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CHARACTERIZATION OF THE GENIC PRODUCTS pUL50 AND pUL53 OF HUMAN CYTOMEGALOVIRUS: FUNCTIONAL INTERACTIONS AND ROLE IN VIRAL MATURATION.

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1. INTRODUCTION

This study is focused on pUL50 and pUL53, two genic products of Human Cytomegalovirus, whose homologs in the Herpesviridae family are described as essential for viral maturation, and on the alterations of cellular components in which the two viral proteins are supposed to be involved.

1.1 HUMAN CYTOMEGALOVIRUS: GENERALITIES

Human Cytomegalovirus (HCMV) belongs to the family Herpesviridae, subfamily β-Herpesvirinae [**1**].
HCMV shares many characteristics with other herpsviruses, including virion and genome structure and the ability to estabilish persistent and latent infections, but presents also distinct characteristics, such as tropism for macrophages and endothelial cells *in vivo*, high species specificity and slow growth in cell culture on epithelial cells and fibroblasts [**2**].

1.1.1 Epidemiology

Cytomegalovirus is easily transmitted, usually through contact with bodily fluids or by placental transfer. Seroprevalence rates vary by socioeconomic class and geographic location, but the overall seroprevalence in developed countries is estimated to be in the range of 30–70% [**3**].
Primary infection in immunocompetent individuals is usually benign, with minimal or no clinical manifestations (although approximately 10% of mononucleosis syndromes are a result of CMV infection). Following primary infection, the virus establishes latency, and viremia is mainly controlled by cell-mediated immunity. A virus reactivation can occur when the

protective immune surveillance fails, for example, as a result of chemotherapy or in patients who have AIDS or who are immunosuppressed for transplantation purposes; such reactivation or primary infection in the context of a disabled immune system can lead to overt disease.

In the case of vertical transmission of CMV, from the mother to the developing fetus, adverse outcomes are most commonly associated with primary infection of the mother, although significant morbidity has also been associated with secondary infection.

- *Congenital HCMV infection*. One of the most important clinical manifestations of primary human cytomegalovirus infection is seen in newborn babies infected during pregnancy[**4**]. In a seronegative mother, the risk of primary infection is 0,7–4,1%, which carries a 40% risk of congenital infection[**5**]. The risk to the infant is greatest if infection occurs in the first trimester, since maternal cytomegalovirus antibodies have a protective role against intrauterine transmission (but not against development of symptoms once infection has arisen)[**6**]. Results of studies on infants with asymptomatic infection indicate that 10–17% develop hearing defects or neurodevelopmental sequelae. More importantly, 5–10% of congenitally infected neonates have symptoms of irreversible CNS involvement in the form of microcephaly, encephalitis, deafness (a solitary finding in 10% of cases), upper motor neuron disorders, psychomotor retardation, and, rarely, myopathy and choroidoretinitis[**7**]. Findings of long-term follow-up studies indicate that most affected infants (up to 80%) display serious life-long neurological abnormalities with severe life-threatening organ dysfunction and death in 10–20% of patients.

- *Perinatal infection.* Cytomegalovirus infection can also be acquired perinatally, which is generally asymptomatic. However, up to 30% of perinatally infected infants display short-term, self-limiting symptoms of hepatosplenomegaly, lymphadenopathy, hepatitis, or pneumonia; neurological sequelae or hearing impairment does not typically arise with this mode of infection.

- *Infection of transplant recipients.* CMV infection is the leading cause of morbidity and mortality for patients who receive solid organ or bone marrow transplantations. The most important risk factor for the development of CMV disease in transplant recipients is the donor's seropositivity coupled with seronegativity of the recipient [**8**]. Although definitive proof is lacking, the degree of viral load within the transplanted organ is probably proportional to risk of subsequent disease. Even secondary infection in a seropositive patient, due to reactivation or super-infection, may lead to CMV disease during immunosuppression, although the risk is substantially smaller and the disease generally less severe.

1.1.2 Antiviral treatment

Currently available drugs consist of molecules able to block the viral replication, mainly inhibiting the replication of viral genome. By now the most effective drugs are the nucleoside analog *gancyclovir*[**9**], the nucleotide analog *cidofovir [10]* and the pyrophosphate analog *foscarnet* [**11**] , which have been licensed for serious for life-threatening HCMV infections in immunocompromised individuals. These drugs have produced clinical improvement in many patients, but suffer from poor oral bioavailability, low potency, development of resistance in clinical practice and dose-limiting toxicities.

- Gancyclovir (GCV) is a competitive inhibitor of viral DNA polymerase (pUL54) and its antiviral activity requires monophosphorylation in the infected cell by the HCMV encoded phosphotransferase pUL97, followed by diphosphorylation by cellular kinases[**12**]. It is usually the front-line drug for the treatment of HCMV infections; however, the emergence of GCV resistant strains due to UL97 and UL54 gene mutations [**13**], mainly in patients during prolonged maintenance therapy, such as in AIDS patients and less frequently in solid organ transplant recipients, is well documented.
- Cidofovir (CDV) is a competitive inhibitor of the HCMV DNA polymerase and has been approved for the treatment of HCMV retinitis. One of its advantages compared with GCV is its long intracellular halflife. Cross-resistance between GCV and CDV could become a concern since they share the same target, the product of the UL54 gene.
- Foscarnet (PFA) is a non-competitive inhibitor of the pyrophosphate binding site of HCMV DNA polymerase, which does not require prior activation by a virally encoded enzyme. It is administered as an alternative to GCV in the event of GCV resistance or severe side-effects, but even long-term exposure to PFA may lead to the emergence of resistant strains due to UL54 mutations.

Double resistance to GCV and PFA in strains from AIDS patients has been reported and high-level GCV-resistant strains with both UL97 and UL54 mutations were found to be cross-resistant to CDV. The emergence of HCMV strains resistant to these conventional drugs induces continuously to the development of new antiviral formulation directed against new targets. An antisense oligonucleotide against the HCMV IE2 mRNA, called *Fomivirsen*, has also been developed for intravitreal applications in patients with retinitis who do not respond to conventional management; moreover, a promising new class of anti-HCMV compounds,

formed of benzimidazole ribosides, which do not inhibit the viral DNA polymerase is object of study.

1.1.3 Virus structure

The virion of HCMV consists of a 100-nm diameter icosahedral nucleocapsid containing a 230-kbp, double stranded linear DNA genome surrounded by a proteinaceous layer defined as the *tegument* or *matrix*, which, in turn, is enclosed by a lipid bilayer, called *envelope*, containing a large number of viral glycoproteins. The mature virion particle is 150–200 nm in diameter (**Fig. 1.1**) .

- *Capsid*. Of the more than 30 viral proteins found in the complete infectious virion, four constitute the capsid: namely, pUL46, pUL48.5, the minor capsid protein (mCP), and the major capsid protein (MCP) encoded by UL85 and UL86, respectively. Three assembly proteins encoded by UL80 associate with capsid and play roles in maturation. The capsid contains the single copy of highly packed DNA genome and two poyamines, spermine and spermidie, which likely counteract the negative charge of packaged DNA.

- *Tegument or matrix*. This amorphous layer between the nucleocapsid and the envelope is still poorly characterized [**14**] and contains about 20-25 structural proteins that may be involved in the maturation of progeny virions or may influence viral and cellular events in the early stages of infection, such as release of viral DNA from disassembling virus particles or the regulation of viral and cellular promoters. Most tegument proteins are phosphorylated and are highly immunogenic. The most abundant are ppUL32 (pp150 or basic phosphoprotein) and ppUL83 (or pp65),which, due to its large amounts, has been chosen as the target antigen in rapid diagnosis assays. Other tegument proteins, such as ppUL69 and ppUL82 (pp71), may play important regulatory roles in both viral and cellular gene expression, acting as transactivators of viral gene expression and dysregulating the cell cycle progression [**15**], [**16**], [**17**], [**18**].

- *Envelope*. The phospholipid envelope originates from the cellular membrane of the host cells and contains at least 8 viral glycoproteins, 6 of which associate to form three highly conserved complexes designated as gCI, gCII, and gCIII [**19**]:

  - gCI, composed of homodimeric gB molecules (gpUL55), plays a crucial role in virus binding and participates in viral entry, cell-to-cell spread, and cell fusion;

- gCII results from the association of gM (gpUL100) and gN (gpUL73). It is the most abundant protein complex on the viral envelope and is involved in the adesion to the host cell binding to heparan sulfate proteoglycans;
- gCIII, a heteroligomeric complex composed of gH, gL, and gO [**20**], is necessary for the final stage of virus entry via pH-independent fusion between the viral envelope and the cell membrane [**21**].

A number of abundant host cell protein, including 2-microglobulin, actin and several cellular enzymes were found to associate with purified virions, suggesting a role during HCMV infection.

1.1.4 Viral life cycle

HCMV replicative cycle reflects the typical viral cycle of Herpesviruses. Four main phases can be distinguished (**Fig.1.2**):

a) Virus attachment and penetration

Virus attachment and penetration are rapid and efficient in both permissive and non-permissive cell types. However, since productive replication is observed in a very restricted range of human cells, a post-penetration block to viral gene expression is thought to restrict replication in nonpermissive cells [**22**]. The poorly characterized receptor(s) for HCMV is widely distributed among host cell types, and contributes to the broad viral tropism observed during natural infections. Viral entry is the result of a cascade of interactions between viral and cellular proteins that culminate in fusion of the virion envelope with the cellular plasma membrane by a pH-independent mechanism. During the initial virus-cell interactions, as observed with other herpesviruses, HCMV attaches to the cell surface by low-affinity binding of gB to heparan sulfate proteoglycans [**23**]. The subsequent interaction of gB with its non-heparin receptor then turns the weak adhesion of the viral particle into a more stable binding or docking state. However, final fusion of the viral envelope with the cell membrane to allow viral penetration is thought to require a further priming event mediated by the heteroligomeric gH-gL-gO complex with as yet unidentified receptors [**20**]. Fusion of the virus and cell membranes is followed by entry into the host cytoplasm of the nucleocapsid and tegument proteins, and their rapid translocation into the nucleus. Interaction of HCMV glycoproteins

with their receptors is enough to generate an intracellular signal transduction pathway, leading to the alteration of cellular gene expression.

b) Viral gene expression

During productive infection, the HCMV genome is expressed in a temporally coordinated and regulated cascade of transcriptional events that lead to the synthesis of three categories of viral proteins described as immediate-early (IE or α), early (E or β), and late (L or γ). Failure in the expression of early gene and subsequent viral DNA replication rather than attachment and/or penetration may be the restricting event in nonpermissive cells. HCMV genes are transcribed in the infected cell nucleus by RNA polymerase II and the associated basal transcription machinery, with the intervention of host-encoded transcription factors whose activity may be stimulated by viral transactivators [**24**], [**25**].

- *Immediate-Early genes (IE or α).* HCMV gene expression initiates from a few IE proteins within l hr p.i. without de novo protein synthesis. The IE genes include the major IE (MIE) UL122/123 genes (IE1 and IE2) and auxiliary genes, such as UL36–UL38, UL115–UL119, IRS1/TRS1, and US3. The MIE proteins, alone or in synergism, are required for subsequent expression by acting as transactivators and autostimulators of viral genes. In addition, these proteins have a deep impact on host cell physiology since they regulate the expression of a large number of host cell genes [**24**].
- *Early genes (E or β).* Expression of E or β genes depends on the presence of functional IE proteins and is unaffected by inhibitors of viral DNA replication [**24**]. They are divided into two subclasses: β1 (E) and β2 (E-L) according to their time of expression. The functional data indicate that E genes encode mostly non-structural proteins, including viral DNA replication factors, repair enzymes, and proteins involved in immune evasion [**26**]. Several E genes, such as UL4, UL44, UL54, and UL112/113, are also transcribed late in infection through several mechanisms, including activation of a promoter different and independent from that transcriptionally active in the E times, initiation of transcription from a new start site, and alteration of the splicing pattern as infection proceeds [**24, 26**]. Both E and L transcripts may have a polycistronic structure due to the relatively few polyadenylation signals in the genome that generate families of 30 co-terminal transcripts. In addition, expression of several E genes studied in some detail is regulated by both transcriptional and post-transcriptional mechanisms [**26**].
- *Late genes (L or γ).* The L proteins are the last class of gene products expressed during HCMV replication. Their transcription begins more than 24 hr p.i. and requires prior

viral DNA replication [**24**]. Late or γ gene expression leads to the synthesis of two subclasses of L proteins (g1 and g2) in accordance with their time of expression and sensitivity to viral DNA replication inhibitors. g1 (leaky late) transcription occurs 24–36 hr p.i., and is reduced by such inhibitors. g2 (true late) transcription occurs 24–48 hr p.i., and is strictly dependent on DNA replication. The L proteins have mainly structural roles and primarily contribute to the assembly and morphogenesis of the virion[**26**].

c) Viral DNA replication

HCMV genome replication, inversion, and packaging occur in the nucleus of the infected cells. Viral DNA synthesis, that begins later than 16 hr p.i., requires the activities of essential and specific viral proteins and the active contribution of several cellular proteins [**26**].

Unlike other herpesviruses, HCMV does not encode deoxyribonucleotide biosynthetic enzymes, such as thymidine kinase, dihydrofolate reductase, thymidylate synthase, and an active form of ribonucleotide reductase [**27, 28**]. Thus, the virus must depend on the host cell metabolism to ensure a sufficient supply of dNTPs for its DNA replication. As a result, it does not shut off host macromolecular synthesis, but stimulates cellular transcription and translation.

Six herpesvirus-conserved ORFs in the HCMV genome provide the core replication proteins for viral DNA replication: among them, the single-stranded DNA-binding protein ppUL57 prevents the reannealing of DNA strands following unwinding by the helicase-primase complex, made of three subunits (pUL70, pUL102, pUL105); the DNA polymerase encoded by UL54 and the DNA polymerase processivity factor UL44 that prevents dissociation of UL54 from the template (Griffiths, 2000; [**26**]. HCMV DNA replication proceeds through initial circularization of the input genome within 4 hr p.i., followed by DNA synthesis via a bidirectional ɵ mechanism from a single origin (oriLyt) of replication that undergoes a switch to a late-phase rolling circle form of DNA replication [**26**]. Packaging of the genome into preformed capsids then follows its cleavage at the essential highly conserved cleavage/packaging signals (pac1 and pac2).

d) Virion assembly, maturation, and egress

Formation of HCMV capsids and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelopment by budding at the nuclear membrane, and further mature through a de-envelopment/reenvelopment process in the cytoplasm before

leaving the cell via an exocytotic-like pathway [**26, 29**]. Nucleocapsid particles accumulate in inclusions that confer the typical ‘‘owl’s eye’’ appearance of the infected cell nucleus. Capsids are initially enwrapped through budding at the nuclear membrane, where they acquire a primary envelope derived from its inner leaflet [**30**]. They then cross the lumen, fuse with the outer leaflet of the nuclear membrane or the ER membrane with which it is contiguous, lose their primary envelope, and move into the cytoplasm. Here, HCMV virion particles further mature by acquiring their tegument. The tegumented capsids then receive their definitive envelope by budding into vesicles of the Golgi apparatus [**31**]. Both tegumentation and reenvelopment are driven by multiple specific protein-protein interactions to secure the integrity of the viral particle [**29**]. These mature particles are retained within the vesicles and transported to the cell surface via the Golgi network, which is enlarged due to the accumulation of nucleocapsids and DB. The Golgi alterations during
the late replication stages create inclusions around the nucleus that result in its characteristic kidney-like appearance [**3**]. Progeny virus accumulates in the cytoplasm, and infectious virus is released into the extracellular compartment beginning at 72 hr p.i. In the very late stages, however, a substantial number of viral particles are still associated with the cell.

1.2 NUCLEAR ENVELOPE AND NUCLEAR LAMINA: STRUCTURE AND IMPAIRMENT DURING VIRAL INFECTION

The nuclear envelope (NE) is composed of three distinct elements: the nuclear membrane, nuclear pore complexes and the nuclear lamina [**32**] (**Fig. 1.3**). The nuclear membrane is a double-unit lipidic layer, in which the outer nuclear membrane (ONM) is continuous with and shares biochemical and functional properties with the endoplasmic reticulum (ER). In contrast, the inner nuclear membrane (INM) is distinct from both the ER and ONM, from which is separated by a narrow lumen, called perinuclear space and is defined by a subset of integral membrane proteins, termed nuclear envelope transmembrane proteins (NETs), that are anchored to the INM during interphase [**33**]. These inner membrane proteins, whose major members are emerin, lamin B receptor (LBR), lamin-associated-polypeptide (LAP)1 and 2 and MAN-1, are arranged with their N-termini facing the nucleoplasm and are modified by various kinases. In addition, nearly all of them associate with the nuclear lamina and/or chromatin and possess hydrophilic end-domains that can drive non-nuclear proteins to the nuclear envelope [**34**].

The nuclear membrane is punctuated by nuclear pore complexes (NPCs), large protein structures of about 125 MegaDaltons (MDa) which regulate the passage of macromolecules between the nucleus and the cytoplasm [**35**]. In principle, molecules of 60 kDa can diffuse freely across the NPC; however, in reality, the trafficking of several such molecules is tightly controlled [**36**]. In general, the nuclear/cytoplasmic trafficking is highly regulated and proteins to be imported or exported from the nucleus contain either a nuclear localization signal (NLS) or nuclear export signal (NES), which is recognized by a receptor and carried through the NPC (**Fig. 1.4**)[**37**]. Once in the correct compartment, the protein cargo is released and the receptor recycled for another round of transport. The transport involves a small Ras-like GTPase, called Ran, crucial to maintain the direction of transport, establishing a gradient essential for nuclear transport in which RanGTP accumulates in the nucleus. Members of a growing family of transport receptors carry proteins into and out of the nucleus and bind preferentially to Ran in its GTP-bound form. For protein export, the exportin CRM1 forms a complex with the NES-containing cargo and RanGTP in the nucleus. Once the complex is translocated into the cytoplasm, hydrolysis of RanGTP to RanGDP occurs, causing the NES containing cargo to be released. The function of CRM-1 has been discovered contemporarly to its inhibitor, Leptomycin B [**38, 39, 40, 41**], an unsaturated branched-chain fatty acid that specifically blocks the association NES-CRM-1, binding covalently CRM1, at

very low molarity [**42**] and preventing the association with NES-containing proteinsm(**Fig. 1.5**).

For the nuclear import of many NLS-containing proteins, the adaptor protein, importin α, recognizes and binds the NLS-containing cargo (Fig. 1). The import receptor, importin β, can then bind to importin α, and this complex translocates across the nuclear envelope through the NPC. Once in the nucleus, RanGTP preferentially binds to importin β, causing the release of the NLS-containing cargo (**Fig. 1.4**). Many proteins for import are not rerecognized by the importin-α or -β receptor, but are recognized by other receptors related to importin β. There are at least 21 potential importin β family members in humans[**43**].

Underneath the INM is the nuclear lamina, a 10 to 50 nm thick meshwork of proteins, whose major components are members of the lamin family of type V intermediate filament (IF) proteins, called lamins [**44**] .

Humans have three distinct lamin genes encoding seven different proteins that, based on their expression pattern, properties and location, can be divided in two broad classes (**Fig: 1.6b**):

- B-type lamis are encoded by two different genes: *LMNB1* originates lamin B1 which is ubiquitous [**45**] and *LMNB2* has two alternatively spliced products, lamin B2 expressed in most cells[**46**] and B3 expressed only in spermatocytes [**47**]. Expression of B-type lamins is essential for nuclear integrity, cell survival and normal development [**48, 49, 16**] and , for these reasons, they are considered fundamental building blocks of the nuclear lamina.
- A-type lamins are all alternatively spliced products of a single gene, termed *LMNA*. Four different proteins have been described: lamin A and C are the major products in most differentiated cells [**44**] and differ the C-terminal domain as lamin C lacks of the last 90 aminoacids; lamin AΔ10 is a lamin A variant with a 30-aminoacid deletion within the C-terminal domain and it is found in tumor and normal cell lines; lamin C2 is germline specific product. In contrast with B-type lamins , A-type lamins are differentially expressed, their appearance in any cell type is correlated with differentiation [**50, 46, 51, 52**] and are dispensable for development, suggesting more specialized functions [**53**]

Lamins share a common primary sequence consisting of globular N-terminal domain, a central rod domain, divided in four a-helical segments and a globular C-terminal domain, containing a nuclear localization signal (NLS) (**Fig. 1.6a**). Through coiled-coil association of the a-helical rod domains, lamins are able to form parallel homodimers[**54**], which, in turn,

associate longitudinally in head-to-tail tetramers [**55, 56, 57**], whose further anti-parallel association originates lamin filaments[**58**].

Post-translational modification of the head and tail domains of lamins is required to control lamin assembly. First of all, lamins have to be processed correctly: all lamins other than lamin C contain a COOH-terminal motif comprising a cysteine, two aliphatic amino acids, and any COOH-terminal amino acid, termed a CaaX box. This motif is the target for a sequence of modifications that lead to isoprenylation and methylation of the COOH-terminal cysteine residue. Addition of a 15-carbon farnesyl isoprenoid to the cysteine occurs initially within the nucleoplasm, and this is followed by proteolytic cleavage of the aaX [**53, 59, 60, 61, 62, 63**]. After this cleavage, the cysteine residue is modified by methylation [**64, 65, 61**]. Isoprenylation and methylation of the COOH-terminal cysteine residues are both necessary for the localization of lamin A and the B-type lamins to the INM. Once at the INM A- and B-type lamins have different fates: while B-lamins are completely processed and thanks to its stable C-terminal farnesylation associates tighly to the INM , A-lamins are further cleaved at the C-terminus, losing the last 18 residues.

Another fundamental modification is phosphorylation as lamins undergo dynamic rearrangements during interphase and mitosis. Lamins contain several target sequence for protein kinases, such as cyclin-dependent kinase 1 (cdk1) [**66, 67**] and nuclear protein kinases (PKC) [**68**], whose phophorylation mediates destabilization and disassembly of lamin filaments. Recruitment of protei phosphatase 1 (PP1) to the INM is essential for lamina assembly at the end of mitosis [**69**].

It had traditionally been thought that the nuclear lamina functions primarily to provide structural support and organization to the nuclear envelope. As major structural components of INM, lamins have indeed a crucial role in maintaining the architecture of the NE, anchoring and guiding the positioning of NCPs via nucleoporin Nup153 [**70, 71**] and supporting other nuclear envelope proteins, but several reports have documented that lamins have multiple and disparate roles (Broers J. L. V., F. C. S. Ramaekers, G. Bonne, R. Ben Yaou and C. J. Hutchison. Nuclear lamins: laminopathies and their role in premature ageing. Physiol Rev 86:967-1008, 2006.; H.J. Worman1, J.-C. Courvalin2 The Inner Nuclear Membrane. J. Membrane Biol. 177, 1–11 (2000)): they are also important to organize cellular cytoskeleton, binding to actin , intermediate filaments [**46, 72**] and in C.Elegans and Drosophila also to microtubules **[73, 74]**; they are critical for DNA replication and, in particular, lamin B seems to participate directly to DNA synthesis, even if contrasting data have reported an involvement at the stage of initiation [**75, 76, 77**]; finally both types of

lamins have functions in transcription regulation, as B-type are involved in the basic process of RNA synthesis, binding RNA polymerase II, while A-type lamins binds and influence the activity of transcription regulator proteins, such as SREBP1[**78**], MOK2 [**79**], c-Fos [**80**] and Rb [**81**] .

