, yeast and mammalian cells have contributed to the identification and characterization of the major components of the fusion and fission machineries involved in mitochondria dynamics and their regulation. A summary of the main proteins involved and characterized is given below.

**Mitochondrial fusion proteins:**

Membrane fusion events require first that the maintained intermembrane distance due to electrostatic repulsion be reduced. To carry out the aforesaid, specific proteins are required to pull the opposed membrane bilayers together to induce bent membranes and/or protein-depleted areas where fusion can proceed. Then, these or other proteins stabilize the stalk intermediates and generate fusion pores thereby bringing about complete fusion. Mitochondrial fusion is different from other intracellular membrane fusions in that is requires the successive fusion of the outer and inner membranes and therefore require specific proteins [116]. The yeast *Fzo1*, and the mammalian *mitofusins1* and *2* which are dynamin related GTPases of the outer membrane, are engaged in mitochondria tethering and outer membrane fusion. Mgm1 and Ugo1 in yeast and the Mgm1 ortholog Opa1 in mammals are believed to coordinate the outer membrane fusion step with the inner membrane, an event thought to be necessary for the inner membrane fusion process itself. Mgm1 and its ortholog Opa1 are dynamin-like GTPases that can have a direct physical involvement in the fusion of inner membranes.

**Mitofusin1 (Mfn1):**

Human Mfn1 and Mfn2 have a 63% of identity, and share the same relevant functional domains. Mfn1 is a transmembrane GTPase protein of 741 residues located in the outer mitochondrial
membrane. The conserved domains are: a carboxy-terminal part which contains a bipartite transmembrane domain (TM) and a coiled-coil domain (also called heptad-repeat domain, HR2) and the amino-terminal part that contains the GTP binding domain and another coiled-coil domain (HR1) [46, 115]. Both the HR2 and GTPase domain are exposed to the cytosol. The HR2 domain is involved in the tethering of two adjacent mitochondria through a dimeric antiparallel coiled-coil structure, which can be homotypic (Mfn1–Mfn1) or heterotypic (Mfn1–Mfn2). The GTPase domain has five GTPase motifs; called G1, G2, G3, G4 and G5 (figure 2.5.2). G1 binds the phosphate of the GTP molecule, G3 coordinates the Mg2+ needed for the hydrolysis, G1, G2 and G3 together form the catalytic center and G4 and G5 provide the specific conformation for GTP binding.

**Mitofusin 2 (Mfn 2):**

Mfn2 is an integral outer mitochondrial membrane protein of 757 residues in humans with the same functional domains as Mfn1 (figure 2.5.2). Both the NH2 –and COOH- terminal parts are exposed to the cytosol and a small part of Mfn2 presumably faces the intermembrane space, and splits the transmembrane domain into two parts [46, 115]. Like Mfn1, the GTPase domain of Mfn2 also contains five G motifs (G1, G2, G3, G4 and G5).

Mitochondrial fusion depends on the GTPase activity of Mfns. Specific alterations of highly conserved residues within the conserved motifs in the GTPase domain affect GTPase activity and consequently mitochondrial fusion. Dependent on the site or nature of the GTPase mutation, mutant Mfn variants with different enzymatic properties can be obtained exhibiting altered rates of nucleotide binding, hydrolysis, and exchange [117]. Taken together, membrane fusion can be considered as a multi-step process starting with the tethering of two different mitochondrial
membranes (HR2 mediated), followed by a GTP required docking step of both membranes before the final fusion occurs.

**Fig 2.5.2: Domains of mitochondrial fusion proteins Mfn1, Mfn2 and OPA1.** GTPase domain is shown in yellow with the distinct motifs (G1-G5 depending on the protein). CC represents the coiled coil domain shown in green, TM the transmembrane domain shown in red and PR the Proline rich region shown in dark blue. MIS represents the mitochondria insertion sequence shown in light blue.