In conclusion, nuclear lamina is an essential and multitask cellular structure which interacts with several cellular proteins both in the nucleus and in the cytoplasm.

In this contest, it comes obvious that nuclear lamina cannot avoid to be involved in the deep cellular re-arrangements that viruses cause during their replicative cycle.

The effects of viral infections on nuclear architecture have been reported in several studies and different viruses are shown to affect both nuclear functions [**82**](DNA transcription and synthesis) and structure (lamins and NET proteins)[**83, 84, 85**].

In the last years, nuclear envelope re-arrangements due to viral infection has become a hot topic especially concerning herpesviruses.

Herpesviruses capsid , in fact, assembly and acquire their genome inside the nuclear compartment, but all the final events of maturation occur in the cytosol of the host cell[**29**]. In this contest, the stage of viral egress from the nucleus to the cytoplasm acquires a fundamental importance for the virus replicative cycle and the major obstacle opposing to this event is essentially the thick protein meshwork that underlines the nuclear envelope membrane: the nuclear lamina[**86, 87, 31**].

Herpesviruses seem to have elaborated a fine strategy to dismantle this "cellular barrier": evidences have demostrated lamina components appear deeply affected during infection and that both viral and cellular factors (but virally induced) are involved in causing the alterations.

The most studied herpesviruses in relation to these events belong to the α-subfamily, whose main representatives are *Herpes Simplex type 1* and *type 2* (HSV-1; HSV-2) and *Pseudorabies Virus* (PrV), but evidences are reported also concerning β- and γ-subfamily of herpesviruses (HCMV and Murine CMV and *Epstein-Barr Virus* (EBV), respectively).

As regards HSV-1, several reports indicate both lamin B and A/C show perforations and distortions at the nuclear rim and a partial dislocation into the cytoplasm [**84, 88, 89**]. Concerning levels of expession during infection, contrasting data have been reported: some studies describe an increase of the solubility and a reduction of the total amount of lamins, due to a uniform thinning or in discrete foci, in order to let the capsids reach the nuclear membrane easily [**89**], while others report only in some cell lines this happens and in a limited degree, as nuclear lamina is important for cellular functions and the virus, relying on the host cell machinery for its replication, affects nuclear lamina, but, at the same time, limits the

disruption, to take advantage [**90**]. Recently, a recruitment of atypical Protein Kinases C (PKC) at the nuclear rim has been reported during HSV-1 infection, resulting in lamin B phosphorylation[**91**]. Moreover, also emerin has been found modified, as new phosphorylated forms of this nuclear transmembrane protein have been discovered in infected cells [**84**]. Similar results emerged from the analysis of cells infected by EBV, a γ-herpesvirus.

Lamins appears to be affected also during CMV infection. In the past, analysis of lysates of HCMV infected samples indicated the quality of the phsphorylation of lamin A/C is altered: using a monoclonal antibody versus a specific phosphorylated epitope of lamin A/C, located within the first 417 aminoacids, data showed a rearrangement of the phosphorylation in lamin A/C and a decrease in that specific site [**34**]. More recently, it has been reported that Murine Cytomegalovirus is responsible of a recruitment of PKCs at the nuclear rim and during infection, both B-type and A-type lamins show an increase of the phosphorylation levels, suggesting a their dissolution [**87**]. Moreover, it has been reported that an increase of HCMV molteplicity of infection causes a reduction of total amount of lamin A/C [**92**].

More similar data among the different *Herpesviridae* subfamilies have been reported by electon microscopy observations: invaginations and large infoldings of nuclear envelope appears evident during infection, suggesting an increase of membrane surface to facilitate viral egress[**83, 93, 94, 86, 95, 96**].

In conclusion, lamina is a nuclear component, fundamental for both structural maintenance and several functional activities essential for cellular life cycle. After infection, a virus has to manage this enourmous barrier to its replication and maturation, mainly operating alterations of nuclear envelope membranes and protein components, but with contained effects, in order to maintain those nuclear essential functions, which important also for the production of viral progeny.

1.3 VIRAL EGRESS FROM THE NUCLEUS: HERPES SIMPLEX TYPE 1 PUL31 AND PUL34 AND THEIR HOMOLOGS.

During herpesvirus infection, viral transcription, DNA replication, formation of capsids, and packaging of viral DNA occur in the nucleus [**30, 97, 98**]. Subsequently, intranuclear capsids have to leave the nucleus to gain access to the cytoplasmic environment, that represents the final site of maturation for viral particles. In this contest, nuclear envelope and, in particular, nuclear lamina provides the major obstacle to movement of herpesvirus virions into the cytoplasm.

This process has been studied in depth in particular in α-herpesviruses, such as *Herpes Simplex type 1* (HSV-1) and, among the different models proposed, several observations strongly support the *envelopment - de-envelopment – re-envelopment* model: in order to achieve the cytoplasmic compartment, viral particles bud through the inner nuclear membrane (INM), reaching the perinuclear space; then the viral primary envelope fuses with the outer leaflet of the nuclear membrane, resulting in the release of un-enveloped and presumably untegumented capsids into the cytoplasm, where final tegumentation and envelopment occur, budding through *trans*-Golgi vesicles.

Electron microscopic analyses indeed demonstrated the validity of this model, as highlighted differences between primary virions and mature extracellular ones in morphology, in tegument composition and in biochemical composition of the envelope [**94**], indicating that the primary envelope has to be lost and replaced by another one originated from extranuclear membranes of the host cell. Moreover electron microscopy evidences reported the presence of enveloped viral particles in the perinuclear space and in the area adjacent to *trans-Golgi* apparatus, while virions localized inside the nucleus and in the cytoplasmic compartment nearby the ONM lacked of envelope [**94, 99, 96**].

Nucleocapsids of primary virions are translocated into the cytosol after fusion of the primary envelope with the outer leaflet of the nuclear membrane (Stackpole, 1969; Severi et al., 1979; Smith, 1980). The molecular mechanism of this fusion process is unknown but two virally encoded proteins have been shown to be crucial. These are the products of the UL31 and UL34 genes of herpes simplex virus 1[**100, 101**] and homologous proteins of PrV ([**102, 103**] and MCMV [**87**]. Interestingly, homologs of these proteins are present in members of all three herpesvirus subfamilies, including varicella-zoster virus[**104**], human cytomegalovirus (HCMV) [**53**], human herpesvirus- 6 [**105**], human herpesvirus-7 [**106**] and Epstein±Barr

virus [**30**], indicating that their function may be conserved throughout the Herpesviridae family [**30, 53, 107**].

UL34 protein represents a type II membrane proteins with a C-terminal transmembrane domain but without a signal sequence [**108, 107, 109, 110**], which is either directly or indirectly phosphorylated by the HSV protein kinase US3 [**40, 111, 109, 112**] UL34 protein is a late gene product and is found to be a component of HSV-2 virions. In transfected cells,the protein is clustered strongly in the perinuclear region and also detected dispersed in the cytoplasm [**103, 87, 113, 110**].

The UL31 gene product is a phosphorylated protein, detectable at late times *post-infection,* that is able to interact with that nuclear matrix[**114**]. When singularly expressed, localizes into the nuclear compartment thanks to a nuclear localization signal (NLS) at its N-terminus, but does not become associated with the nuclear membrane.

In cells infected with either HSV-1, PrV or MCMV, both pUL34 and pUL31 are concentrated in the nuclear membrane, indicating that they need other viral protein(s) for their distinct perinuclear localization: it has indeed been shown that when co-expressed the UL34 proteins physically interact with the respective gene products of the UL31 genes (Bjerke et al., 2003; Fuchs et al., 2002a; Muranyi et al., 2002; Reynolds et al., 2001)and this interaction is sufficient for their re-localization at the nuclear rim [**115, 102, 87, 116, 117**]

Thus, complex formation between the UL31 and UL34 proteins apparently is required for efficient transport of the UL34 protein into or retention in the nuclear compartment by UL31, and for nuclear membrane localization of UL31 by interaction with UL34, that inserts into the INM and functions as an anchorage for UL31. The domains responsible of the interaction are different in different herpesviruses: in HSV-1 pUL34 the binding region to pUL31 is located in the centre of the sequence[**118**], while in M50, the homolog in MCMV, resides at the N-terminus[**119**]. However, it has been demonstrated that even if the positions of the binding domain are different, homolog proteins are able to complement each other in interact with pUL31 and homologs, indicating the function is conservated. In contrast, as regards pUL31, the variability seems to be higher, as complementation assays among the homologs failed[**120**].

In the absence of either protein, primary envelopment is inhibited and capsids accumulate in the nucleus as shown for respective deletion mutants of HSV-1, reducing viral titers by several log. These data suggest their function is very important, though not strictly essential for formation of infectious virions [**100, 102, 103, 101**].

The UL31 and UL34 gene products have been shown to be constituents of primary enveloped virions but not mature virus particles [**102, 108, 121**], whereas other tegument and envelope proteins present in mature virions have not been detected in primary virus particles[**122**].

Only one virally encoded protein, the product of the US3 genes of PrV and HSV-1, has been identified which plays a role in this fusion process. US3 homologous proteins are present only in the alphaherpesviruses and they represent protein kinases [**123, 98**]. Strikingly, in the absence of US3, primary virions accumulate in the perinuclear space [**124, 121, 125**]. Apparently, primary envelopment occurs in the absence of the US3 protein, but de-envelopment is impaired: deletion of the US3 gene reduces infectivity only about 10-fold, indicating that the US3 function in nuclear egress is beneficial but not essential. This correlates with the fact that US3, in contrast to UL31 and UL34, is not conserved in all herpesvirus subfamilies. The UL34 protein of HSV-1 has been proposed as a substrate for the US3 kinase, which could indicate that the role of US3 in nuclear egress may be indirect by modulating UL34 activity via phosphorylation [**112**]. However, in PrV the UL34 protein is not phosphorylated, at least not exclusively, by the US3 kinase [**124**], although localization of UL34, and consequently of pUL31, in the nuclear membrane is influenced by the presence of the US3 protein [**124, 121**]: in the absence of U_S3, in fact, U_L31 and U_L34 still co-localize but loose their smooth and homogeneous distribution, localizing within punctate aggregates that accumulate predominantly at the nuclear rim [**121**].

As previously reported, HSV has been shown to alter nuclear architecture [**126**], inducing perforations and distortions and to increase nuclear lamina phosphorylation and disassembly by recruiting cellular PKC at the nuclear rim.

pUL31 and pUL34 are reported to be essential for nuclear lamins modifications occurring during infection, as recombinant viruses deleted for either pUL31 or pUL34 maintain integral the nuclear envelope and its components [**127**] and, in this way, do not enable nuclear capsids to access the INM (HSV-1 [**100, 101**] and PrV [**102, 103**]).

In MCMV, it has been shown UL34 homolog is responsible of cellular PKC recruitment, playing a role in the phosphorylation of nuclear lamins [**87**]. Moreover, the two viral proteins seem to interact with lamin A/C *in vitro* [**88**] and the homologs in EBV are able to co-immunoprecipitate with lamin B [**93**]. Anyway, most of data reported support the hypothesis that pUL31 and pUL34 are necessary to affect lamins but do not act directly[**88**].

Recently, a protein kinase pUL97 of HCMV has been reported to be partially involved in lamina dissolution, as its over-expression induces a decrease of total amount of lamin A/C[**92**], even if infection with a recombinant virus deleted for UL97 showed the viral protein

kinase is not essential for HCMV replication and lamins still appear alterated . A synergic action of pUL97 and pUL31 and pUL34 homologs has been proposed, as HCMV pUL31 homolog, called pUL53, has been found to co-localize with lamin A/C and lamin B [**128**].
Finally, all these reports describe UL31 and UL34 products and their homologs like viral factors essential for nuclear capsid egress and, in particular, for primary envelopment: their action at the nuclear rim is necessary to induce those nuclear lamina modifications that allow virions to reach the INM and begin the budding events that will lead them to maturation.

1.4 RNA INTERFERENCE: A POTENT TOOL FOR GENE SILENCING

Post-transcriptional gene silencing (PTGS) was initially considered a rare phenomenon limited to few plant species but in the last years, it has become clear that PTGS occurs in both plants and animals and has roles in viral defense and transposon silencing mechanisms. PTGS and, in particular, RNA interference (RNAi) — PTGS initiated by the introduction of double-stranded RNA (dsRNA) — are considered a tool to knock out expression of specific genes in a variety of organisms [**129, 130, 131**]. Although transgene-induced silencing in some plants appears to involve gene-specific methylation (transcriptional gene silencing, or TGS), in others silencing occurs at the post-transcriptional level (post-transcriptional gene silencing, or PTGS): the homologous transcript is made, but that it is rapidly degraded in the cytoplasm and does not accumulate [**129, 131, 132**] Both biochemical and genetic approaches have led to the current models of the RNAi mechanism.that consists of an initiation and an effector step [**131, 133**]. In the initiation step, input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs), which have also been called "guide RNAs" [**133, 134**]. Evidences indicate that siRNAs are produced when the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, processively cleaves dsRNA (introduced directly or via a transgene or virus) in an ATP-dependent processive manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs [**135, 133**]. In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, called RISC. An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA [**131, 134**]. Although the mechanism of cleavage is not completely clear, evidences indicate that each RISC contains a single siRNA and an RNase, called Slicer, that appears to be distinct from Dicer[**133**]. Because of the remarkable potency of RNAi in some organisms, an amplification step within the RNAi pathway has also been proposed. Amplification could occur by copying of the input dsRNAs, which would generate more siRNAs, or by replication of the siRNAs themselves. Alternatively or in addition, amplification could be effected by multiple turnover events of the RISC [**131, 133, 134**].

While the natural presence of RNAi had been observed in a variety of organisms (plants, protozoa, insects, and nematodes), evidence for the existence of RNAi in mammalian cells

took longer to establish. Transfection of long dsRNA molecules (>30 nt) into most mammalian cells causes nonspecific suppression of gene expression, as opposed to the gene-specific suppression seen in other organisms. This suppression has been attributed to an antiviral response, which takes place through one of two pathways. In one pathway, long dsRNAs activate a protein kinase, PKR, that in turn phoshorylates and inactivates the translation initiation factor, eIF2a, leading to repression of translation [**136**]. In the other pathway, long dsRNAs activate RNase L, which leads to nonspecific RNA degradation [**137**]. Interestingly, dsRNAs less than 30 nt in length do not activate the PKR kinase pathway. This observation, as well as knowledge that long dsRNAs are cleaved to form siRNAs in worms and flies, prompted researchers to test whether introduction of siRNAs could induce gene-specific silencing in mammalian cells [**138**]. Indeed, siRNAs introduced by transient transfection were found to effectively induce RNAi in mammalian cultured cells in a sequence-specific manner. The effectiveness of siRNAs varies — the most potent siRNAs result in >90% reduction in target RNA and protein levels [**139, 48, 140**]. The most effective siRNAs turn out to be 21 nt dsRNAs with 2 nt 3' overhangs. Sequence specificity of siRNA is very stringent, as single base pair mismatches between the siRNA and its target mRNA dramatically reduce silencing [**48, 141**]. Unfortunately, not all siRNAs with these characteristics are effective. The reasons for this are unclear but may be a result of positional effects [**140**] Jarvis RA, and Ford LP. (2001) The siRNA Target Site Is an Important Parameter for Inducing RNAi in Human Cells. *TechNotes* **8(5)**: 3-5., Brown D, Jarvis R, Pallotta V, Byrom M, and Ford L. (2002) RNA Interference in Mammalian Cell Culture: Design, Execution and Analysis of the siRNA Effect. *TechNotes* **9(1)**: 3-5.). In early experiments with mammalian cells, the siRNAs were synthesized chemically. So far, injection and transfection of dsRNA into cells and organisms have been the main method of delivery of siRNA. And while the silencing effect lasts for several days and does appear to be transferred to daughter cells, it does eventually diminish. Recently, however, a number of groups have developed expression vectors to continually express siRNAs in transiently and stably transfected mammalian cells [**142, 143, 144, 145, 146, 147, 148**] As recent findings in *C. elegans*, *Drosophila*, plants and Trypanosomes demonstrate RNAi can be induced by an RNA molecule that folds into a stem-loop structure [**131**], some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which get processed in vivo into siRNAs-like molecules capable of carrying out gene-specific silencing [**142, 145, 148**].

In the last years, RNAi is acquiring importance as it is considered a potential effective tool, with also anti-viral therapeutic potentials. Several studies, concerning a variety of viruses,

have shown RNA interference has given good result inhibiting viral replication, using as targets the transcripts of essential viral genes. A part from this potential and important application, RNA interference remains a useful instrument for basic research, as post-transcriptional silencing allows to modify the pattern of gene expression without manipulating genic sequences, an important advantage, in particular, to silence genes that are inverted on the genome sequences and share nucleotides with adjacent open reading frames.

2. MATHERIALS AND METHODS

2.1 Cell culture and viruses.

Primary human lung fibroblasts (HELFs) were cultivated in MEM, containing 10% foetal bovine serum; COS7 cells in DMEM containing 5 % foetal bovine serum. HCMV laboratory strain AD169 was propagated in HELFs. Infections were performed at a MOI of 0,5 as briefly described: cells

were washed once with MEM without serum and inoculated with the appropriate dilution of viral stock; after an incubation of 1h at 37°C in gentle agitation, the inoculum was removed and replaced with fresh MEM containing 10% foetal bovine serum.

2.2 Plasmids encoding pUL53 and pUL50.

Plasmid pc53FLAG has been previously described (Dal Monte et al., 2002). To express pUL53 as a fusion protein with 6XHis tag in bacteria, UL53 ORF has been cloned in pCR T7/CT-TOPO (Invitrogen, Carlsbad, CA, USA). UL53 ORF has been amplified by PCR using the upper primer UL53-U21 and the lower UL53-L21 (see **Table 2.1**), lacking of the STOP codon, contained in the vector, downstream the sequences encoding 6XHis and V5 epitopes. The PCR product has been incubated with the linearized vector for 5 min at room temperature and the resulting plasmid, pCR TOPO-UL53, was used to transform the bacterial strain E.Coli BL21(DE) for bacterial expression and JM109 for plasmid propagation.

UL50 ORF was amplified by PCR from HCMV strain AD169 genome using the primers UL50-U18 and UL50-L16 (see **Table 2.1**). The PCR product has been inserted in pCR T7/CT-TOPO, originating the plasmid pCR TOPO-UL50. To obtain the eukaryotic expression vector pcDNA-UL50V5, pCR TOPO-UL50 was cut with the restriction enzymes

XbaI and PmeI and the resulting sequence, containing UL50 ORF in frame with 6XHis and V5 epitopes, was subcloned in the EcoRV site of pcDNA3 plasmid (Invitrogen, Carlsbad, CA, USA).

2.3 Expression and purification of pUL53-6XHIS fusion protein.

The construct pCR T7 TOPO-UL53 described above was used to chemically transform the *Escherichia Coli* BL21(DE) strain, a bacterial strain optimized for protein expression. For production of pUL53-6XHIS, 10 ml of fresh stationary-phase culture was inoculated into 500 ml of Luria broth (LB) supplemented with ampicillin (50 μg/ml). The bacterial culture was grown at 37°C until an optical density at 600 of nm 0.6 and protein expression was induced with 5 mM isopropylthiogalactopyranoside (IPTG) for 6 h at 28°C, in gentle shaking. The protein was purified by affinity chromatography using the *Invitrogen ProBond Purification System* for 6XHis tagged proteins (Invitrogen, Carlsbad, CA, USA), according to manifacturer's instructions. Briefly, the bacterial pellet was lysed in denaturing conditions with Guanidinium Lysis Buffer (6 M Guanidine Hydrochloride; 500 mM NaCl; 20 mM Sodium Phosphate, pH 7.8) and sonicated 3 times with 10 sec pulses at medium intensity with sonicator Bandelin Sonoplus (Bandelin Electronic, Berlin, Germany). After a 30 min centrifugation at 3000xg, the supernatant was harvested, loaded on purification columns, previously washed with water and equilibrated with Denaturing Binding Buffer (8 M Urea; 500 mM NaCl; 20 mM Sodium Phosphate pH 7.8), and incubated at room temperature for 30 min in gentle agitation. After two washes with Denaturing Binding Buffer and Denaturing Wash Buffer (8 M Urea; 500 mM NaCl; 20 mM Sodium Phosphate, pH 6 and pH 5.3 respectively), pUL536XHis was eluted using Denaturing Elution Buffer (8 M Urea; 500 mM NaCl; 20 mM Sodium Phosphate; pH 4.0).

2.4 Production of anti-pUL53 antibody.

The pUL53 affinity purified protein was denatured in SDS and separated by electrophoresis on 10% denaturing polyacrylamide gels, stained with Coomassie blue or transferred on nitrocellulose membrane and revealed for the expression of V5 epitope. As Western Blot analysis revealed two specific bands with molecular weight of about 40 KDa and 28 kDa, the most abundant was chosen to obtain the antibody. The purified protein was separated on 9% denaturing polyacrylamide gel, stained with $CaCl_2$, cut out of the gel and electroeluted. After a step in PD-10 Desalting Columns (Amersham Biosciences, Buckinghamshire, UK), the recombinant protein has been concentrated by evaporation at 4° C, using UniVapo 150H

(UniEquip, Martinsried, Germany), to the final concentration of a final concentration 0.45µg/µl and used to immunize laboratory mice.

To produce the polyclonal anti-serum anti-pUL53, six BALB\c mice have been immunized with the purified protein and three with water as negative control. The immunogen (45µg in 100µl of water) an the negative control were emulsioned with an excess of complete Freund's adjuvant and injected once a week, for six weeks into the intraperitoneal cavity of the BALB\c mice (Cevenini et al., 1991). The ascytes containing the polyclonal antibody against pUL53 and the controls were prelevated and tested to evaluate the specificity by Western Blot on lysates of mock and pc53Flag-transfected COS7 cells.

2.5 Plasmids encoding GFP fusion proteins (Gateway Technology®).

Every construct expressing fusion proteins between pUL53 or pUL50 fragments and GFP or DsRed was generated using Gateway Technology® (Invitrogen, Carlsbad, CA, USA) (see paragraph *2.6 The gateway Technology*). The sequences were amplified by PCR with *AmpliTaq Gold* polymerase (Roche, Branchburg, New Jersey, USA), using appropriate primers containing AttB1 and AttB2 sites (listed in **Table 2.1)** and the plasmids pcDNA50V5 and pc53Flag as templates. After electrophoretic separation in 1% agarose gel, PCR fragments were purified using the commercial extraction kit *Cleanmix* (Talent, Trieste, Italy), according to manifacturer's instructions, and cloned in the donor vector pDONOR207 (Invitrogen, Carlsbad, CA, USA) by BP reaction, obtaining the *entry* clones listed in **Table 2.2**. All entry clones were sequenced by PRIMM Srl (PRIMM Srl, Milan, Italy). To obtain expression clones (see **Table 2.2**), LR reactions were performed using pDONOR vectors and the destination vectors pDEST53 (Invitrogen, Carlsbad, CA, USA) and pBKdsRed2CMV (Soboleva et al., Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme. J Biol Chem, 2005. 280(1): 382-52). The integrity of expression vectors was verified by enzymatic digestion with appropriate restriction enzymes.

2.6 The Gateway Technology.

The Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways: the components of the lambda recombination system are modified to allow an intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded recombination proteins.

Lambda recombination occurs between site-specific *att*achment (*att*) sites, *att*B on the *E. coli* chromosome and *att*P on the lambda chromosome, which represent the binding site for recombination proteins. Upon lambda integration, recombination occurs between *att*B and *att*P sites to give rise to *att*L and *att*R sites. The actual crossover occurs between homologous 15 bp core regions on the two sites, but surrounding sequences are required as they contain the binding sites for the recombination proteins.

Lambda recombination is catalyzed by a mixture of enzymes that bind to specific sequences (*att* sites), bring together the target sites, cleave them, and covalently attach the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel form. The recombination proteins involved in the reaction differ depending upon whether lambda utilizes the lytic or lysogenic pathway The lysogenic pathway is catalyzed by the bacteriophage λ Integrase (Int) and *E. coli* Integration Host Factor (IHF) proteins (included in BP Clonase™ enzyme mix) while the lytic pathway is catalyzed by the bacteriophage λ Int and Excisionase (Xis) proteins, and the *E. coli* Integration Host Factor (IHF) protein (included in LR Clonase™ enzyme mix) (**Fig. 2.1**).

The Gateway® Technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified *Att* sites) from an *entry* vector to a variety of *destination* vectors, generating specific expression clones (**Fig. 2.2**). Two recombination reactions constitute the basis of the Gateway® Technology:

- BP Reaction: facilitates recombination of an *att*B substrate (*att*B-PCR product or a linearized *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing *entry* clone (**Fig. 2.3**). This reaction is catalyzed by BP Clonase™ enzyme mix.
- LR Reaction: facilitates recombination of an *att*L substrate (*entry* clone) with an *att*R substrate (*destination* vector) to create an *att*B-containing expression clone (**Fig. 2.4**). This reaction is catalyzed by LR Clonase™ enzyme mix.

2.7 Transfection.

Transient transfection of subconfluent COS7 cells and HELFs was performed at a confluency of 75 %, using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) or Fugene6 (Roche) according to the manufacturers' instructions. Transfected cells were fixed or harvested after 24-48 hours after transfection and analized by Immunofluorescence assay and ImmunoBlotting assay.

2.8 Immunofluorescence assay.

96-120 h *post-infection* or 48h *post-transfection*, cells were fixed in 4% formaldehyde in PBS for 10 min at 4°C and permeabilized with methanol at -20°C for 10 min. After 3 washes in PBS, cells were blocked with BSA 4% in PBS for 30 min and then incubated for 1 hour at 37°C with the following primary antibodies: mouse monoclonal anti–Flag M2 (Sigma-Aldrich, St. Louis, MO, USA) dilution 1:250; mouse monoclonal anti-V5 epitope (Invitrogen, Carlsbad, CA, USA) 1:500; mouse polyclonal anti-pUL53 1:100; goat polyclonal anti-lamin A/C (S. Cruz Biotechnology, Santa Cruz, CA, USA) 1:10; goat polyclonal anti-lamin B (S. Cruz Biotechnology, Santa Cruz, CA, USA) 1:10; mouse monoclonal IgM SW2-30 pure (kindly provided by Professor Radsak, Marburg, Germany); mouse monoclonal IgG anti-gB pure (kindly provided by Professor Mach, Erlangen, Germany); mouse monoclonal IgM 14-16 anti-gN pure (kindly provided by Professor Mach, Erlangen, Germany). After 3 washes with PBS, cells were incubated for 1 hour at room temperature with fluorochrome conjugated secondary antibodies Alexa Fluor (Invitrogen, Carlsbad, CA, USA) diluted 1:1000 in PBS.
Chromatin staining was performed using DAPI (4'6'-diamidino 2-phenylindole) diluted 0,1 ng/ml for 3 min at room temperature. After 3 washes, the coverslips were mounted with Polyvinyl Alcohol Mounting Media with DABCO (Sigma-Aldrich, St. Louis, MO, USA) and analyzed with a fluorescence microscope Nikon Eclipse 600 (Nikon, Enfield, CT, USA) with a 100X oil objective. The images were acquired using Lucia Image software.

2.9 Leptomycin B treatment and Confocal laser microscopy on living cells.

The subcellular localization of the fusion proteins with GFP has been analized in living cells 24 h post- transfection and after 4 h of treatment with Leptomycin B (LMB) with a final concentration of 4ng/ml by confocal laser scanning microscopy (CLSM) using a *Bio-Rad MRC 600* system with a fluorescence microscope *Nikon* and a 40X objective. The ratio between nuclear and cytoplasmic fluorescence for every fusion protein has been calculated as previuosly, using *Image J 1.6* software (National Institute of Health, USA) and the mathematical formula $Fn/c=(Fn-Fb)/(Fc-Fb)$, where *F* stands for fluorescence, *n* for nuclear, *c* for cytoplasmic and *b* for background..

2.10 Sub-cellular fractioning.

Sub-cellular fractioning of mock-infected and infected HELFS was performed to separate cytoplasmic and nuclear proteins. Cells were resuspended lysis buffer containing 10 mM Tris-HCl pH 7.8, 1% Nonidet P-40 (NP-40), 10 mM 2-mercaptoethanol and protease inhibitors.

The separation was achieved by hypotonic shock and shearing; nuclei were obtained as pellet by a 300x*g* centrifugation at 4°C and the supernatant representing the cytoplasmic fraction was clarified by centrifugation at 600xg. Pellets containing purified nuclei were resuspended in RIPA buffer (10mM Tris-HCl; 150mM NaCl; 1mM EDTA; 0.1% SDS; 0.5% sodium deoxycolate; 1% TritonX-100; 1mM Na_3VO_4; 1mM PMSF), sonicated at low intensity for 15 sec and cleared from nuclear debris by a 14000 rpm centrifugation at 4°C for 10 min. Cytoplasmic and nuclear samples were additioned with Laemmli's sample buffer and loaded on 5-20% gradient polyacrylamide gels.

2.11 Immunoprecipitation.

To perform immunoprecipitation assay, COS7 cells were harvested 48h post-transfection, while infected and mock-infected HELFs 96-120 h post-infection. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in Triton-X lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton-X , protease inhibitor cocktail [Roche]) for 10 min in ice and sonicated at low intensity for 10 seconds. After 20 min of centrifugation at 14000 rpm, the supernatants were collected and mixed with the appropriate antibody (4 µg/sample) and incubated overnight at 4°C while rocking gently. The day after, 30µl of protein A/G-beads (S. Cruz Biotechnology) were added and incubated at 4°C while rocking gently. After 4h, the beads were collected b centrifugation for 1 min at 1000 rpm and washed three times with Triton-X Lysis buffer for 15 min at 4°C while rocking gently. After the last washing, the beads were resuspended in Laemmli's buffer 2X, boiled at 95°C and centrifugated at 14000 rpm for 5 min, before loading the supernatant on 8% or 10% SDS-PAGE gel.

2.12 Western blotting.

After SDS-PAGE electophoresis, proteins were transferred on PROTRAN Nitrocellulose Transfer Membrane (Schleischer & Schuell Biosciences, Dassel, Germany). The membranes were blocked with non fat milk (Bio-Rad, Hercules, CA, USA) 5% in PBS-0,1% Tween20, washed three times with PBS-0,1% Tween 20 and probed with the following antibodies: mouse monoclonal anti-Flag (Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-V5 mouse (Invitrogen, Carlsbad, CA, USA); polyclonal anti-pUL53, dilution 1:150; goat polyclonal anti-lamin A/C (S. Cruz Biotechnology, Santa Cruz, CA, USA), 1:100; goat polyclonal anti-lamin B (S. Cruz Biotechnology, Santa Cruz, CA, USA), 1:100. After washes with PBS-0,1% Tween20, the membranes were incubated with secondary antibodies

conjugated to horseradish peroxidase (DAKO, Glostrup, Germany), diluted 1:10,000 and washed again 3 times. The signal was detected with enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Buckinghamshire, UK) on standard X-ray film (Kodak, Rochester, NY, USA).

2.13 Plasmid for RNA interference against UL53 ORF transcript.

To perform RNA interference versus UL53 transcript during infection, the plasmid pG-SUPER has been used (**Fig.2.1**): the plasmid contains two human promoters, leading the transcription of GFP encoding gene, as marker of transfection, and the transcription of a small hairpin RNA directed against UL53 ORF [**142, 149**]. The gene-specific insert is designed to specify a 19 nucleotide sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 19 nucleotide sequence. The resulting transcript is predicted to fold back on itself to form a 19-base pair stem-loop structure, that will mediate gene silencing. Three specific sequences of 19 nucleotides were individuated on UL53 ORF sequence, using a sequence design on-line software (*http://bioinfo.clonetech.com/rnaidesigner/sirnaSequenceDesign.do*) and selected for the ability to not cross-react with other viral and cellular transcripts, using *BLAST* software (*http://www.ncbi.nlm.nih.gov/BLAST*). The sequences and the derivating oligonucleotides and plasmids are listed in **Table 2.3**.

The phosphorylated oligos were chemically synthesized (Geneworks Pty Ltd) and annealed at 94°C for 4 min, then at 70°C for 10 min and decreasing the temperature 0,1°C/sec until it was 20°C. Once annealed, the duplexes were inserted in the vector pG-SUPER, digested with the restriction enzymes BglII and HindIII (New England Biolabs). After a ligation with T4 Ligase (Roche, Branchburg, New Jersey, USA) and bacterial transformation, the colonies were screened by enzimatic digestion and sequencing.

2.14 RNA interference against UL53 ORF transcript.

To silence UL53 ORF expression during viral infection, subconfluent HELFs have been transfected separately with the plasmids pG-SUPER, as negative control, pG-SUPER-UL53-224, pG-SUPER-UL53-757 and pG-SUPER-UL53-782 encoding for GFP, as marker of transfection and a small hairpin RNA with interfering properties. The day after, cells have been infected with HCMV AD169. 120 h post-infection, cells were fixed and stained for pUL53 and gN.

2.15 Sequence analysis and statistical analysis

All sequence analysis of pUL53 and pUL50 proteins have been performed manually and using the on-line softwares *PSORTII*, *NetNES* and *THMM*.

Statistical analysis of data have been performed using the software *Sigma Plot 9.0.*

3. RESULTS

3.1 Nuclear lamina remodelling during HCMV infection

To determine eventual nuclear alterations occurring during HCMV infection, nuclear lamina and, in particular, its major components, lamin A/C and lamin B, were chosen as targets of the analysis. The observations were performed on mock- and infected HELFs using commercial antibodies directed against lamin B and lamin A/C and a mouse monoclonal antibody reacting versus the viral glicoprotein gB, that during infection localizes in the cytoplasmic area, called "assembly compartment".

In interphasic mock-infected HELFs, nuclei presented a normal ovoid morphology and lamin A/C and lamin B stainings revealed an homogeneous pattern at the nuclear periphery (**Fig. 3.1a - b)**, with the occasional presence of small dots corresponding to normal invaginations of the nuclear membrane. Immunofluorescence assays on infected fibroblasts revealed remarkable modifications of the nuclear shape and a strong alteration of nuclear lamins. In details, while at early stages of infection, no change is detectable, at later stages (72-120 h *post-infection*) lamin B showed a ruffled staining at the nuclear rim with the formation of invaginations which increased in number and dimensions during the progression of infection (**Fig. 3.1a)**; a similar pattern was observed for lamin A/C (**Fig. 3.1b)**.

3.2 Antiserum anti-pUL53 specificity.

To performe analysis of pUL53 during HCMV infection, a novel antibody directed against the viral protein was necessary. Big amounts of protein were obtained inducing the expression of pUL53, as a fusion protein with 6XHis epitope, in the bacterial strain E. Coli BL21 (DE), transformed with the plasmid pCR TOPO-UL53. The recombinant protein has been purified in several steps: as purification by affinity chromatography resulted insufficient, the fractions have been electrophoretically separated, then the band of interest has been excised and

electroeluted. The purified fraction has been desalted and concentrated to a final concentration of 0.45µg/µl and used to immunize laboratory animals.

The antiserum against pUL53 and the controls have been prelevated and tested for specificity by WB on lysates of mock- and transfected COS7 cells with the plasmid pc53Flag (Dal Monte et al, 2002). The antiserum versus pUL53 recognized a specific band in transfected fractions, with a specificity comparable to the monoclonal antibody anti-Flag (**Fig. 3.2**), indicating an high efficiency and high signal to noise ratio.

3.3 pUL53 during HCMV infection.

To perform the analysis of pUL53 during infection, a novel highly specific antibody was generated as detailly described in Materials and Methods. IIF assays performed on mock- and infected HELFs confirmed that, at late stages of infection (96-120h *p.i.*), pUL53 localizes mostly at the nuclear rim forming aggregates, previously described as "punctuate patches" (Dal Monte et al., 2002), although at minimal levels in the cytoplasm of HCMV infected cells(**Fig. 3.3a**). The analysis of the phases of HCMV infection showed that pUL53 is undetectable in cells at early stages of infection and appears at 48h pi, at the nuclear rim with a slightly discontinuous distribution (**Fig. 3.3b**). The formation of distinct aggregates follows at later stages (**Fig. 3.3b'**), suggesting a temporal organization of pUL53 expression and localization.

To confirm pUL53 distribution, nuclear and cytoplasmic extracts were obtained from mock- and infected HELFs and analyzed by Western Blotting: a clear band at about 42 kDas was strongly detectable in infected nuclear fractions, while a very low signal was detectable in infected cytoplasmic extracts (**Fig. 3.4**). Nuclear and cytoplasmatic extracts of mock-infected HELFs did not show any positivity.

3.4 pUL53 colocalizes with nuclear lamina modifications.

To further investigate the role of pUL53 during HCMV infection, co-staining experiments of pUL53 with d the different components of nuclear lamina were performed on HELFs late in infection, when the viral protein is organized in nuclear aggregates.

The analysis revealed that the HCMV-induced invaginations in lamin B and in lamin A/C correspond to pUL53 aggregates on the nuclear rim(**Fig. 3.5a – b,** respectively).

To further characterize the invaginations and distortions observed, a monoclonal antibody raised against a phosphorylated epitope at the N-terminus of lamin A/C, called SW2-30 (Radsak), has been used for co-staining IIF assay with anti-pUL53 anti-serum. Differently from lamin A/C, its N-terminal phosphorylated form presented, in mock-infected cells, an irregular and sometimes punctuate distribution, associated to the nuclear boundary. Infected cells resulted strongly affected: the overall fluorescence signal resulted decreased, but aggregates of phospho-lamin A/C were detectable at the nuclear rim co-localizing with pUL53 punctuate patches (**Fig. 3.5c**).

3.5 pUL53 does not co-immunoprecipitate with nuclear lamins

To investigate if a strong interaction exists among pUL53 and nuclear lamina components, an immunoprecipitation has been performed on lysates of nuclear extracts of mock- and infected HELFs, using the anti-pUL53 anti-serum. The viral proteins has been efficiently immunoprecipitated (**Fig. 3.6**), but both lamin A/C and lamin B were detectable only in the lysates lanes (**Fig. 3.6c** and **Fig. 3.6b,** respectively), indicating co-immunoprecipitation did not occurr.

3.6 Singularly expressed pUL53-Flag localizes into the nucleus of transfected cells.

To study the behaviour out of the viral contest, pUL53 has been expressed as a fusion protein with the epitope Flag in COS7 cells. IIF assays have revealed a wide-spread nuclear distribution with a low cytoplasmic staining, in contrast with the observations during infection (**Fig. 3.7a)**. WB analysis on lisates of mock- and trasfected cells has confirmed the predicted molecular weight of about 41 kDas (**Fig. 3.7b)**.

3.7 Singularly expressed pUL50V5 localizes into the cytoplasm of transfected cells.

pUL50 has been studied during transfection in COS7 cells, when singularly expressed as a fusion protein with epitope V5. IIF analysis showed the protein localizes predominantly in the cytoplasmic compartment and in perinuclear area, with a predominant ER pattern(**Fig. 3.8a)**, in accordance with the predictions. The putative molecular weight of about 43 kDas has been confirmed by WB analysis on lysates of mock- and transfected COS7 cells (**Fig. 3.8b)**.

3.8 pUL50V5 and pUL53Flag interact at the nuclear rim of transfected cells and co-immunoprecipitate.

As the observations of the localization of pUL53 during infection and when singularly expressed showed such a discrepancy, pUL53 has been co-expressed with pUL50V5 in COS7 cells to study a potential interaction between them. IIF assays has revealed a complete re-localization of both protein at the nuclear rim with the formation of aggregates (**Fig. 3.9a)**, resembling the pattern of pUL53 observed in HCMV infected cells. The interaction has been further investigated by immunoprecipitation assay, that revealed pUL50V5 and pUL53Flag are able to interact and co-immunoprecipitate (**Fig. 3.9b)**. All these data indicate pUL50 and pUL53 interact and need each other to acquire a punctuate distribution at the nuclear rim, that is the physiological organization of pUL53 during viral infection.

3.9 pUL53 sequence analysis.

Sequence analysis of pUL53, performed manually and using several on-line softwares, revealed important putative characteristics of the viral protein:

- 1 putative nuclear localization signal (NLS) with sequence RERRSALRSLLRKRRQR[27] at the N-terminus that could be responsible of its translocation into the nucleus when singularly expressed.
- 1 C-terminal region with two sequences highly rich in basic aminoacids, typical of nuclear export signals (NESs): VTLNIVRDHVVISVL(230-244) and VKIDVTILQRKIDEMDI (253-269) (basic aminoacids are underlined). Manual analysis individuated other two short sequences, rich in leucine LTLHDL(59-64) and LELKYL(74-79), at the N-terminus, downstream the NLS.

To study the role all these characteristics on the behaviour of pUL53 (**Fig. 3.10a),** the corresponding domains have been cloned singularly and expressed as fusion proteins with GFP, to analyse them in fixed and living cells. Six different plasmids were generated (see **Table 2.2)**, encoding fusion proteins (**Fig. 3.10b)** between N-terminal GFP and

- pUL53 wild type (residues 2-376);
- N-terminus of pUL53 (residues 2-29), containing the putative NLS;
- pUL53 lacking the N-terminus (residues 30-376), containing the putative NESs, but not the NLS;
- pUL53 lacking the C-terminus with the putative NES (residues 2-242)
- pUL53 lacking both the N-terminus with the NLS and the C-terminus (residues 30-242)
- C-terminal domain of pUL53(residues 220-376).

3.10 Analysis of the localization pUL53 domains as fusion proteins with GFP: pUL53 contains a functional nuclear localization signal (NLS) at the N-terminus.

Classic approaches to identify an NLS are the generation of deletions corresponding to the signal sequence in the protein of interest and the generation of fusion proteins between the putative NLS with reporter proteins, such as β-galactosidase and GFP, that when singularly expressed have cytoplasmic and ubiquitous localization, respectively. In this study, the putative NLS of pUL53 has been expressed as a fusion protein with GFP, transfecting COS7 cells with the plasmid pDEST53-UL53(2-29) and the localization has been observed both in fixed and living cell: GFP(2-29) is localized into the nucleus, accumulating frequently in the nucleoli and the signal in the cytoplasm is almost undetectable, indicating pUL53 NLS is functional (**Fig. 3.11b** and **3.12b**, respectively).

The analysis has been performed also expressing the plasmids pDEST53-UL53(2-242), encoding for N-terminal GFP fused with pUL53, deleted of the hydrophobic C-terminal domain but still containing the putative NESs and pDEST53-UL53(2-376) encoding for N-terminal GFP fused with full-length pUL53: both proteins displayed the same localization pattern of GFP-UL53(2-29) (**Fig. 3.11a-3.12a** and **Fig. 3.11c-3.12c**, respectively), indicating the NLS is able to translocate into the nucleus also proteins with a higher molecular weight.

To confirm the N-terminal NLS is responsible of pUL53 nuclear distribution, pDEST53-UL53(30-376) encoding the N-terminal deletion mutant of pUL53 as a fusion protein with GFP has been expressed in COS7 cells and observed by fluorescence microscopy on living and fixed cells. The expected result was the exclusion of the fusion protein from the nuclear compartment, as free diffusion through the membrane is allowed only for small proteins (the cut-off is about 50 kDas), while, for large proteins, a NLS is required. Despite the relatively molecular weight (about 66 kDas) and the absence of the N-terminal NLS, GFP-UL53(30-376) showed an ubiquitous distribution (**Fig. 3.11d-3.12d**).

3.11 Analysis of the localization pUL53 domains as fusion proteins with GFP: pUL53 contains nuclear export signals (NESs).

Primary sequence analysis of pUL53 revealed the presence of one sequence rich in basic aminoacids, typical of nuclear export signals (NES), located at the C-terminus of the viral protein. Moreover, other two short leucine-rich sequences are present at the N-terminus. To study the functionality of these sequences, pUL53 domains have been expressed in COS7 cells as fusion protein with GFP in presence or not of a specific nuclear export inhibitor, Leptomycin B, and the analysis has been performed by CLSM on living cells and using

ImageJ software. Ratio between nuclear and cytoplasmic fluorescence (Fn/c) revealed GFP-UL53(2-376) full-length is significantly sensitive to the inhibitor of the nuclear export (**Fig. 3.13**); the same result has been obtained for GFP-UL53(30-376) and GFP-UL53(30-242), indicating that the C-terminal putative NES is functional. Surprisingly, also GFP-UL53(30-242) showed a significant increase of Fn/c value after treatment, indicating the two sequences individuated by manual analysis could act as NESs.

The analysis could not be performed for the proteins containing the N-terminal domain, but lacking of the C-terminus, GFP-UL53(2-29) and GFP-UL53(2-242), as the nuclear fluorescence was too intense with respect to the cytoplasmic one, preventing an accurate calculation of the Fn/c values. However, this observation suggests that the two NES-like sequences at the N-terminus are not sufficiently strong to confer cytoplasmic translocation in the presence of the NLS and in the absence of the C-terminal region.

3.12 pUL50 sequence analysis.

Similarly to pUL53, also pUL50 primary sequence has been analyzed to identify its characteristic domains. On-line softwares revealed the presence of a cluster of hydrophobic aminoacids, indicated as a putative transmembrane domain, located at the C-terminus (residues 359-381) **(Fig. 3.14)**.

To study the role of the putative characteristics of pUL50 (**Fig. 3.15a),** the corresponding domains have been cloned singularly and expressed as fusion proteins with GFP and analyzed in fixed and living cells by fluorescence micoscopy. Four plasmids have been generated (see **Table 2.2)**, encoding fusion proteins (**Fig. 3.15b)** between N-terminal GFP and

- pUL50 full length (residues 2-397);
- pUL50 deleted of the first 70 aminoacids (residues 71-397),but containing the putative transmembrane region;
- pUL50 deleted of the C-teminal transmembrane domain(residues 2-358);
- C-terminal transmembrane domain of pUL50 (residues 215-397).