**Optic atrophy factor 1 (Opa1):**

OPA1 was discovered as the gene whose mutations were responsible for the autosomal dominant optic atrophy (ADOA). OPA1 is a dynamin-related GTPase that controls mitochondrial fusion and cristae remodeling [118]. The OPA1 gene encodes a mitochondrial protein localized in the inter-membrane space (IMS) and associated to mitochondrial membranes. It consists of three conserved regions: a GTPase domain, a middle domain and a carboxy-terminal coiled coil domain (CC-II) also called GTPase effector domain (GED) (figure 2.5.2). The latter is involved in the oligomerization and activation of the dynamins. The amino terminal region of OPA1,
preceding the GTPase domain, displays a mitochondrial import sequence (MIS) followed by a predicted transmembrane domain (TM1) and a coiled-coil domain (CC-I) located downstream of alternatively spliced exons [119]. In humans OPA1 ORF is built from 30 exons, 3 of which (4, 4b and 5b) are alternatively spliced leading to 8 mRNA. Exon4 that is evolutionary conserved does not present any remarkable domain whereas both exons 4b and 5b that are specific to vertebrates encode hydrophobic domains (TM2a and TM2b). Precursors translated from the eight OPA1 mRNA are targeted to mitochondria via their MIS which is removed upon import by the mitochondrial processing peptidase (MPP) to give rise to long isoforms (l-OPA1). Each l-OPA1 isoform is then subjected to a limited proteolysis generating short isoforms (s-OPA1). Both short and long isoforms of OPA1 are associated to mitochondrial membranes, and it is proposed that l-OPA1 is anchored to the IM while s-OPA1, lacking TM1, is peripherally attached to the IM, a fraction of it having the possibility to diffuse in the IMS and to associate to OM [119]. OPA1 cleavage not only occurs under basal conditions but also induced by apoptosis and dissipation of mitochondrial membrane potential. Both long and short isoforms are required for membrane fusion.

**Mitochondrial fission proteins:**

Four major proteins contribute to mitochondrial membrane fission in yeast: the outer membrane protein Fis1 and the three cytosolic proteins Caf4, Dnm1 and Mdv1. Fis1 and its human ortholog named hfis1 are tailed-anchored proteins considered as receptors for the recruitment of cytosolic components of the outer fission machinery. Dnm1 and its mammal ortholog DRP1/DLP1 are dynamin-like proteins containing an N-terminal GTPase domain and a C-terminal GTPase effector domain. Caf4 and Mdv1 are related proteins with redundant functions. They have two N-terminal α-helices which interact with Fis1, a C-terminal WD40 repeat domain that may bind
Dnm1 and a coiled-coil region that may participate to self-oligomeric interactions. No orthologs of these two proteins have yet been identified in mammals. Dnm1 seems to be the scission protein of the outer mitochondrial membrane (recruited by Fis1 and Caf4/Mdv1), and its GTP hydrolysis-dependent activity in driving membrane fission is similar to what is known for dynamin-dependent membrane fission in endocytosis. The inner membrane proteins Mdm33 in yeast and Mtp18 in mammals are believed to favor inner membrane fission because overexpression or depletion of these proteins induces either a fragmentation of the mitochondrial network or the accumulation of giant mitochondria [46].

**Dynamin Related Protein 1 (Drp1):**

Drp1 is the principal actor of mitochondrial fission. It consists of a highly conserved NH2-terminal GTPase domain followed by a conserved middle domain and a putative helical domain called the assembly domain (figure 2.5.3). It is a cytosolic protein and is recruited to the mitochondrial outer membrane where it interacts with Fis1 during fission. Thereafter it oligomerizes around mitochondria and constricts the organelle in a GTP dependent manner. The process of mitochondrial fission is likely to require several steps, including the recruitment of Drp1/Dnm1 (and any accessory proteins) to sites on the sides of mitochondrial tubules, the constriction of mitochondrial tubules at these sites, and the coordinated division of the outer and inner mitochondrial membranes to generate new tubule ends. By immunogold labeling, Drp1 was detected to be clustered on the outer membrane of mitochondrial tubules that are not constricted. These were later found to represent sites of future constriction and fission. Drp1/Dnm1 was also found concentrated at constriction sites in mitochondrial tubules representing sites of fission [120]. Drp1 forms punctuate foci on the mitochondria but the mechanism of initial Drp1 recruitment is largely unknown. These punctuate foci of Drp1 appear to migrate towards a site of
mitochondrial fission, where they assemble into a functional scission complex [121]. That mitochondria are the principal target of Drp1 action is established by the fact that collapse of mitochondrial network is induced by mutant Drp1 as in this case the balance between fission and fusion is lost. Drp1 establishes mitochondrial morphology through a role in the distribution of mitochondrial tubules throughout the cytoplasm.

**Mitochondrial Fission 1 Protein (Fis1):**

Fis1 and its human ortholog named hfis1 are tailed-anchored proteins considered as receptors for the recruitment of cytosolic components of the outer fission machinery [45]. Fis1 mutations have been shown to suppress mitochondrial fission and fragmentation. [116]Fis1 is required for Dnm1 to assemble into the functional oligomers that drive the scission event. Fis1 is a small protein of 17kDa anchored in the outer mitochondrial membrane and it does not contain domains with any known enzymatic activity. It does not show any GTPase activity or contains any domains related to the dynamin family. It contains six alpha helices and a transmembrane domain (figure 2.5.3). Four of these helices conform two TPR (tetratricopeptide repeat) motifs that are involved in protein-protein interactions [122]. This observation suggests that Fis1 fission activity is mediated through its binding to Drp1 or to other mitochondrial fission proteins that remain to be determined. Drp1 and Fis1 topology suggests that these proteins mediate outer mitochondrial membrane fission.
Functions of mitochondrial dynamics:

Mitochondrial dynamics plays an important role in both cell survival and cell death such as ATP production, apoptosis, autophagy, synaptic transmission, metabolism, embryonic development etc.