3.13 Analysis of the localization pUL50 domains as fusion proteins with GFP in living and fixed cells.

pUL50 domains fused with GFP have been expressed in COS7 cells and analyzed by fluorescence microscopy in living and in fixed cells. Like pUL50V5, GFP-UL50(2-397)

displayed a typical RE pattern with a cytoplasmic localization, associated to the nuclear membrane (**Fig. 3.16-3.17a**). A similar distribution resulted for both GFP-UL50(71-397) and GFP-UL50(215-397), confirming the transmembrane domain is located at the C-terminus of the protein (**Fig. 3.16-3.17 b** and **c**, respectively). In particular, UL50(215-397) showed a strong association with the cellular membranes. In contrast, GFP-UL50(2-358), lacking of the C-terminal domain, showed an ubiquitous distribution, with a slight accumulation in the nucleus and the occasional formation of small intranuclear speckles (**Fig. 3.16-3.17d**).

3.14 Analysis of the interaction between pUL50 and pUL53 by fluorescence assay on living and fixed cells.

To study pUL50 and pUL53 interaction and individuate the domains responsible of their binding, COS7 cells have been transfected with the constructs encoding the protein fragments as fusion proteins with GFP and DsRed2 and observed by microscopy on living cells.

Initially, DsRed2-UL53(2-376) full-length has been co-transfected with the different domains of pUL50 fused with GFP. The analysis showed DsRed2-UL53 co-localizes with GFP-UL50(2-397) in punctuate patches at the nuclear rim and with GFP-UL50(2-358), as both of them are dispersed into the nucleus **(Fig. 3.18 a-b)**. In contrast, GFP-UL50(71-397) and GFP-UL50(215-397) are retained in the cytoplasmic compartment, while DsRed2-UL53(2-376) maintains its nuclear distribution, displaying the absence of co-localization (**Fig. 3.18 c-d)**. These data suggest that the domain of pUL50 to bind pUL53 resides at the N-terminus, as the deletion of the first 70 aminoacids is sufficient to abolish their co-localization.

The reverse experiment has been performed co-transfecting COS7 cells with DsRed2-UL50(2-397) full-length in combination with pUL53 fragments fused with GFP. The observations showed that DsRed2-UL50(2-397) co-localizes forming aggregates at the nuclear boundary with GFP-UL53(2-376) (**Fig. 3.19a)**, as expected; forms aggregates in the cytoplasm and at the nuclear rim with GFP-UL53(30-376) (**Fig. 3.19d)** and with GFP-UL53(30-242) (**Fig. 3.19e)**, while seems to co-localizes in the same cytoplamic compartment with GFP-UL53(220-376) without a strict association (**Fig. 3.19f).** GFP-UL53(2-242) displayed a distribution mostly in the nucleus, but a interaction at the nuclear rim, forming small aggregates with DsRed2-UL50(2-397) was detectable(**Fig. 3.19c)**. In contrast, no co-localization is detectable in cells co-tranfected with DsRed2-UL50(2-397) and GFP-UL53(2-29), as pUL50 appeared distributed in the cytoplasm and the N-terminal pUL53 domain only inside the nucleus(**Fig. 3.19b)**.

All these data suggest the N-terminus of pUL53 is not required to bind pUL50 and that the domain immediately downstream (as showed by GFP-UL53(2-242) is already sufficient to establish an interaction. The results about GFP-UL53(220-376) were not clear as displayed colocalization without any indication of interaction (the proteins did not change their original distribution).

All these observations were performed also on fixed cells by IIF, using different plasmids: pc53Flag instead of pBKdsRed2CMV-UL53(2-376) and pcDNA-UL50V5 instead of pBKdsRed2CMV-UL50(2-397). The results were completely superimposable. (**Fig. 3.20** and **Fig. 3.21**, respectively)

3.15 Analysis of pUL50-pUL53 interaction by co-immunoprecipitation assay.

To confirm the data obtained by microscopy on living cells and IIF and study in depth pUL50-pUL53 interaction, COS7 cells were transfected with the same different combinations of vectors (using pc53Flag and pcDNA-UL50V5 instead of pBKdsRed2CMV-UL53(2-376) and pBKdsRed2CMV-UL50(2-397), respectively) and the cell lysates were used to perform immunoprecipitation assays. The analysis by WB of the immuno- and co-immuno-precipitated proteins confirmed that:

pUL50V5 interacts with GFP-UL53(2-376) full length, GFP-UL53(30-376), GFP-UL53(2-242) and GFP-UL53(30-242), but not with GFP-UL53(2-29) and GFP-UL53(220-376), indicating that the N-terminal and C-terminal domains of pUL53 are not necessary to bind pUL50 (**Fig. 3.22**); the essential binding domain of pUL53 is comprised between the aminoacids 30 and 220; pUL53Flag co-immuniprecipitates with GFP-UL50(2-397) and GFP-UL50(2-358), but not with GFP-UL50(71-397) and GFP-UL50(215-397 (**Fig. 3.23**) confirming the binding domain of pUL50 to pUL53 is included in the first 70 residues.

3.16 The complex pUL50-pUL53 induces nuclear lamina modifications and co-localizes with the alterated sites during transfection

The remarkable alterations of nuclear lamins observed during infection, co-localizing with the aggregates of pUL53, and the observation of interaction and co-localization between pUL50 and pUL53 reproducing the same pattern at the nuclear rim, reported for pUL53, opened the question if the viral proteins were able to induce those modifications even out of the viral contest, during transfection. To perform this study, COS7 cells were transfected singularly

with the plasmids GFP-UL53(2-376) and GFP-UL50(2-397) singularly and to co-expressed pUL50 and pUL53 the plasmids GFP-UL53(2-376) and pcDNA-UL50V5 were used. The samples have been analyzed by IIF for lamin B, lamin A/C, phospho-lamin A/C.

When singularly expressed, pUL50 did not determine any significant alterations on lamin B (**Fig.3.24a**); similar results have been obtained for lamin A/C, both in its not- and phosporylated form, a part from a partial and occasional mislocalization in the cytoplasm of transfected cells(**Fig.3.24b** and **c**, respectively).

Singularly expressed GFP-UL53(2-376) showed occasionally showed to cause a dissolution of both lamin B and lamin A/C. Moreover, phospho-lamin A/C showed a decreased staining at the nuclear rim (**Fig.3.25a, b** and **c**, respectively).

The sample co-expressing GFP-UL53(2-376) and pUL50V5 gave results completely superimposable with the observations during infection: in contrast with their homogeneous pattern in untransfected cells, both lamin B and lamin A/C showed an irregular staining, with formation of invaginations corresponding to the aggregates of GFP-UL53(2-376) (**Fig. 3.26a** and **b**). In contrast, phospo-lamin A/C displayed a decrease of overall signal in co-transfected cells, as observed during infection, but the aggregates less frequently co-localized with the complex of transfected proteins, indicating their only expression is unsufficient to reproduce the same pattern (**Fig. 3.26c**).

3.17 UL53 ORF can be post-transcriptionally silenced by RNA interference.

To study the effects of the silencing of UL53 on viral cycle and on HCMV induced modifications of nuclear lamina during infection, silencing of the gene expression was necessary. UL53 ORF is inverted in HCMV genome sequence with respect to adjacent genes and shares about 50 nucleotides with UL54 ORF, encoding for the viral DNA polymerase. Moreover, polyadenilation signal of other genes are localized in UL53 sequence. For these reasons, the manipulation of HCMV genome and deletion of UL53 gene was not considered a good choice for gene silencing.

Another options was represented by post-transcriptional gene silencing by RNA interference (RNAi) . To perform RNAi directed against UL53 transcript, pG-SUPER plasmid [**142, 149**] has been used (**Fig. 2.1**): this vector, after the insertion of the sequence directed against the gene of interest, produces a small hairpin RNA able to silence the target transcript. It also encodes for GFP, as marker of transfection.

Three construct have been generated: the plasmids, called pG-SUPER-UL53-224, pG-SUPER-UL53-757 and pG-SUPER-UL53-782 have different target sequences on UL53 gene (see **Table 2.3**)

HELFs were transfected with the constructs separately and infected the day after. 120h *post-infection*, cells were fixed and stained for pUL53, to verify if the silencing occurred, and the glycoprotein gN, to individuate HCMV positive cells at late time *post-infection*, as gN has been reported to be a *late* protein. The results have been contrasting: the constructs pG-SUPER-UL53-757 and pG-SUPER-UL53-782 displayed a poor efficiency in silencing the expression of pUL53, as the viral protein was still detectable in GFP- and gN-positive cells, even if the intensity of pUL53-staining showed a decrease (**Fig.3.27c** and **d**, respectively); on the contrary, pG-SUPER-UL53-224 exhibited a good interfering activity as shown in (**Fig.3.27e**), with respect to the negative control transfected with pG-SUPER and infected. This result is promising for further analysis of the role of pUL53, during infection.

4. DISCUSSION

This study has been focused on fundamental events of HCMV replicative cycle: the capsidic maturation through the nuclear envelope to enter the cytoplasm and the modifications of involved cellular components. As previously reported, the egress of virions from the nuclear compartment is essential to reach the cytoplasm, which is the final site of maturation and tegumentation; however, during this passage, viral particles face a strong obstacle, consisting in a thick protein network that underlines the INM, called nuclear lamina, that opposes to the egress.

Our results show that, during HCMV infection, lamins, the main components of nuclear lamina, undergo remarkable modifications: analysis by IIF assay of mock- and infected fibroblasts, co-stained for the major components of nuclear lamina, lamin B and lamin A/C and the viral glycoprotein gB as a marker of infection, showed that both lamins assume an irregular distribution at the nuclear rim of infected cells, with respect to the smooth and homogenuous pattern observed in mock-infected cells, and form deep invaginations, increasing in number with the progression of infection .

In the herpesvirus family, two highly conserved viral products, pUL31 and pUL34, are described as essential "guides" for the passage through the INM: the two viral proteins form a complex at the INM and are strictly correlated to nuclear envelope alterations.

Thus, the study has been focused on pUL53 and pUL50, homologous to pUL31 and pUL34,respectively, and their role in viral infection.

To perform analysis during infection, a novel, highly specific antibody has been raised against the purified protein. The antibody α-pUL53 allowed to follow the expression of the viral protein during infection: pUL53 is weakly detectable at 48h *post-infection*, while, at later stages, is strongly expressed and localizes at the nuclear rim, at first with a discontinuous but homogenous pattern, later forming aggregates or "punctuate patches", suggesting a temporal rearrangement of the protein at the nuclear boundary.

The involvement of pUL53 in lamina rearrangement has been investigated by IIF on mock- and infected HELFs: pUL53 aggregates resulted to co-localize with lamin B and lamin A/C invaginations at the nuclear rim, suggesting a role for this protein in inducing such alterations.
Modification of lamin A/C was further studied using a monoclonal antibody versus a N-terminal phosphorylated form of lamin A/C, in which the phosporylated epitope is located within the first 417 aminoacids: in uninfected cells, phospho-lamin A/C is distributed at the nuclear boundary with a clear even if inhomogeneous pattern, while in infected pUL53-positive HELFs, a decrease of the intesity of total phospho-laminA/C staining has been detected, consistent with the previously published data that reported a quantitative decrease of this form of phosporylated lamin A/C in HCMV infected fibroblasts, as shown by Western blotting; interestingly, the phosphorylated lamin appeared to form small aggregates strongly co-localizing with pUL53 patches.
These data support the observation performed with lamin A/C and B and indicate lamins undergo alterations in structure and phosphorylation at the same sites where pUL53 is distributed and the degree of damage increases with the progression of the infection and the organization of pUL53 in larger aggregates.
Immunoprecipitation (IP) assays have been performed to evaluate if an interaction among pUL53 and lamina components exists: IP against pUL53 did not reveal a stable interaction nor with lamin B neitehr with lamin A/C.
The next step in this study has been the characterization of pUL53 out of the viral contest. The protein has transiently been expressed in mammalian cells and the analysis of its sub-cellular localization by IIF revealed a different pattern, as compared to the one observed during infection: when singularly expressed, pUL53 displays a wide-spread nuclear distribution with a low cytoplasmic staining.
This result induced to enlarge the study to pUL50, homolog of HSV-1 pUL34 that is the counterpart of pUL31, homolog to HCMV pUL53: pUL34 and pUL31 are reported to need each other to interact and co-localize at the nuclear rim of infected cells, where the complex can direct the egress of nuclear virions.
pUL50 is predicted to be a type II trans-membrane protein, similarly to the other members of pUL34 family, and, as expected, IIF analysis on singularly transfected mammalian cells showed that the protein localizes predominantly in the cytoplasmic compartment and in perinuclear area, with a predominant ER pattern.
To study their potential interaction, pUL50 an pUL53 have been co-expressed and heir localization has been analyzed by IIF, that showed both proteins undergo a marked re-

localization at the nuclear rim with the formation of aggregates, resembling the pattern of pUL53 observed in HCMV infected cells. The interaction has been confirmed by IP and Western blotting.

All the results reported are consistent with data in literature that describe the homolog protein to HCMV pUL50, pUL34, as a cytoplasmatic product with a ER-like localization and the homolog to HCMV pUL53, pUL31 as nuclear protein; also for the homologs, the interaction is necessary and sufficient for the re-localization at the nuclear boundary. In contrast to pUL50 and pUL53, that organize in distinct aggregates, pUL31 and pUL34 exhibit a smooth pattern on the nuclear rim, differently from pUL50 and pUL53 that organize in distinct aggregates. Notably, it has been also reported that pUL31 and pUL34 form aggregates only in absence of a non-essential viral encoded kinase, called Us3, that is supposed to phosphorylate preferentially pUL34: in HCMV an homolog kinase to Us3 is not present and it is possible to postulate that in this case the virus evolved without this accessory product.

As, during infection, nuclear lamins modification are detectable mainly when native pUL53 forms aggregates at the nuclear rim, the analysis of nuclear components has been performed also during transient expression of pUL50 and pUL53, singularly and simultaneously.

When singularly expressed, pUL50 did not determine any significant alterations on lamin B; similar results have been obtained for lamin A/C, both in its not- and phosporylated form, a part from a partial and occasional mislocalization in the cytoplasm of transfected cells.

In contrast, pUL53-positive cells showed an occasional dissolution of both lamin B and lamin A/C. Moreover, phospho-lamin A/C showed a decreased staining at the nuclear rim.

In cells co-expressing pUL50 and pUL53 in form of aggregates at the nuclear boundary, laminA/C and lamin B displayed a pattern superimposable to the distribution observed during viral infection: lamin A/C and B showed an irregular staining and invaginations, in correspondence of the punctuate patches of pUL50-pUL53 complex. Phospho-lamin A/C displayed a decrease in fluorescence intensity, but only a partial co-localization with the complex was detectable.

The present data suggest pUL50 and pUL53 might have a direct and fundamental role in modifying nuclear lamina and that their co-expression is sufficient to induce structural alterations, comparable to the ones occurring during HCMV infection. As over-expression of pUL50 and pUL53 was not sufficient to determine the re-arrangement of phospho-lamin A/C as induced upon infectionN-term phosphorylated lamin A/C did not show, out of the viral contest, the same pattern observed in infected cells, we can hypothesize that, during infection, other factors can be involved in the biochemical alterations of lamina, acting in sinergy with

pUL50 and pUL53. This hypothesis is also consistent with the observation during infection that pUL53 and nuclear lamins lack of a stable interaction.

The results obtained are in agreement with data published for the homologous proteins to pUL50 and pUL53 in other herpesviruses, that report the homolog proteins are able to induce conformational changes at the nuclear rim and to recruit cellular protein kinases to phosphorylate nuclear lamina components.

The primary sequences of pUL50 and pUL53 have been analyzed to characterize their putative properties and to map the domains responsible of their reciprocal interaction and, as a consequence, of their re-localization at the nuclear rim, where the complex can carry out its functions.

pUL53 presents a putative nuclear localization signal at the N-terminus (residues RERRSALRSLLRKRRQR[27]), while at the C-terminus a region highly rich in basic residues has been individuated, suggesting the presence of a nuclear export signal (NES). Moreover, other two short basic sequences are located at the N-terminal domain, downstream the NLS.

pUL50 present a putative transmembrane domain at the C-terminal end.

These putative properties have been studied expressing the correspondent domains as fusion proteins with GFP and analyzing both living and fixed cells by fluorescence microscopy. Both full-length proteins fused with GFP displayed a pattern superimposable to the one observed when expressed as fusion protein with smaller tags (Flag for pUL53 and V5 for pUL50). The expression of GFP-UL53(2-29), carrying only the N-terminal domain of pUL53, has demonstrated that the putative NLS is functional as the protein displayed a completely nuclear distribution, with frequent nucleolar accumulation. Also GFP-UL53(2-242) showed a nuclear pattern, but it was excluded from the nucleoli. Consistent with the finding that pUL53 NLS is licalized at its N_terminus, GFP-UL53(30-376) distributed both in the nucleus and in the cytoplasm. The central fragment GFP-UL53(30-242) and the C-terminal fragment GFP-UL53(220-376) localized ubiquitously, but mainly in the cytoplasm of transfected cells, indicating the potential presence of NES in their sequence.

To analyze the putative NESs, transfected cells have been treated with Leptomycin B (LMB), a specific inhibitor of CRM1, which is an exportin that mediates the translocation of proteins carrying NESs, from the nucleus to the cytoplasm. Living transfected cells, treated and un-treated with LMB, have been analyzed by CLSM and tocalculate nuclear to cytoplasmic fluorescence ratio(Fn/c). GFP-UL53(2-376) full-length, GFP-UL53(30-242) and GFP-UL53(220-376) showed a significant Fn/c increase upon treatment with LMB, indicating that the NES-like sequences individuated by primary sequence analysis are functional. The

analysis could not be performed for the proteins containing the N-terminal domain but lacking of the C-terminus, as the nuclear fluorescence was too intense with respect to the cytoplasmic one, preventing an accurate calculation of the Fn/c values.

The analysis on living and fixed cells of pUL50 domains fused with GFP revealed that GFP-UL50(2-397) full-length, GFP-UL50(71-397), lacking of the N-term domain and GFP-UL50(215-397), carrying only the C-terminus of pUL50 localize in the cytoplasm of transfected cells, with a typical ER distribution. In contrast, GFP-UL50(2-258) localized ubiquitously, with a slight accumulation in the nucleus and and the occasional formation of small intranuclear speckles.

All these data suggest pUL53 is a shuttling protein with a functional NLS at the N-terminus of its sequences, responsible of its nuclear localization and NES sequences at the C-terminus.

The analysis of the sequence of pUL50 suggested only the presence of a transmembrane region at the C-terminus (residues 358-381), whereas nor NLS neither NES putative sequences were identified.

Our fluorescence analysis confirmed pUL50 carries a C-terminal transmembrane region as the domain (215-397) is necessary and sufficient to confer a ER localization pattern to GFP.

The analysis of pUL50 fragments fused with GFP has dem nstrated the presence of a transmembrane region at the C-terminus, that is necessary to acquire the right cellular localization.

The expression of the different fragments of pUL50 and pUL53 fused with GFP has been used also to study their interaction domains in living and fixed cells by fluorescence microscopy. Firstly, all pUL50 fragments fused with GFP have been expressed with pUL53 full length fused with DsRed2 (DsRed2-UL53(2-376)): GFP-UL50(2-397) full length showed co-localization and interaction with DsRed2-UL53(2-376), as expected; co-localization has been observed also for GFP-UL50(2-258), while both GFP-UL50(71-397) and GFP-UL50(215-397), lacking of the N-terminus of pUL50 did not show any co-localization.

The same analysis has been performed expressing DsRed-UL50(2-397) full length in combination with pUL53 fragments fused with GFP: only GFP-UL53(2-29) failed to co-localized with DsRed-UL50(2-397), even if for GFP-UL53(220-376) the co-localization seemed more due to a distribution in the same compartment, than a real interaction.

The same experiments were performed in fixed cells by IIF, expressing pUL53Flag and pUL50V5, instead of DsRed2-UL53(2-376) and DsRed-UL50(2-397), respectively. The results in fixed cells were superimposable to the data obtained with living cells.

Finally, co-IP assays were performed on lysates of cells co-expressing pUL50V5 in combination with pUL53 fragments fused with GFP and pUL53Flag in combination with pUL50 fragments fused with GFP to confirm the observations in fluorescence microscopy.
The results allowed to identify the region of both proteins required for the reciprocal interaction, in residues 1-70 and 30-242 for pUL50 and pUL53, respectively.

5. CONCLUSIONS

Human Cytomegalovirus infection causes severe pathologies in newborn babies and immunocompromised patients, worldwide. The pharmacological therapies available contrast viral replication by blocking the activity of the viral encoded DNA polymerase. The compounds with such anti-viral activity are indeed highly effective, but the emergency of new resistant viral strains is growing, raising the necessity to develop alternative antiviral treatments to associate with the classic ones. In this contest, the comprehension of essential mechanisms for a productive viral cycle and the research of new antiviral targets are acquiring importance.

In this work, we performed a study of pUL50 and pUL53, two still uncharacterized proteins of Human Cytomegalovirus (HCMV), whose homologs in other herpesviruses are reported to have a fondamental role in viral maturation, promoting the egress of the newly formed virions from the nucleus to the cytoplasm of the host cell.

In parallel, also nuclear lamins, the major cellular components constituting nuclear lamina, have been examined: lamins, in association with other nuclear proteins, form a thick proteic meshwork underlying the inner nuclear membrane and, in the viral contest, represent an physical obstacle to the viral egress from the nuclear compartment.

Observations performed during infection showed marked rearrangements of nuclear lamins: lamin B and A/C displayed a ruffled distribution and deep invaginations at the nuclear rim and a N-terminal phosphorylated form of lamin A/C showed a decrease in infected cells, with the formation of inhomogeneous aggregates at the nuclear rim. .

The novel high specific anti-serum anti-pUL53, which has been raised in this work, has allowed to observe the localization of the viral protein in punctuate patches at the nuclear rim and that its aggregates strongly co-localize with the alterations on nuclear lamina, suggesting its involvement in inducing such modifications.

The analysis by IIF and Western blotting of pUL53 and its counterpart, pUL50, out of the viral contest, has shown that, when singularly expressed, pUL53 localizes into the nucleus and pUL50 in the cytoplasm, with a typical ER pattern, while when co-expressed, the two

protein are able to interact and to re-localize at the nuclear rim, resembling the distribution pUL53 acquires during infection. This data demonstrates pUL50 and pUL53 are able form a complex and their interaction is necessary and sufficient to each other to aquire a distribution in aggregates at the nuclear boundary, which is the physiological pattern of pUL53.

The analysis of nuclear lamins in the presence of pUL50 and pUL53, expressed singularly or co-expressed out of the viral contest, strongly suggests the two HCMV-encoded proteins might have a role in the alterations of nuclear lamina. At the same time, the observation that their complex is not sufficient to determine the modifications in the form of phopshorylated lamin A/C revealed in infected cells and that , during infection, they do not establish a stable interaction with lamins suggests the hypothesis that other viral factors can be involved and act in sinergy with pUL50 and pUL53.

This study has also demonstrated that pUL53 presents a functional nuclear localization signal (NLS) at its N-terminus (RERRSALRSLLRKRRQR^{27}) and two functional nuclear export sequences (NES) at the C-terminus (VTLNIVRDHVVISVL^{244} and VKIDVTILQRKIDEMDI^{269}), indicating pUL53 is a shuttling protein and that pUL50 presents a transmembrane region in its C-terminal domain (residues 359-381).

Moreover, the analysis of different portions of the two viral proteins has allowed to indentify the domains of their reciprocal interaction, in residues 1-70 and 30-242 for pUL50 and pUL53, respectively.

Moreover, RNA interference experiments directed against pUL53 transcript during infection indicated that UL53 ORF can be efficiently silenced during infection, opening the way to new investigations on the mechanisms of viral maturation

This study provides a characterization of pUL50 and pUL53 properties, interaction, both reciprocal and with the host cells nuclear proteins that is promising to understand the mechanism of viral egress from the nucleus.

6. TABLES

OLIGO NUCLEOTIDE	SEQUENCE (Sequences are indicated 5'-3'; *Att* sites are underlined; STOP codons are in italics.)
PRIMERS for UL53	
UL53-U21	ATGTCTAGCGTGAGCGGCGTG
UL53-L21	AGGCGCACGAATGCTGTTGAG
AttB1-UL53-1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTCTAGCGTGAGCGGTGTCCGCACGCCGCG
AttB1-UL53-86	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCCAGCAAAGTGGCGTCGACGGTGAA
AttB1-UL53-242	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGAAGATGGCCATCACGGGCAA
AttB1-UL53-677	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGACTACAGCGTCACGCTCAACATCG
AttB2-UL53-87	CGCAAGCGCCGCCAACGCGAGCTG*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL53-240	CCGAACTGGAGCTCAAGTACCTTAAC*TAG*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL53-726	AACATCGTGCGCGACCACGTCGTTATCAGC*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL53-1128	GTTTCTCAACAGCATTCGTGCGCCT*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
PRIMERS for UL50	
UL50-U18	ATGGAGATGAACAAGGTT
UL50-L16	GTCGCGGTGTGCGGAG
AttB1-UL50-1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGAGATGAACAAGGTTCTCCATC
AttB1-UL50-215	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATCTTTAAAAACACTGGCTG
AttB1-UL50-647	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCCCCCTCCTCCTCCTCGTCA
AttB1-UL50-1073	GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCTGCCCGCGCGCTTTGCGACTCTG
AttB2-UL50-213	CACAGACCACGTGCCTTGT*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL50-651	CTCTCGGACCGCATCTCCC*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL50-1074	CGCGACCGCAGTCTGGGCCGTGG*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC
AttB2-UL50-1191	ACACGCTCCGCACACCGCGAC*TGA*ACCCAGCTTTCTTGTACAAAGTGGTCCCC

Table 2.1. List of the oligonucleotides used in this study for the generation of expression vectors.

Constructs generated by Gateway Technology	
pDONOR207-UL53(2-376)	pDEST53-UL53(2-376) pBKdsRed2CMV-UL53(2-376)
pDONOR207-UL53(2-29)	pDEST53-UL53(2-29)
pDONOR207-UL53(2-242)	pDEST53-UL53(2-242)
pDONOR207-UL53(30-376)	pDEST53-UL53(30-376)
pDONOR207-UL53 (30-242)	pDEST53-UL53 (30-242)
pDONOR207-UL53 (220-376)	pDEST53-UL53 (220-376)
pDONOR207-UL50(2-397)	pDEST53-UL50(2-397) pBKdsRed2CMV-UL50(2-397)
pDONOR207-UL50(2-358)	pDEST53-UL50(2-358)
pDONOR207-UL50(71-397)	pDEST53-UL50(71-397)
pDONOR207-UL50(215-397)	pDEST53-UL50(215-397)

Table 2.2. Constructs generated by Gateway Technology, used in this study.

STARTING NUCLEOTIDE IN UL53 ORF	TARGET SEQUENCE	PLASMID
224	**GCTCAAGTACCTTAACATG**	pG-SUPER-UL53-224
757	**TCAAGATCGACGTGACTAT**	pG-SUPER-UL53-757
782	**ACGCAAGATTGACGAGATG**	pG-SUPER-UL53-782

Table 2.3. Plasmids for UL53 RNA interference. The target sequences were chosen distributed in all UL53 gene. Oligos consist in a 19-nucleotides sequence separated by a short spacer from its reverse complement and were inserted in pG-SUPER vector.

7. FIGURES

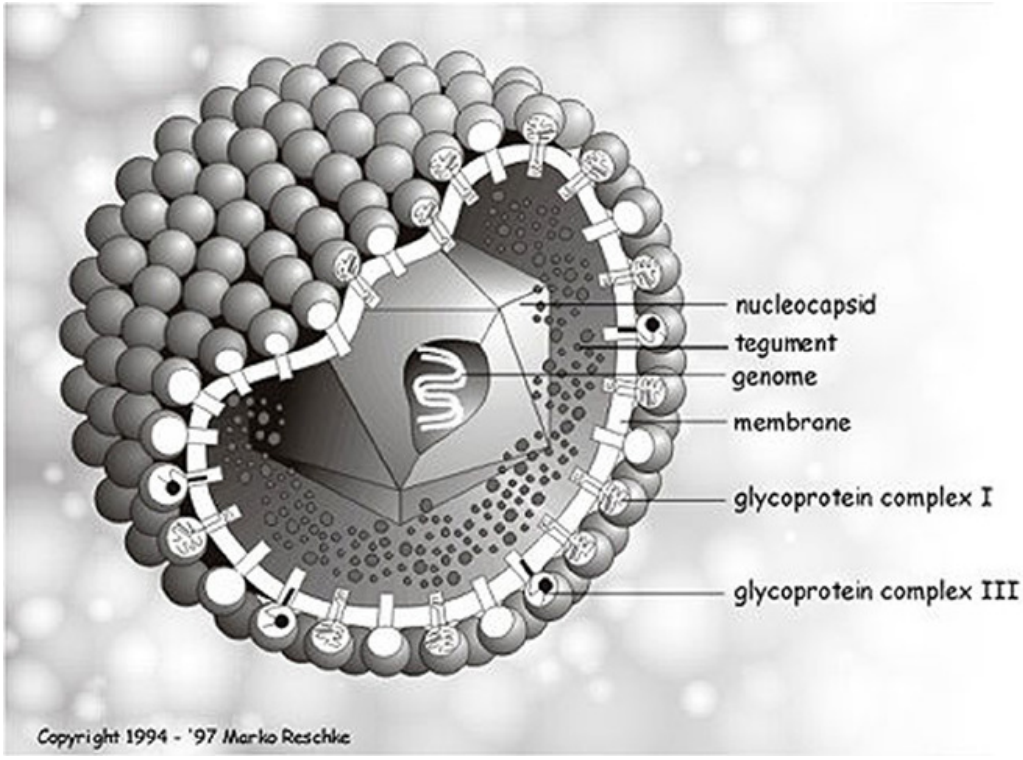


Fig.1.1. Schematic drawing of Human Cytomegalovirus structure. (Adapted from Marko Reschke).

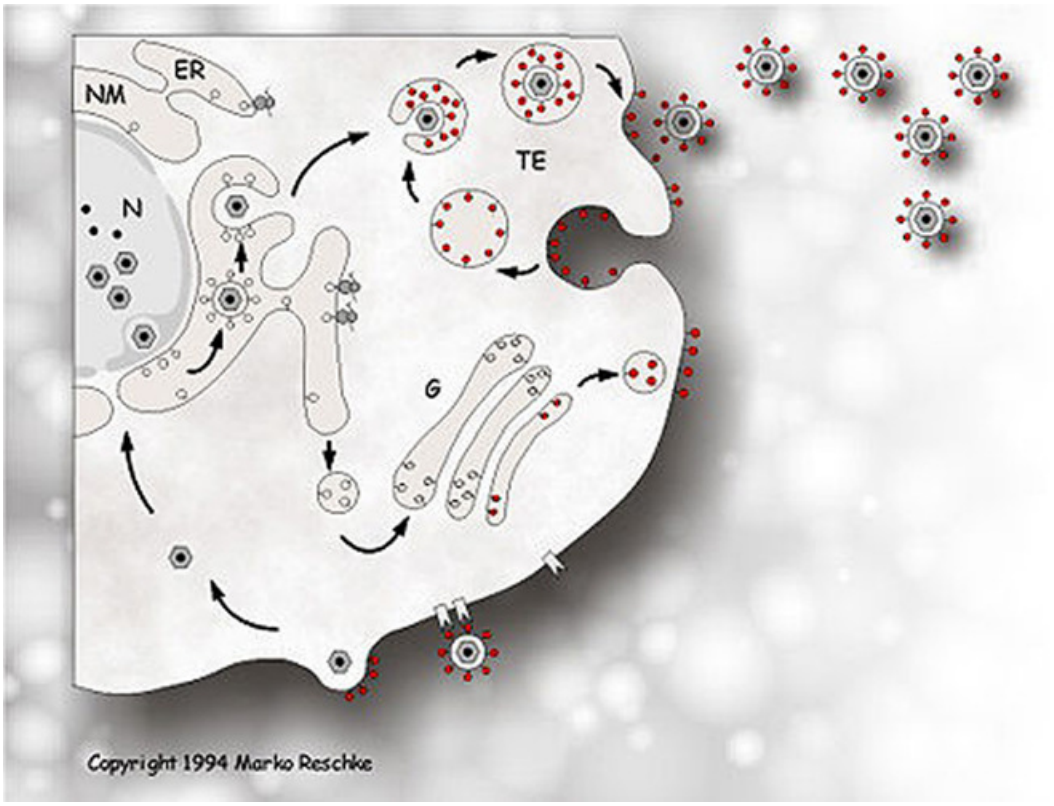


Fig.1.2. Schematic representation of Human Cytomegalovirus replication cycle. N = nucleus; C = cytoplasm; ER = endoplasmic reticulum;G = Golgi(Adapted from Marko Reschke).

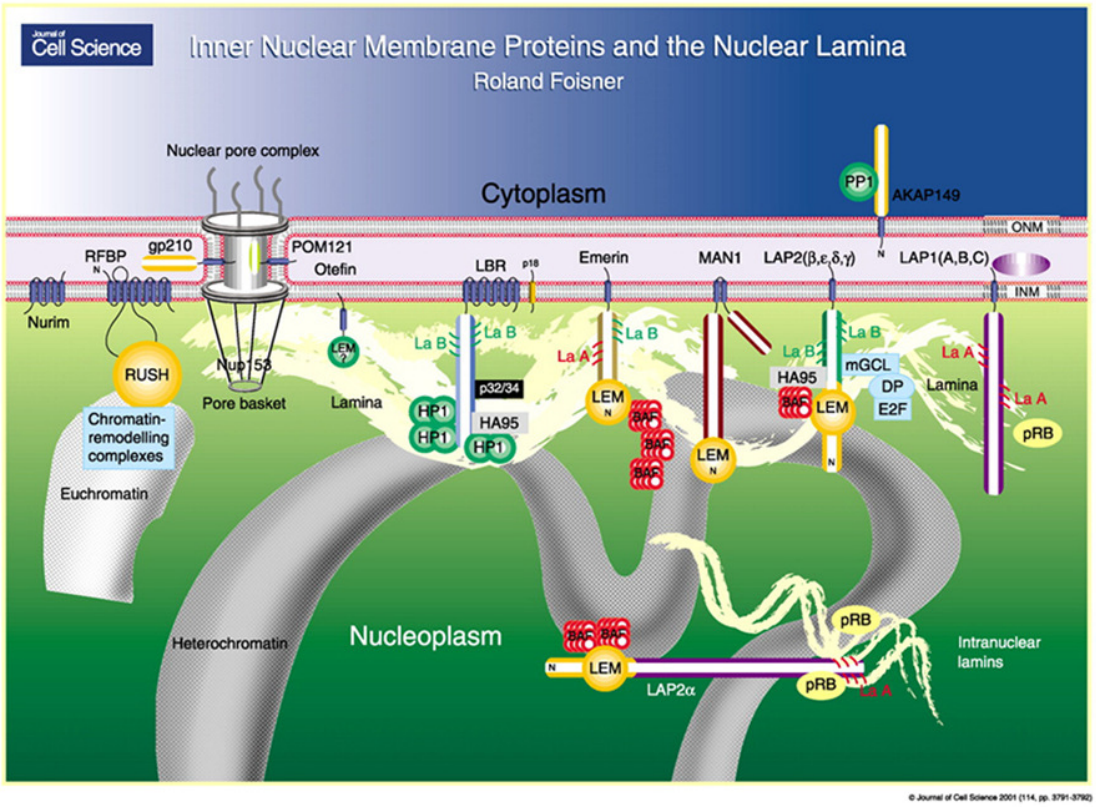


Fig.1.3. Schematic view of the nuclear envelope. Lamin A/C and B underline INM and estabilish interactions with several nuclear membrane proteins and chromatin (Adapted from Foisner et al., Journal of Cell Science, 2001).

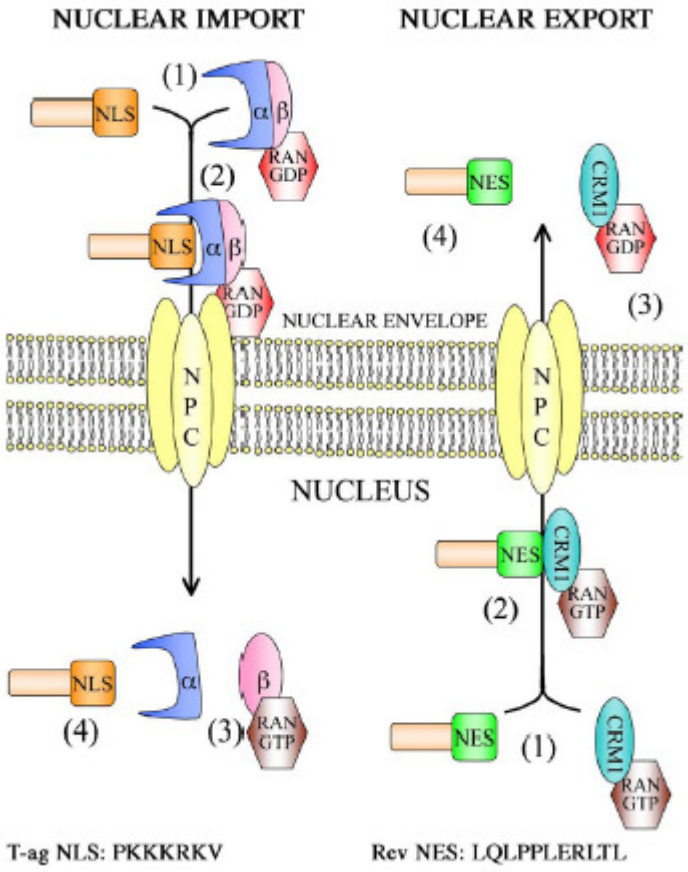


Fig. 1.4. Schematic representation of nucleocytoplasmic transport. (A) Nuclear import occurs when IMP1, either alone or through IMP, recognizes an NLS on the cargo protein in the cytoplasm (1) and mediates the docking of the complex to the NPC (2) followed by translocation into the cell nucleus. Binding of RanGTP to IMP1 (3) results in the cargo being released into the nucleus (4). (B) Nuclear export occurs when cellular export receptors, such as the IMP homologue CRM-1, recognize a NES on the cargo protein in the presence of RanGTP (1) and mediate the docking of the complex to the NPC and its translocation to the cytoplasm (2). Upon hydrolysis by Ran of GTP to GDP (3), facilitated by Ran GTPase-activating protein (not shown), the cargo is released into the cytoplasm (4).(Adapted from Alvisi *et al*., *Drug Resistance Update*, 2006.)

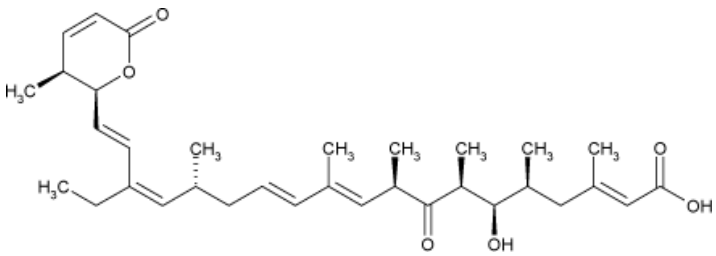


Fig. 1.5 Chemical structure of Leptomycin B. Leptomycin B (LMB) is a specific inhibitor of nuclear export, binding covalently CRM1 and preventing its interaction with proteins provided of NESs.

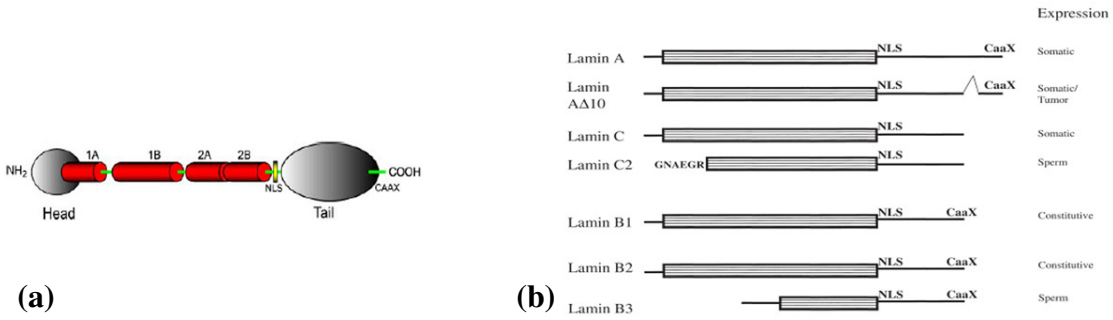


Fig.1.6. Schematic representation of lamins. (a) Structure of lamin proteins. Main characteristics are four central rod domains (1A, B, 2A, B), flanked by a globular head and tail domain. In the tail domain a nuclear localization signla can be identified, as well as a CaaX motif, which is present in both B-type and A-type lamins, other than lamin C. (Adapted from Broers *et al., Physiol Rev*, 2006); (b) Different lamin isotypes found in mammalian tissues. Lamin A, C, AΔ10 and C2 result from differentialsplicing of *LMNA* gene; lamins B2 and B3 originate from *LMNB2* gene ans lamin B1 is the only transcript of *LMNB1* gene. (Adapted from Moir et al.,Cellular and Molecular Cell Science, 2001).

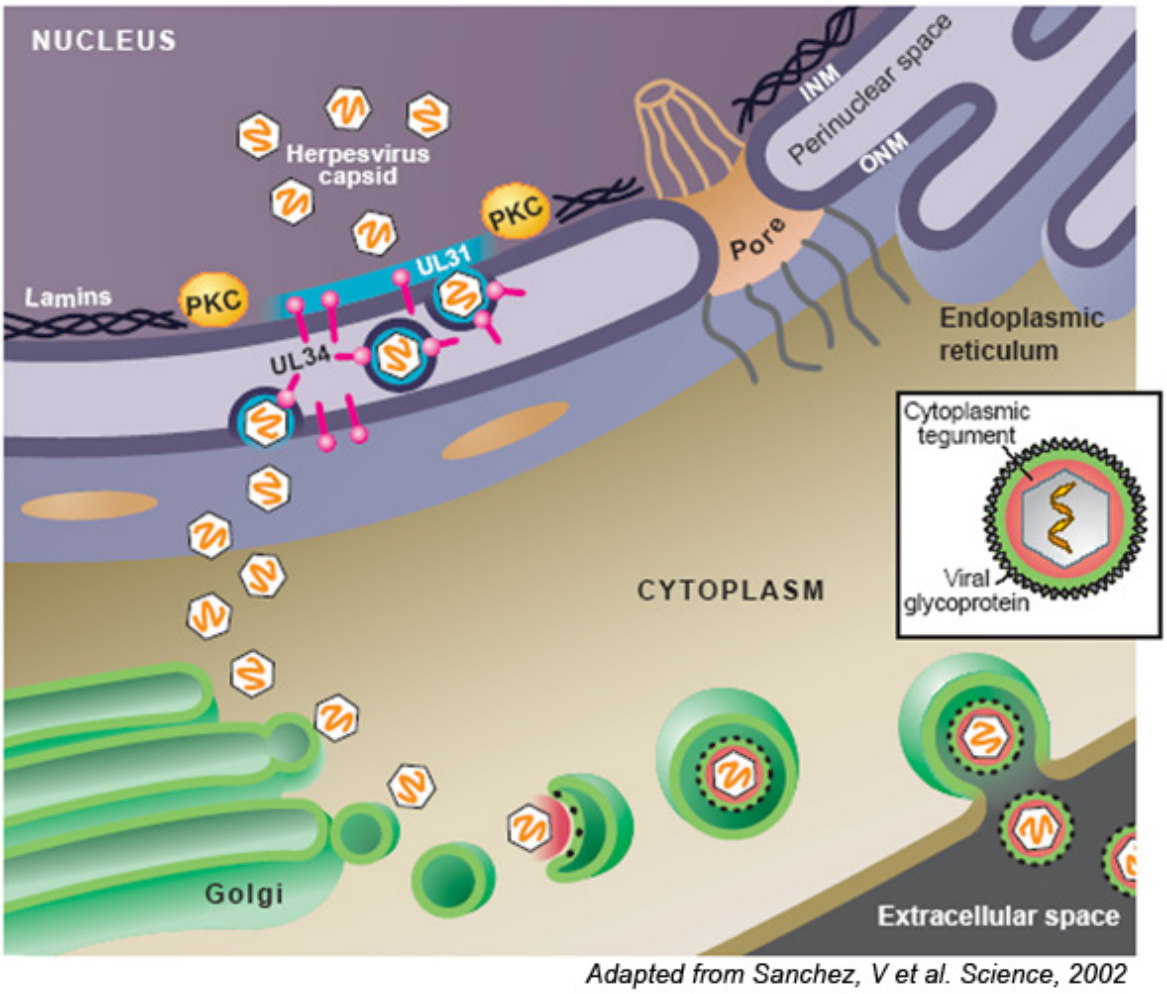


Fig. 1.7. Schematic representation of herpevirus egress from the nucleus. Newly replicated herpesvirus DNA is inserted into preformed capsids in the nucleus of the host cell. To reach the inner nuclear membrane (INM) of the nuclear envelope, the subviral particles must transit through the physical barrier of the nuclear lamin meshwork. The viral protein UL34 (pink) of HSV-1 (the homolog of M50/p35 in murine CMV) is a type II transmembrane protein that is localized at the INM in infected cells via its interaction with UL31 (turquoise), a viral protein in the nucleus. UL34 and UL31 recruit protein kinase C (PKC) to the nuclear membrane, resulting in an increase in phosphorylation of the lamins. This causes partial disruption of the lamin network, thereby facilitating envelopment of subviral particles at the INM. The particles acquire some of their tegument proteins and a primary envelope by budding through the INM. They then lose their primary envelope as they bud through the outer nuclear membrane (ONM) of the nuclear envelope and are released into the cytoplasm. The DNA-containing capsids move to the Golgi complex, where they acquire additional tegument proteins and a mature envelope. Finally, they travel in Golgi-derived vesicles to the plasma membrane, where they are released from the host cell.