ATP production, synaptic and neuronal activity:

Mitochondrial distribution is generally believed to adapt to cellular physiology. Mitochondria move towards active growth cones of developing neurons, and their proper distribution is critically needed for maintenance of adequate synaptic transmission [123]. The control of mitochondrial movements has been long assumed to be mediated by ATP which is consumed to maintain transmembrane ion gradients across neuronal membranes. ATP depletion is expected to be biggest at the sites of intense activity such as synapses. ATP usage is the highest at post synaptic ending, then comes a pre-synaptic ending and the soma where mitochondria migrate to meet the energy demand created.

Fig 2.5.3: Domain structure of human Drp1 and Fis1. The GTPase domain is shown in yellow and the middle domain in brown. PR represents the proline rich domain shown in dark blue, GED represents the GTPase effector domain shown in green, TM the transmembrane domain. The TPR represents the tetratricopeptide domain consisting of six α helices.
Mitochondria morphology and metabolism:

Balanced mitochondrial dynamics is required for the maintenance of the tubular shape of normal mitochondria [111]. Also mitochondrial fusion is required for mtDNA maintenance as loss of mitofusins or OPA1 leads to mtDNA loss and mitochondrial fragmentation. Metabolites within the mitochondria may become concentrated within the intercristal membranes, allowing their efficient use by the respiratory chain. However, should the regulation of cristae assembly be compromised, then these stores may become depleted leading to altered metabolism [45].

Apoptosis:

One of the best identified roles of mitochondrial dynamics is in apoptosis. Apoptosis mediates the catabolism of eukaryotic cells that is crucial for metazoan development, adult tissue turnover, host defense pathways, and protection from cancer. All pathways of apoptosis converge upon the activation of caspases; proteases that orchestrate the efficient and non inflammatory demolition of cells. Proteins involved in mitochondrial fission and fusion actively participate in apoptosis induction. Prior to, or simultaneous with, cytochrome C release and upstream of caspase activation, mitochondria fragment into multiple small units. Blocking of this mitochondrial fission inhibits cytochrome C release and delays cell death, linking the morphogenesis machinery of this organelle to cell death induction. Upon apoptotic stimulation, Drp1 is recruited to the mitochondrial outer membrane where it colocalizes with Bax and Mfn2 at fission sites. Drp1 association with mitochondria is stabilized in a Bax/Bak-dependent manner after mitochondrial fragmentation but prior to cytochrome C release. Drp1, in concert with other components of the fission or fusion machinery, contributes to the process that mediates mitochondrial membrane permeabilization. Levels of Fis1 also regulate apoptosis, as down regulation of Fis1 inhibits Bax translocation and conformational change thereby inhibiting
apoptosis. Moreover, experiments have demonstrated that overexpression of Fis1 induces fission and apoptosis [124].

Apart from the fission machinery, the fusion machinery also participates in this process as overexpression of Mfn1 increases mitochondrial connectivity, results in delayed Bax activation, cytochrome C release, and apoptotic death. Loss of OPA1 (GTPase and GED domains) induces spontaneous apoptosis of cells, providing another important link between apoptosis and mitochondrial morphogenesis. Down regulation of OPA1 not only leads to mitochondrial fragmentation but also disrupts normal cristae structure. Bulk of cytochrome C resides in the cristae and cristae junctions are held in position by OPA1 oligomers. Pro-apoptotic protein tBID brings about the disruption of OPA1 oligomers and cytochrome C release [125].

**Functional complementation:**

Individual mitochondria in single cells can exchange genetic contents via fusion and fission, and they can function as a single dynamic cellular unit in conforming to “one for all, all for one” principle. Mitochondrial fusion enables inter-mitochondrial cooperation by allowing exchange of both membrane and matrix contents and therefore may help to restore local depletions and maintain mitochondrial function [113]. Therefore, complementation allows a cell that contains defective mitochondria to survive normally provided the cell contains a threshold of healthy mitochondria as the gene products deficient in the defective mitochondria are complemented from the normal ones via mitochondrial dynamics. There are two types of mitochondrial complementation. One is mitochondrial positive complementation, which occurs when the number of normal mitochondria is more abundant than that of respiration deficient mitochondria. The other is mitochondrial negative complementation, which occurs when the number of
respiration deficient mitochondria is more abundant than that of normal mitochondria. Mitochondrial diseases comprise just the latter condition. The mitochondrial positive complementation is mitochondria specific system preventing individual mitochondria from expression of disease phenotypes by mutated mtDNAs [113].