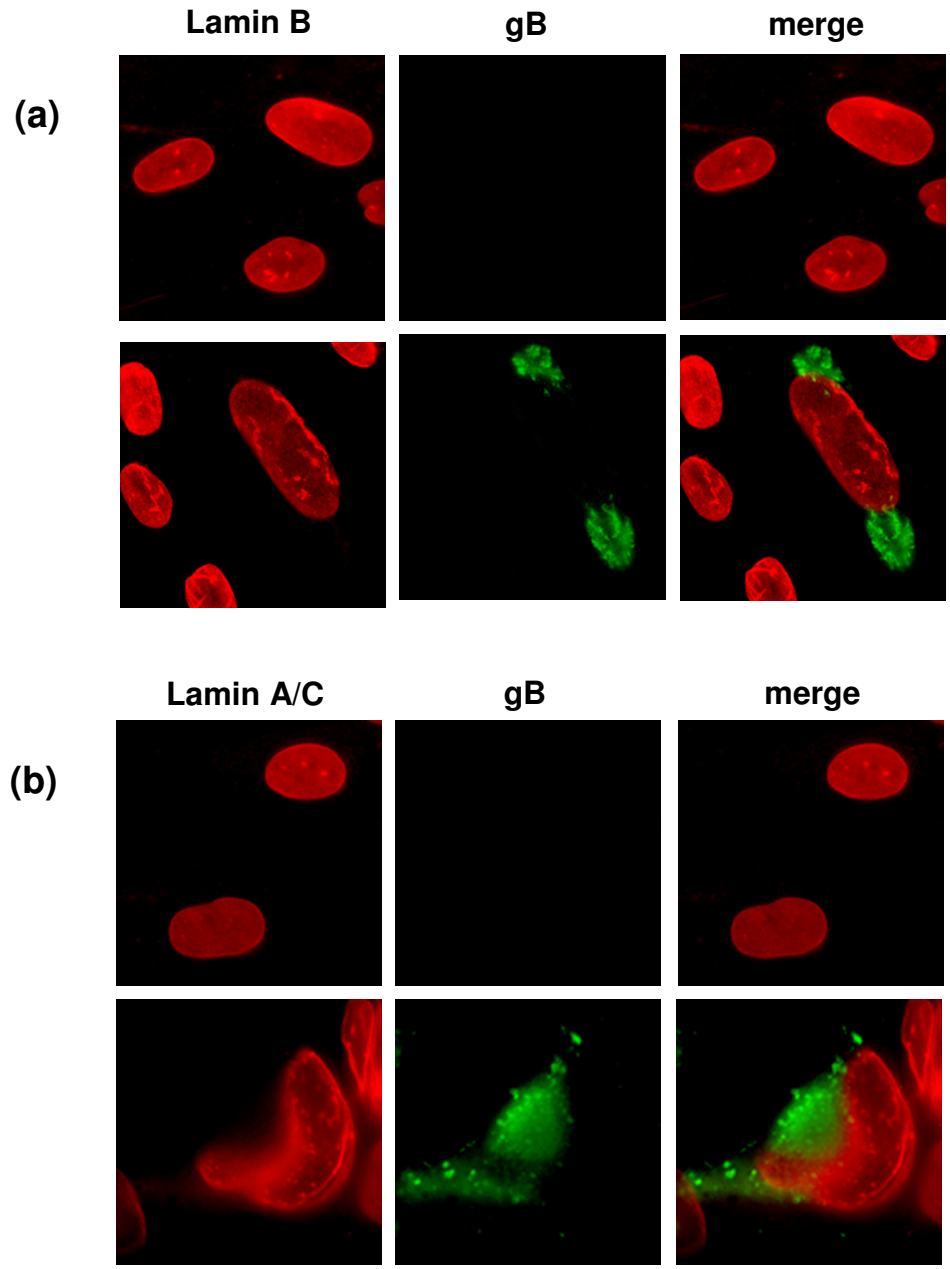


Fig. 3.1. HCMV infection induces remarkable modifications on nuclear lamina. Infected human fibroblast have been co-stained for gB, as marker of infection, and **(a)** lamin B or **(b)** lamin A/C. Both lamins showed an irregular staining with an aberrant formation of invaginations.

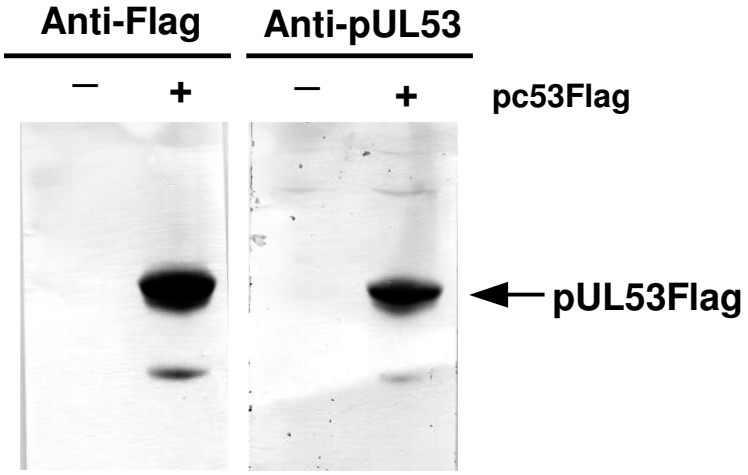


Fig. 3.2 Test of the specificity of the anti-serum raised against pUL53 in this study. The antiserum against pUL5 and the controls have been prelevated and tested for specificity by WB on lysates of mock- and transfected COS7 cells with the plasmid pc53Flag. Anti-pUL53 anti-serum displayes a reactivity similar to the commercial monoclonal antibody anti-Flag

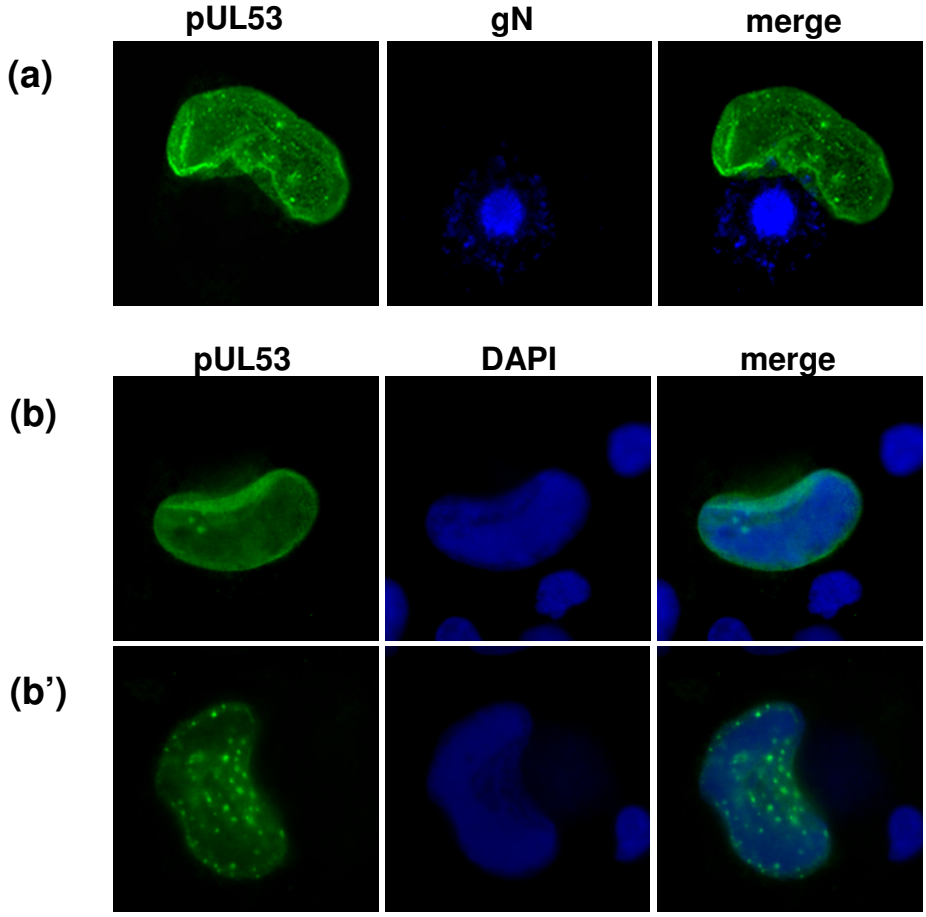


Fig. 3.3. pUL53 during HCMV infection: IIF assay on infected HELFs.
(a) Fixed cells were co-stained for gN, as marker of infection, and pUL53 thanks to the novel antibody raised for this study: pUL53 localizes at the nuclear boundary, forming aggregates. **(b)** Fixed cells were stained for pUL53 at different times after infection: at 48-72h *post-infection*, pUL53 shows mainly a slighty discontinuous pattern at the nuclear rim, while, 120h *post-infection,* distinct aggregates of the viral protein are clearly detectable.

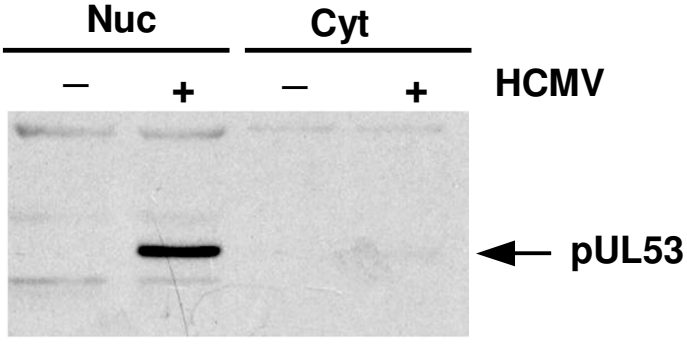


Fig. 3.4. pUL53 during HCMV infection: WB analysis on nuclear and cytoplasmic extracts of infected HELFs. Nuclear and cytoplasmic extracts were obtained from mock- and infected HELFs and analyzed by WB assay: a clear band at about 42 kDas was strongly detectable in infected nuclear fractions, while a very low signal was detectable in infected cytoplasmic extracts. No signal was detectable in nuclear and cytoplasmatic extracts of mock-infected HELFs.

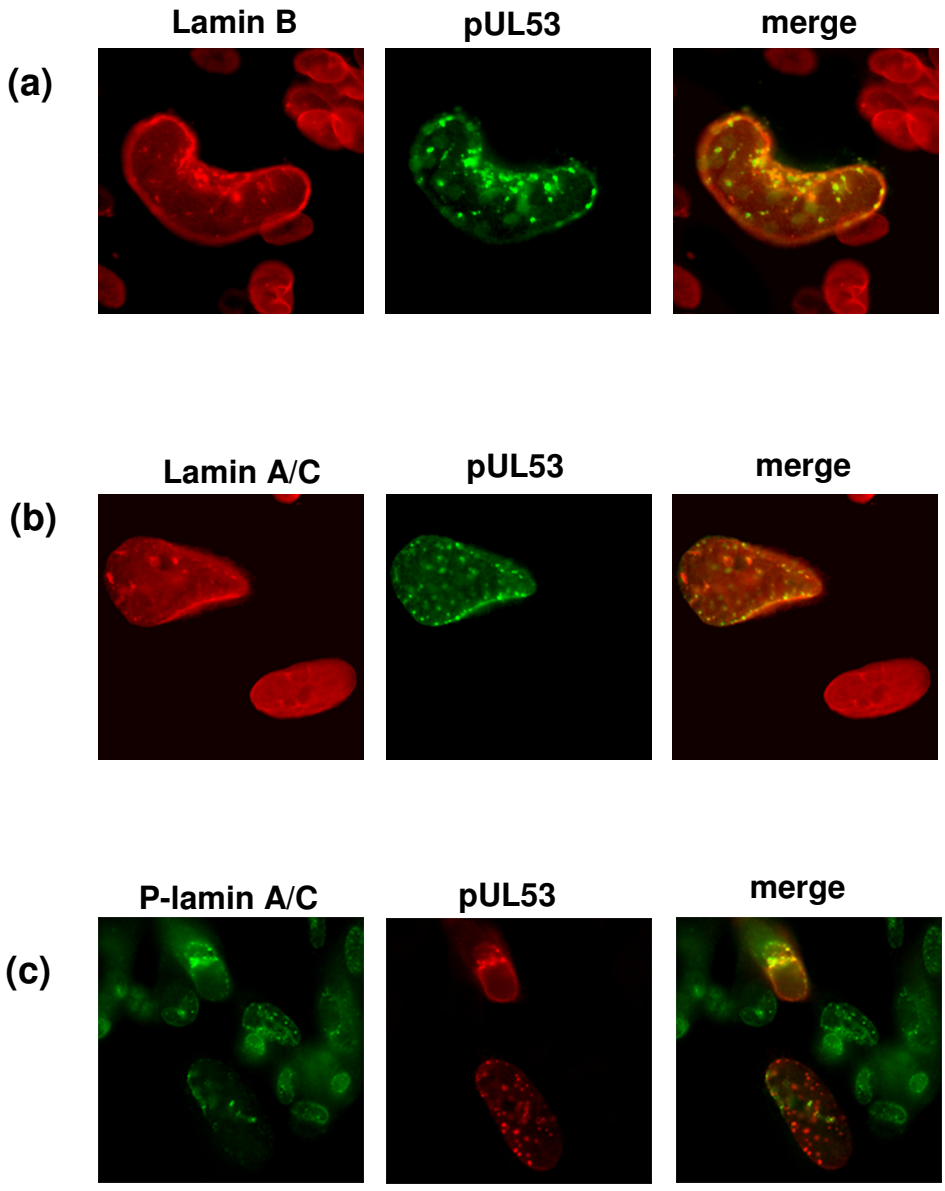


Fig. 3.5. pUL53 colocalizes with the modifications occurring on nuclear lamina during HCMV infction. Infected human fibroblast have been co-stained for pUL53 with **(a)** lamin B, **(b)** lamin A/C or **(c)** phospho-lamin A/C. pUL53 co-localizes with the invaginations of lamin B and lamin A/C and with the aggregates of phospho-lamin A/C.

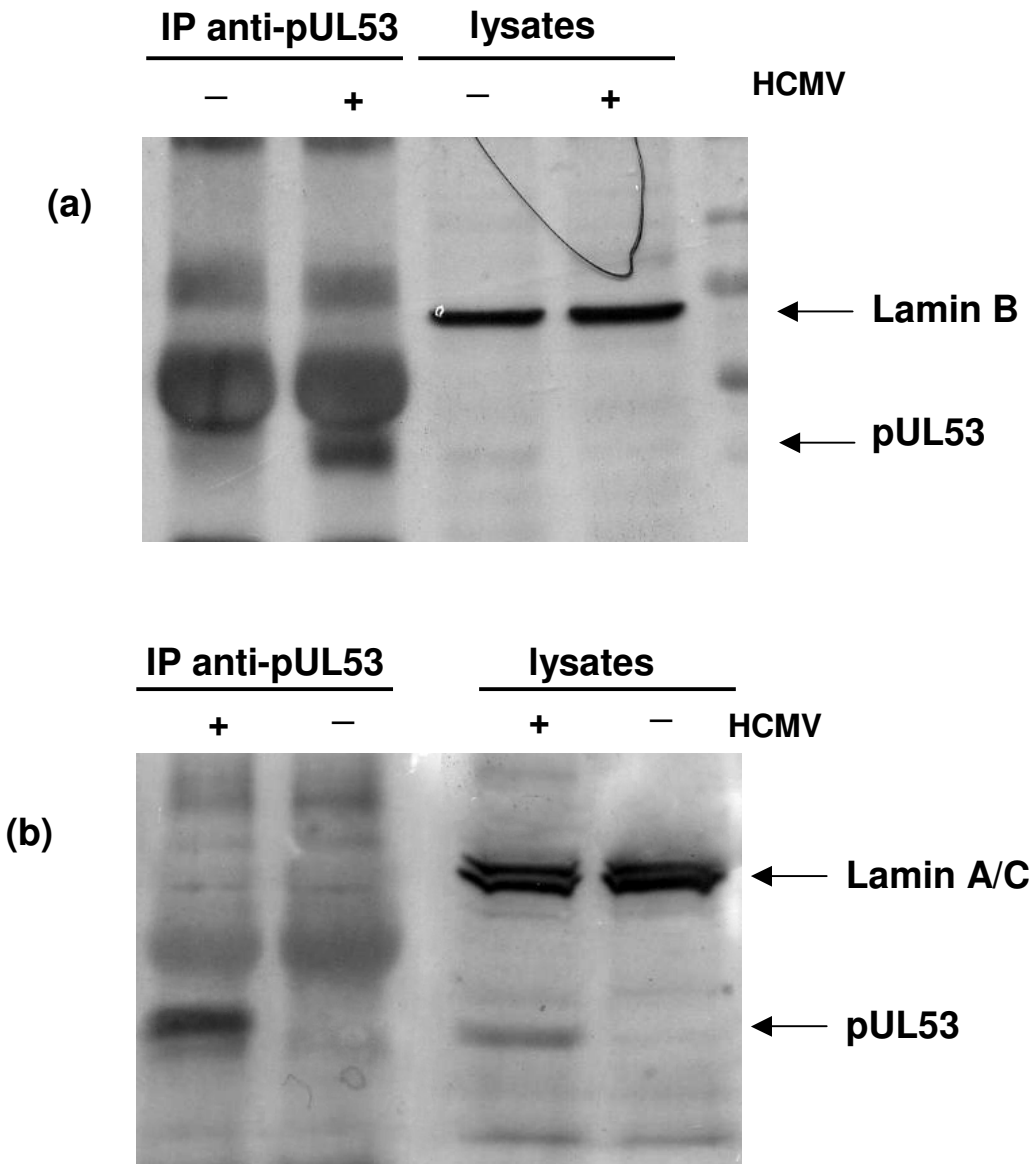


Fig. 3.6. pUL53 and nuclear lamins do not strongly interact. Lysates of nuclear extract of mock- and infected HELFs were used to immunoprecipitate pUL53 with anti-pUL53 anti-serum. pUL53 has been immunoprecipitated, but not nuclear lamins indicating the absence of a strong interaction between nuclear lamina and viral protein.

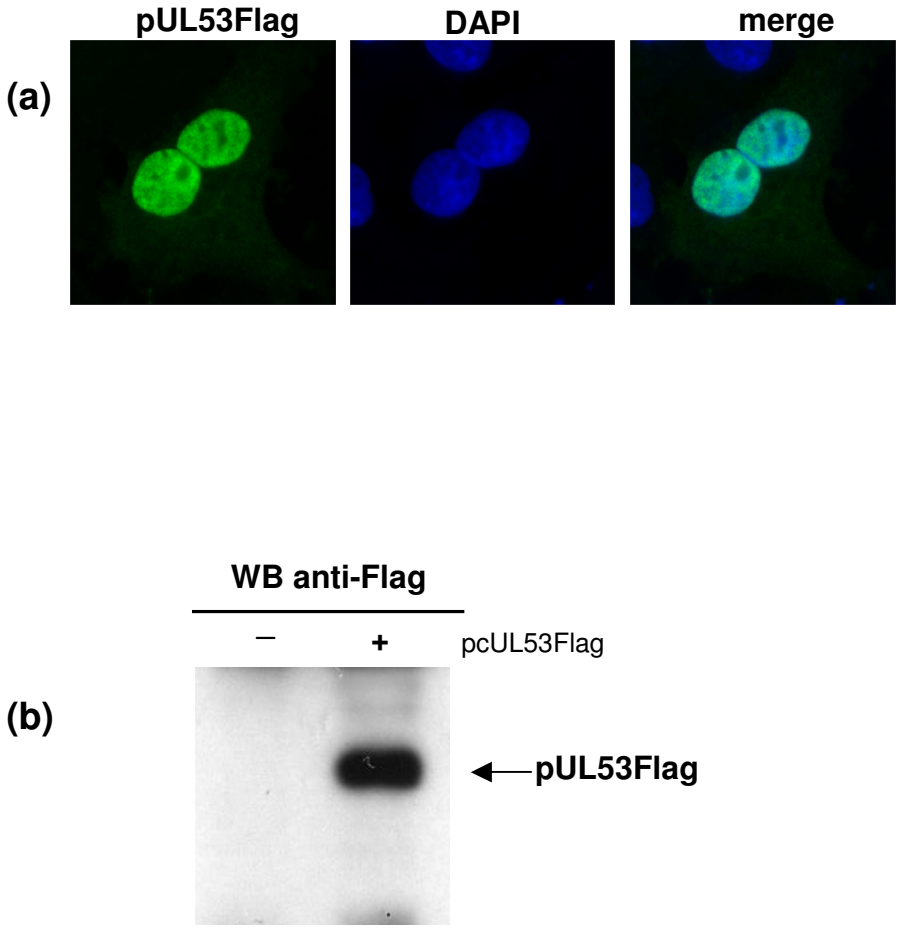


Fig 3.7. pUL53Flag localizes in the nucleus of transfected cells. **(a)** In IIF analysis, pUL53Flag shows a wide-spread nuclear distribution with a low cytoplasmic staining, in contrast with the observations during infection; **(b)** WB analysis on lisates of mock- and trasfected cells has confirmed the predicted molecular weight of about 41 kDas

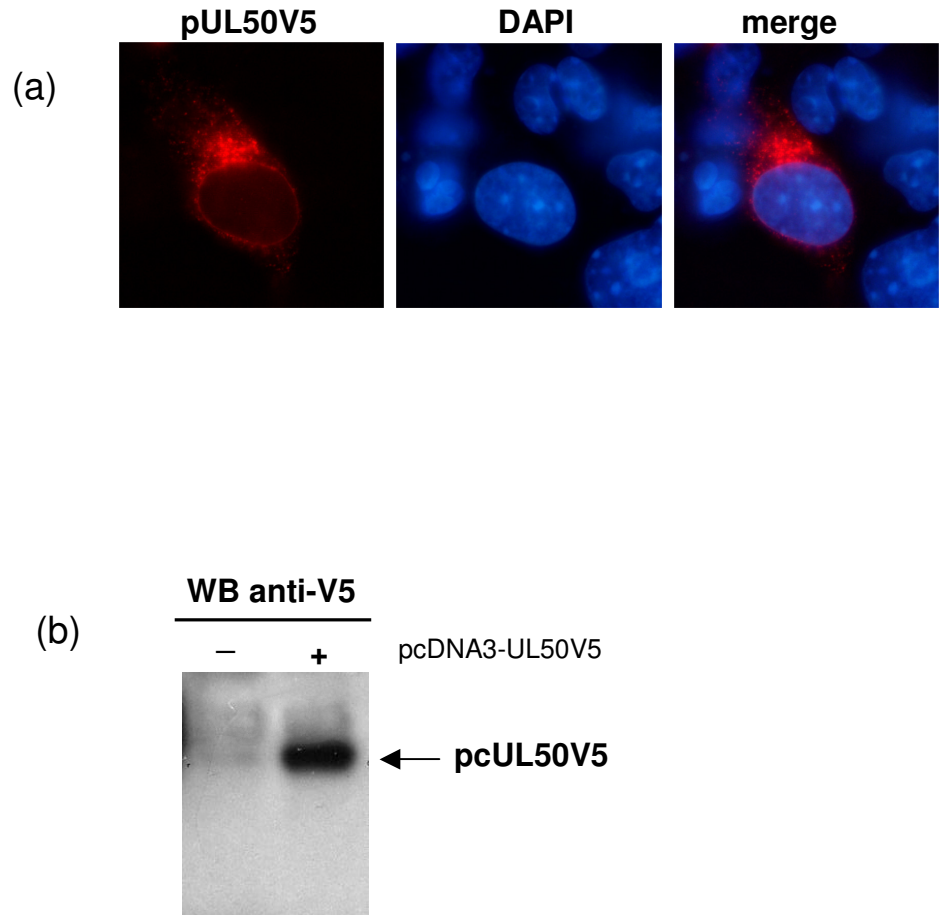


Fig 3.8. pUL50V5 localizes in the cytoplasm of transfected cells. (a) In IIF analysis, pUL50V5localizes predominantly in the cytoplasmic compartment and in perinuclear area, with a predominant ER pattern ; (**b**) WB analysis on lisates of mock- and trasfected cells has confirmed the predicted molecular weight of about 43 kDas

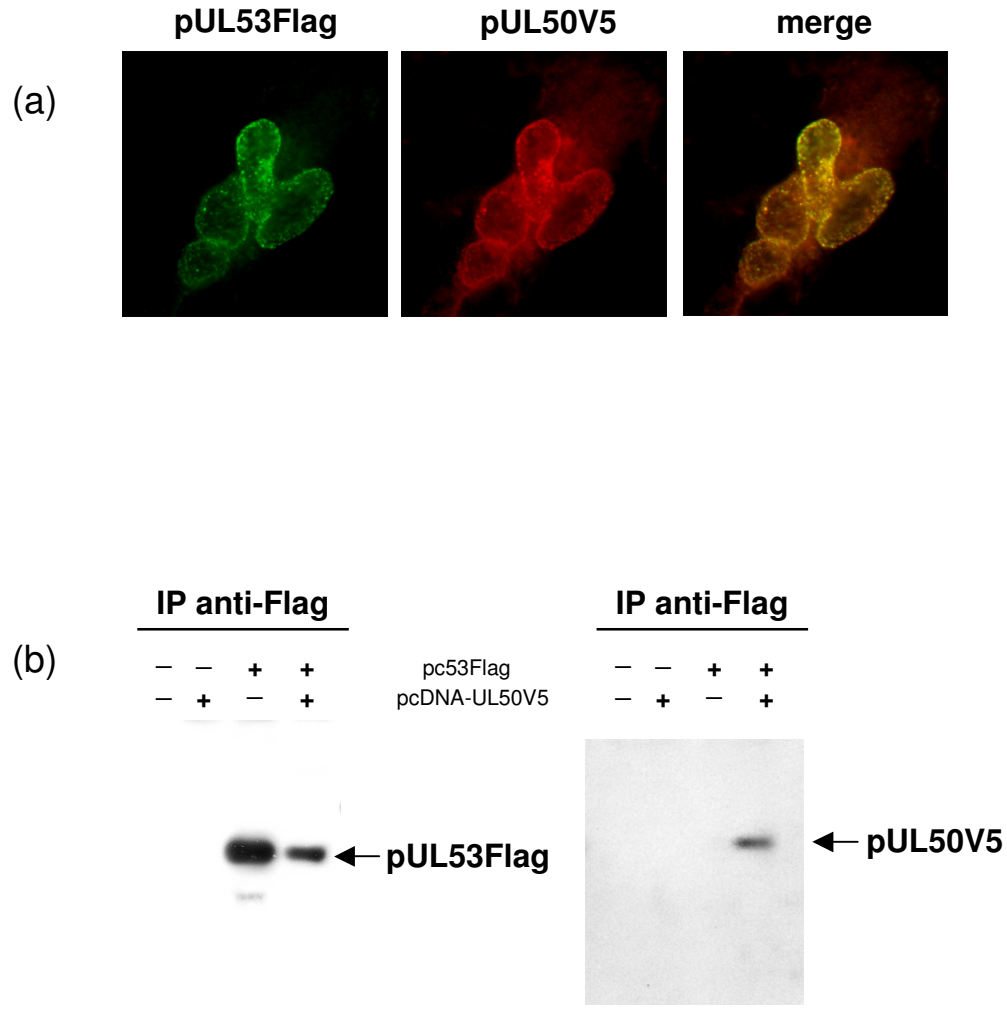


Fig. 3.9. pUL50 and pUL53 interact and colocalize at the nuclear rim. (**a**) IIF assay on COS7 expressing both pUL53Flag and pUL50anti-Flag V5 revealed the proteins interact and completely re-localize at the nuclear rim; (**b**) IP assay on lysates of mock- and co-transfected COS7 cells indicates the two viral proteins interact directly.

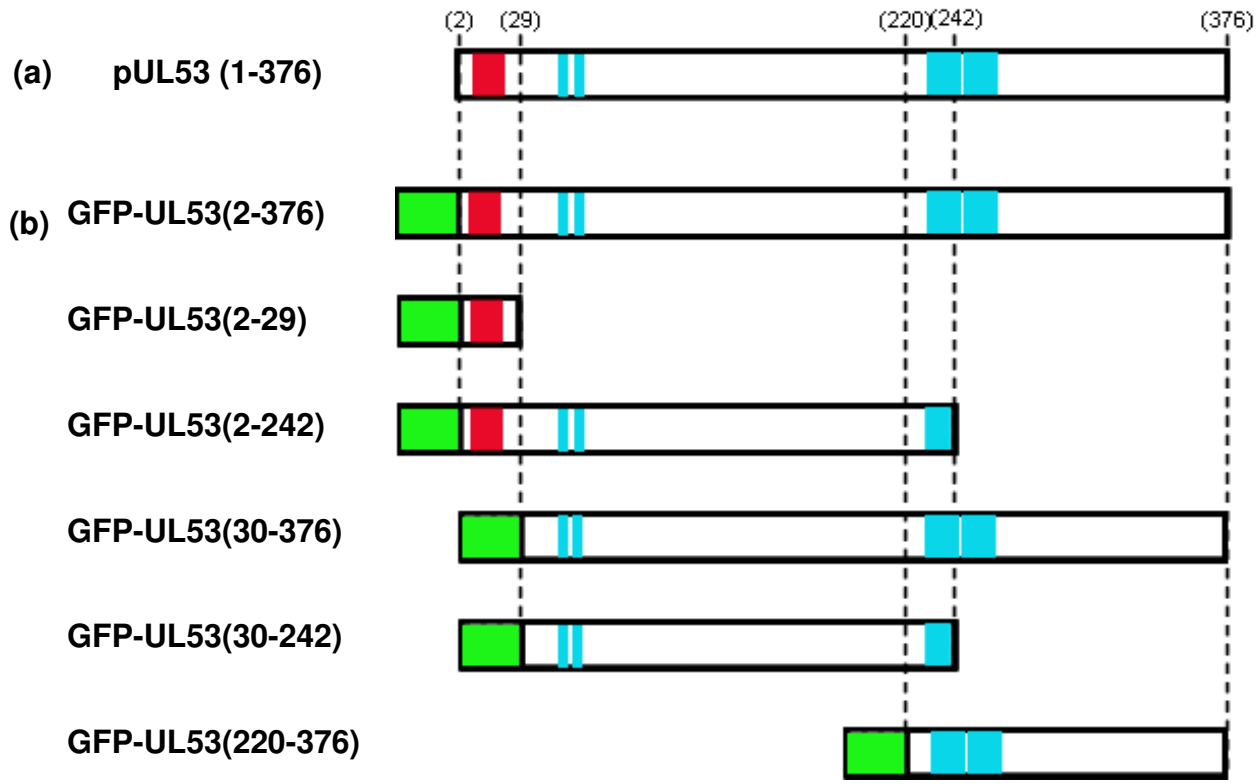


Fig. 3.10. Schematic representation of pUL53 properties. (**a**) pUL53 wild type presents a putative N-terminal NLS (red) (residues 10-27) and putative NESs (blu) at its N-terminus (residues 59-64 and 74-79) and C-terminus (residues 230-244 and 253-269 **(b)** different domains of pUL50 expressed as fusion proteins with GFP, used in this study.

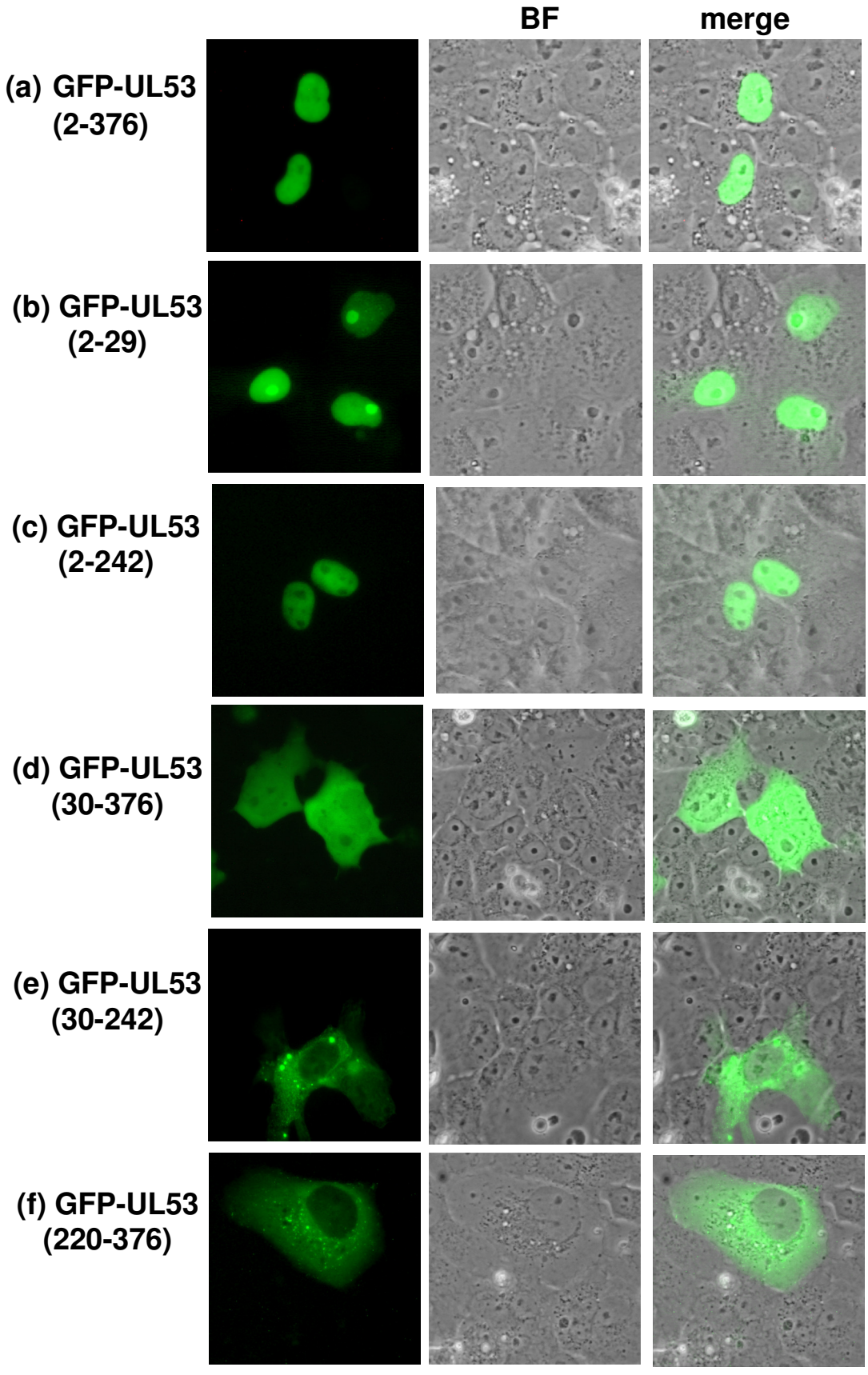


Fig. 3.11. Imaging of living cells expressing pUL53 different domains fused with GFP. (BF: brightflield). The N-terminal NLS mediated the protein nuclear localization (**a**, **b**, **c**); pUL53 domains lacking of N-terminus localizes ubiquitously (**d**) or in the cytoplasm(**e**, **f**).

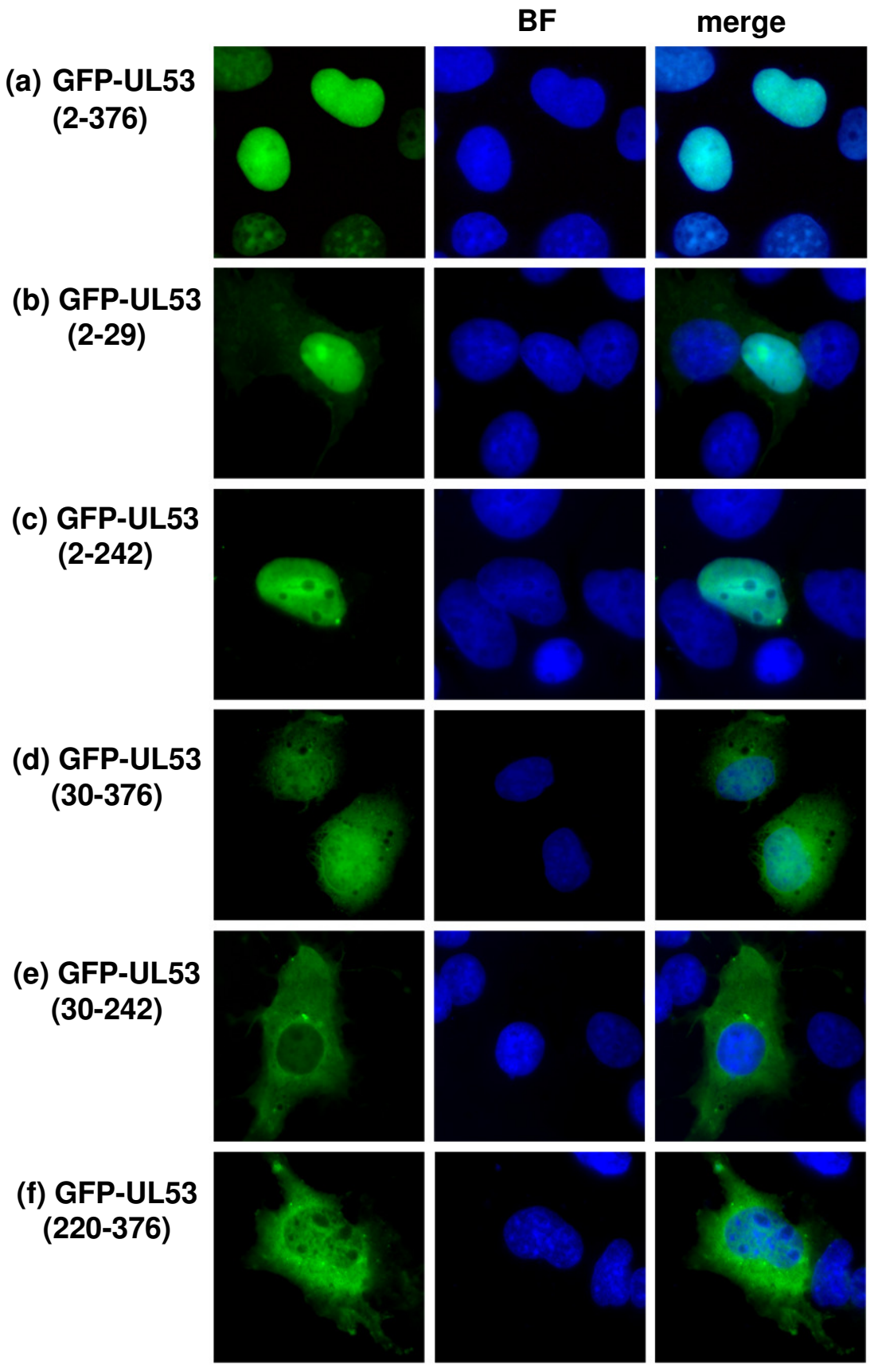


Fig. 3.12. Imaging of fixed cells expressing pUL53 different domains fused with GFP. Nuclear staining has been performed with DAPI. The results are superimposable to the one obtained with living cells (**Fig. 3.11**)

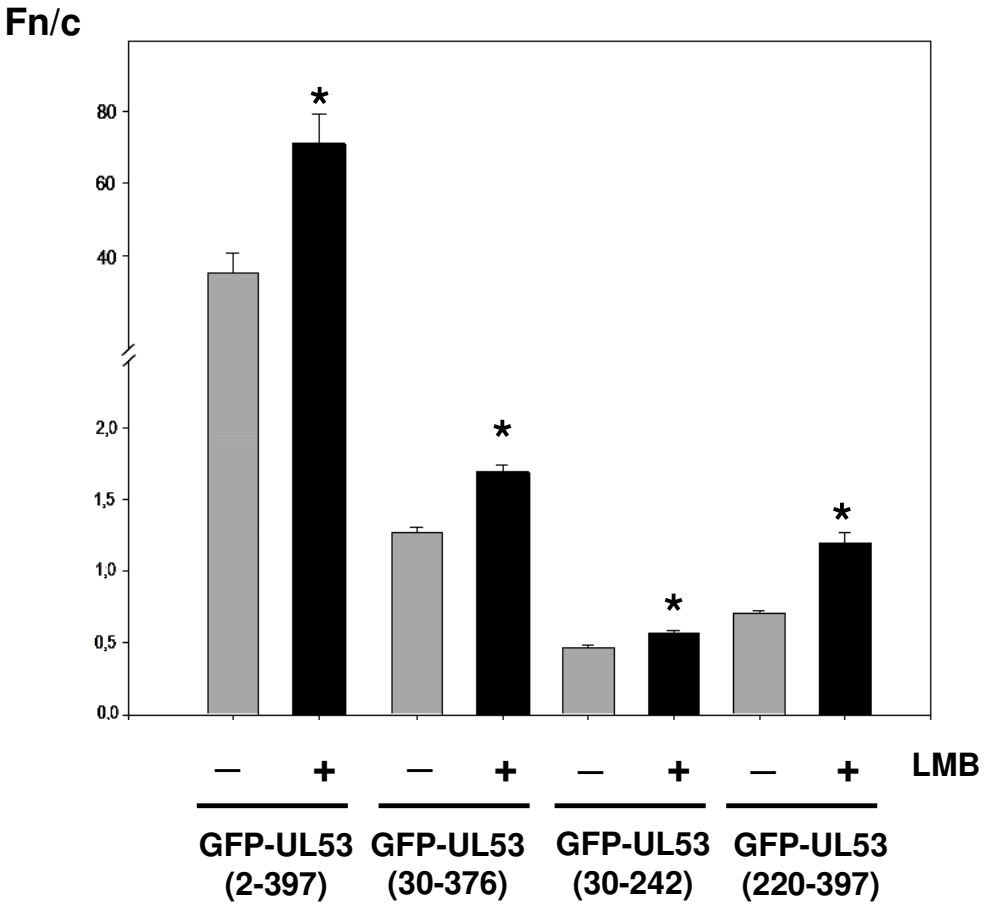


Fig. 3.13. Statistical analysis of Fn/c ratio of pUL53 domains localization in absence or presence of Leptomycin B (LMB). Images have been analiyzed using *ImageJ* software and data using *Sigma Plot 9.0* software. The columns represent the mean values +/- standard errors. Significative differences are indicated with (*)

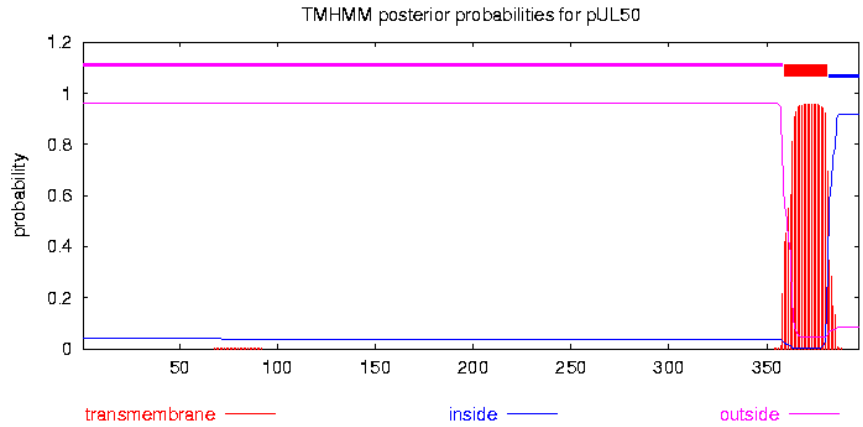


Fig. 3.14. The on-line software *THMM* predicts a transmembrane region at the N-terminus of pUL50 (residues 358-381)

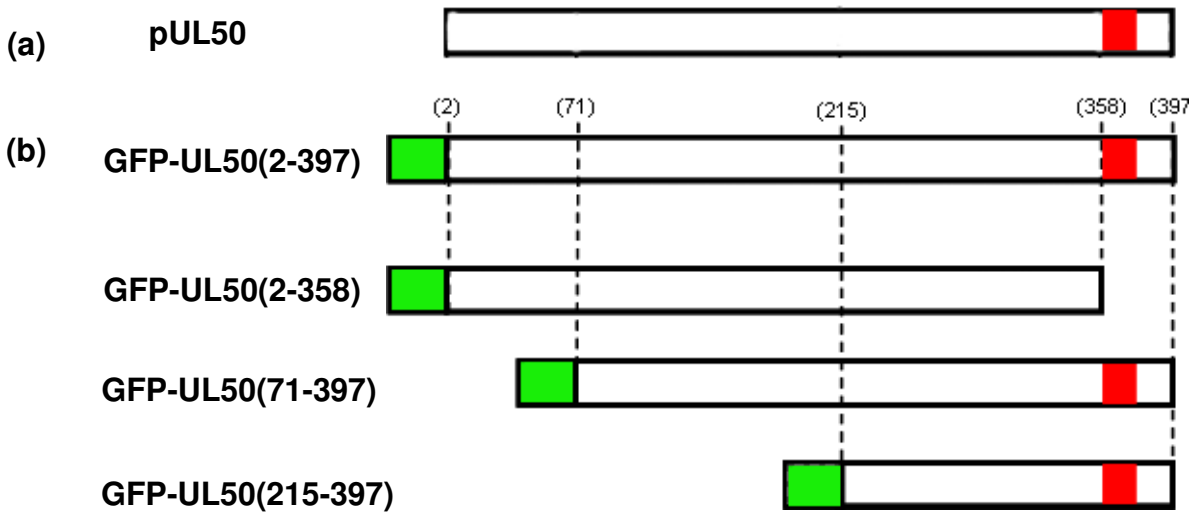


Fig. 3.15. Schematic representation of pUL50 properties. (**a**) pUL50 wild type presents a putative transmembrane region at its C-terminus (residues 358-381); (**b**) different domains of pUL50 expressed as fusion proteins with GFP, used in this study.