**Autophagy:**

Autophagy also called mitophagy in this case is a mechanism that targets depolarized mitochondria for digestion and elimination. Fission events often give rise to one depolarized daughter mitochondrion. If the depolarized daughter does not repolarize swiftly its chances of a second fusion event is usually six times lower compared to the hyperpolarized daughter. These mitochondria are then targeted for autophagy where they are engulfed by autophagosomes and thereafter digested [126].

**Embryonic development:**

Mitochondrial fusion is essential for embryonic development. Genetic studies carried out in mouse models have shown that both the Mfn1 and Mfn2 knockouts demonstrate full viability and fertility in heterozygous animals but result in embryonic lethality of homozygous mutants [127]. In addition, the mitochondria from these mice show decreased fusion rates and decreased membrane potential. In conclusion, in cells with continual cycles of fusion and fission, the mitochondrial population is essentially functionally homogeneous. However, at any given time point individual mitochondria are functionally distinct entities. Therefore, without mitochondrial fusion, the stochastic differences between distinct mitochondria can accumulate to affect the well-being of the cell. The mammalian mitofusins, Mfn1 and Mfn2, function in three distinct molecular complexes to promote mitochondrial fusion and thus protect mitochondrial function.
**Lymphocyte chemotaxis:**

Mitochondrial dynamics appears to be important for proper mitochondrial redistribution in lymphocytes during chemotaxis. Mitochondria are concentrated in the trailing edge in lymphocyte cell lines that migrate in response to chemical attractants [128]. Modulation of mitochondrial fusion or fission affects both mitochondrial redistribution and cell migration. Fragmentation enhances mitochondrial redistribution and cell migration, whereas conditions that promote fusion have the opposite effect. Therefore, as in neurons, mitochondrial shape in lymphocytes can affect the recruitment of mitochondria to local cellular areas.

**Cell migration and invasion:**

Cell migration and invasion are among the early events of metastasis which is associated with poor prognosis in tumors, especially epithelial cancers. Mitochondrial dynamics, specifically mitochondrial fission has been shown to be one of the factors responsible for this effect in breast carcinoma cells [129]. Mitochondria assumes a more fragmented morphology accompanied by Drp1 elevation in invasive and metastasizing breast cancer compared to non-metastatic cells. Silencing Drp1 or overexpressing Mfn1 resulted in reduced migration of these cells.

**2.6: EBV oncoproteins and cell migration:**

Cell migration and invasion are among the hallmarks of metastasis and Epstein Barr Virus oncoproteins especially LMP2A has been related to these effects. EBV induces expression of αv integrins on transformed B lymphocytes which brings about cell growth and matrix invasion [130]. LMP1, LMP2A and EBNA2 were shown to selectively transactivate the αv integrin promoter. Role of LMP2A in epithelial cells invasiveness and motility is well established [25, 49]. In NPC cells, LMP2A upregulated matrix metalloproteinase 9 (MMP9), a metastasis-
associated protease [38]. The EBV protein latent membrane protein 2 (LMP2) is expressed in NPC tumor tissue and has been shown to induce transformation, inhibit differentiation, and promote migration of epithelial cells. This LMP2A mediated migration is mediated by the ITAM domain and is Akt dependent. LMP2A also induced phosphorylation of the Akt target GSK3, a Wnt signaling mediator that has been shown to regulate the activity of focal adhesion kinase (FAK), a tyrosine kinase activated by clustering and ligand interaction of integrins. Inhibition of either FAK or its signaling mediator Src kinase inhibited LMP2A-induced migration [25]. Moreover, LMP2A has been shown to induce migration and EMT in HaCaT cells [37]. EBV LMP2A has also been reported to induce primary epithelial cell migration and invasion in tonsil epithelial cells [48].

EBV pathogenesis has been associated with epithelial carcinomas. Mitochondrial dynamics is important for many cellular outcomes. Altered balance of mitochondrial fission and fusion has been implicated in various diseases. Studies have also extended so as to implicate imbalanced dynamics in cancers. Therefore it is possible that mitochondrial dynamics may have a role to play in tumor progression. Cell proliferation, chemoresistance and metastasis are some of the events associated with EBV tumorigenesis. Hence, strategies to prevent the above are currently required to combat EBV-associated malignancies. Such efforts need a deeper understanding of the functions of EBV-oncogenes and are likely to be lead to improved cancer treatments.