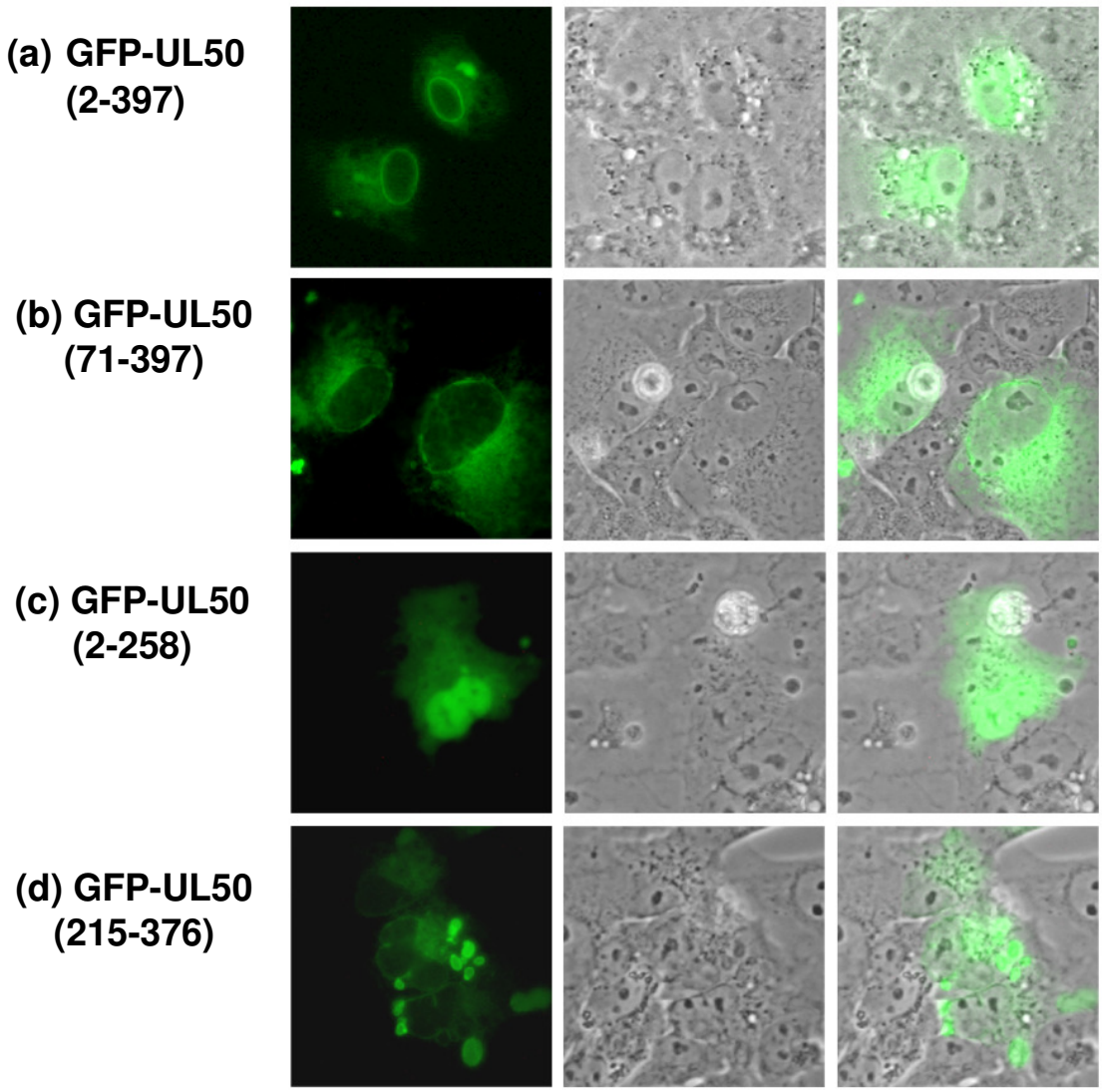


Fig. 3.16. Imaging of living cells expressing pUL50 different domains fused with GFP. All the fusion proteins containing the C-terminal domain localize in the cytoplasm with a ER pattern **(a**, **b**, **c); ** GFP-UL50 (215-376) is strongly associated to cellular membranes **(c).** GFP-UL50, lacking of the C-terminus, localizes in the all cell, accumulating in the nucleus and forming occasionally intanuclear speckles.

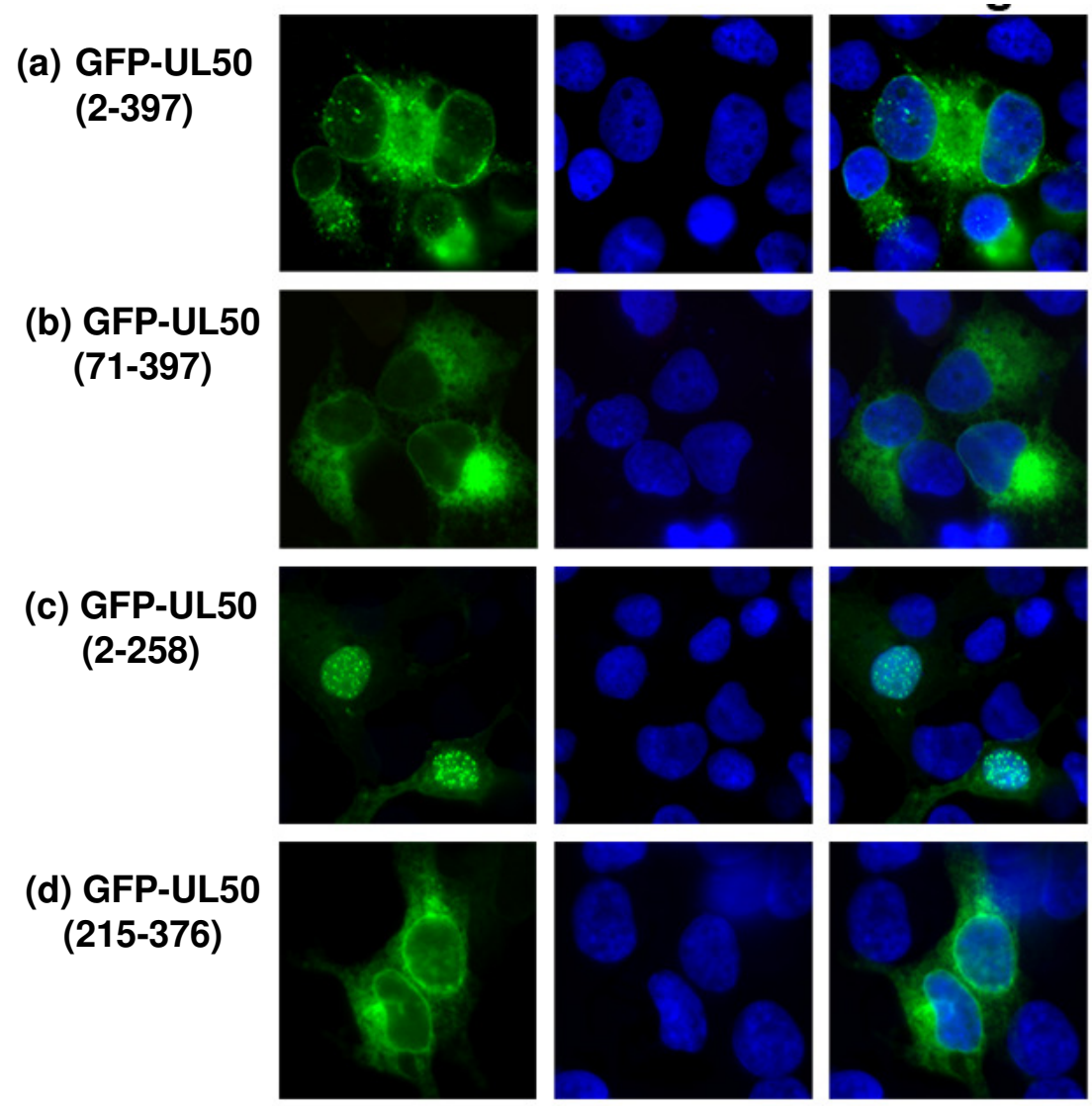
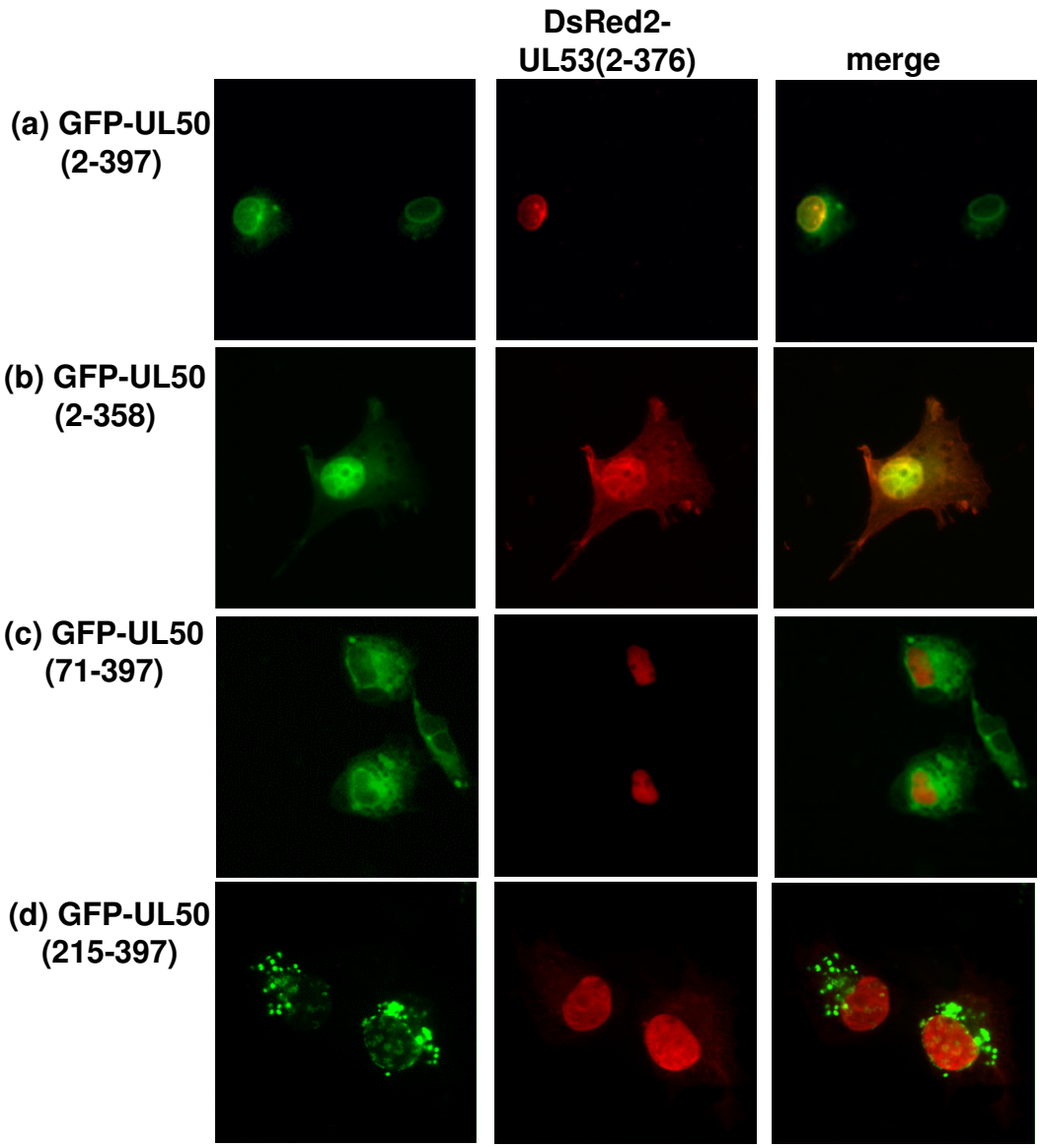
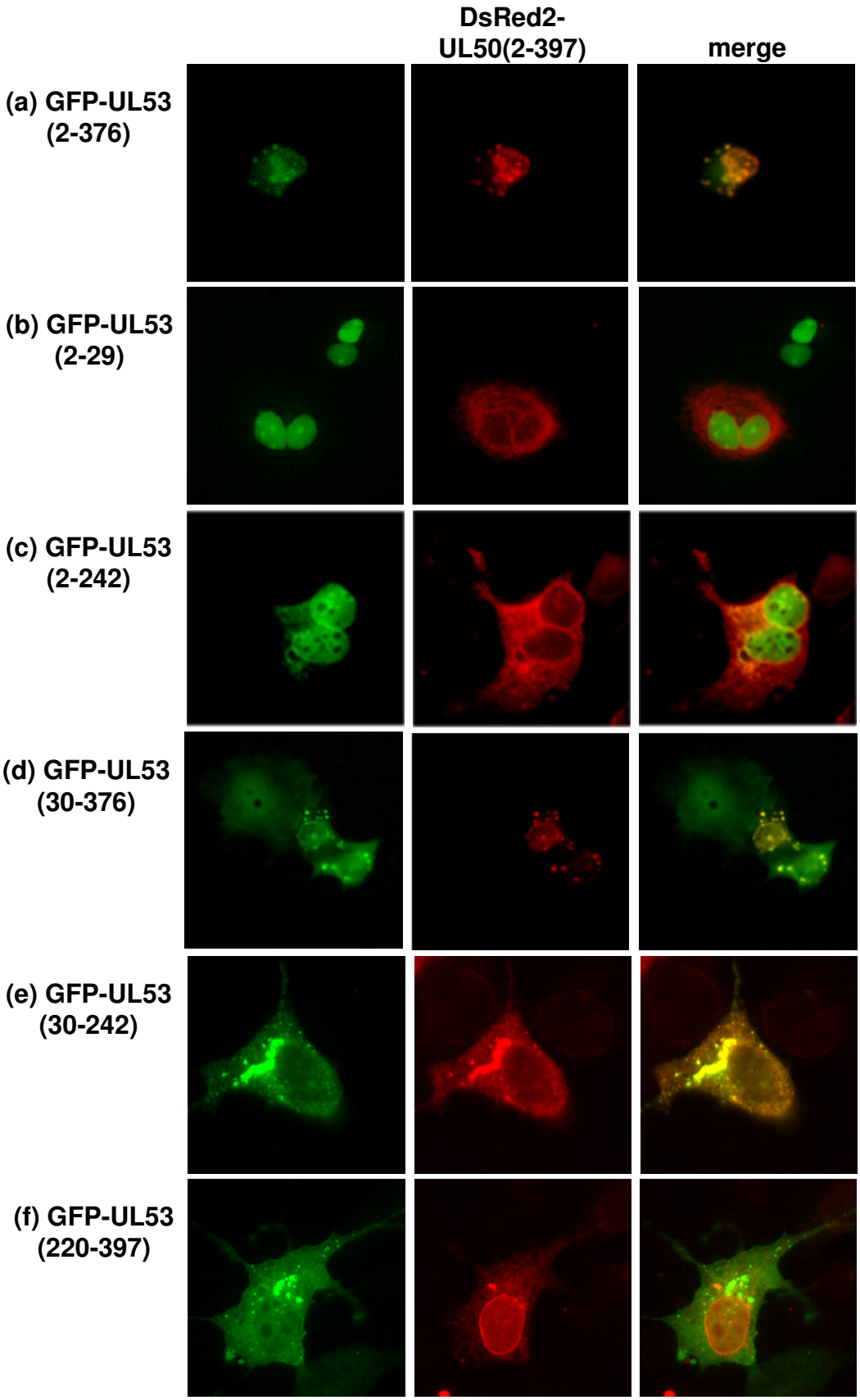


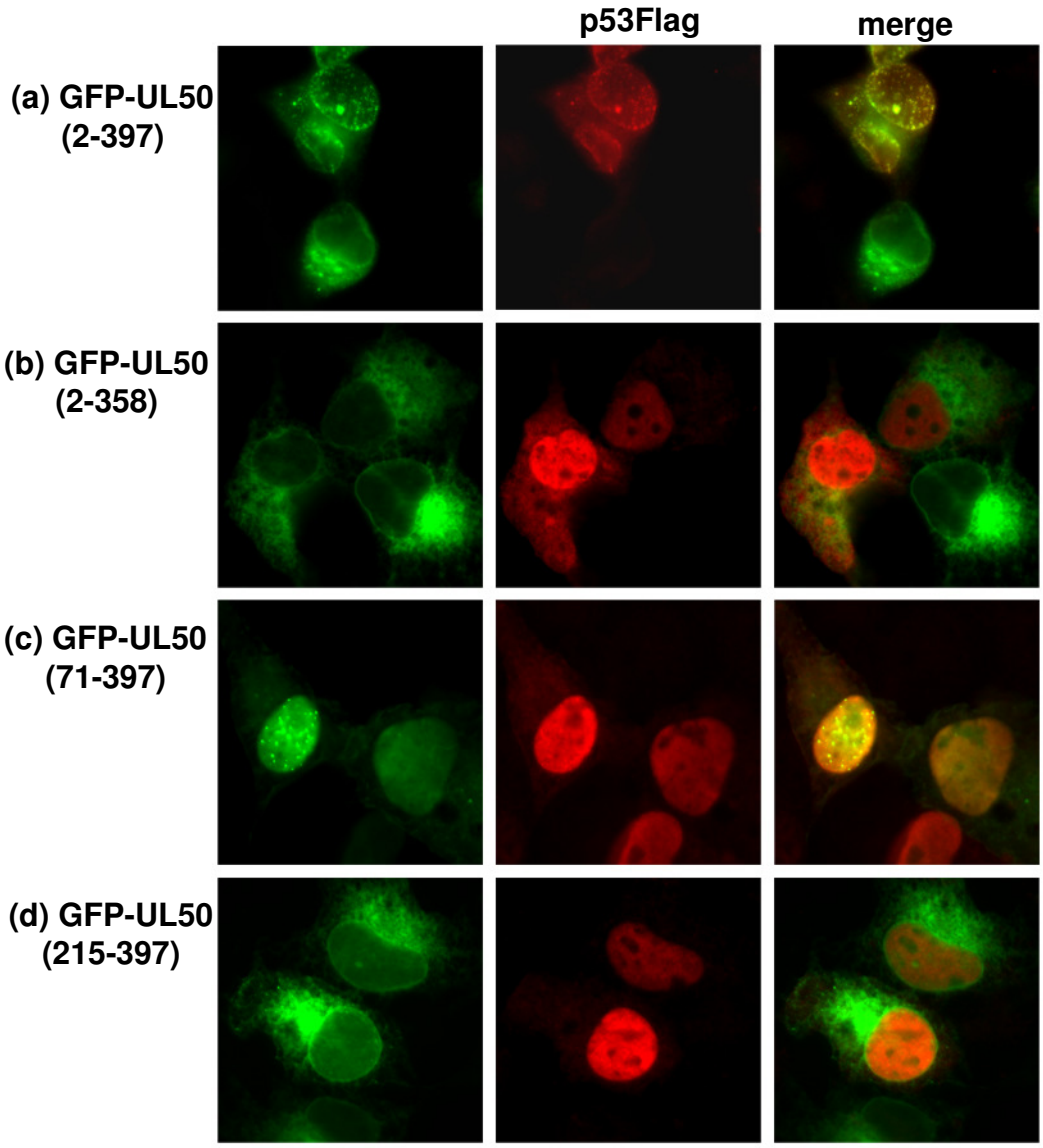
Fig. 3.17. Imaging of fixed cells expressing the different pUL50 domains fused with GFP. Nuclear staining has been performed with DAPI. The results are superimposable to the one obtained with living cells (**Fig. 3.1?**)



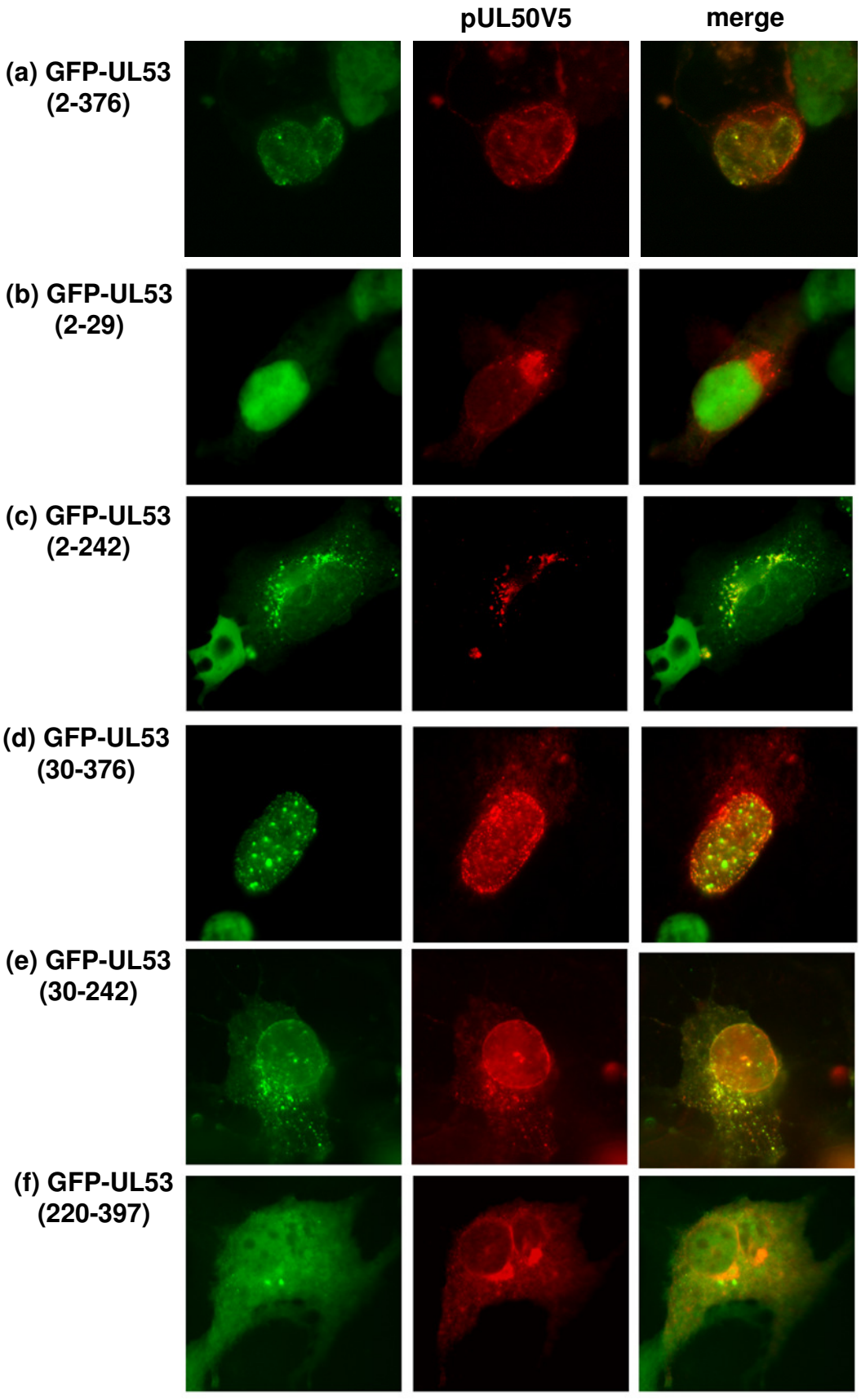
3.18 Analysis of pUL50 binding domain to pUL53 in living cells: DsRed2-UL53(2-376) full length has been espressed with all the different domains of pUL50 fused to GFP. Living cells were analyzed by fluorescent microscopy.



3.19 Analysis of pUL53 binding domain to pUL50 in living cells. DsRed2-UL50(2-397) full length has been espressed with all the different domains of pUL53 fused to GFP. Living cells were analyzed by fluorescent microscopy.



3.20 Analysis of pUL50 binding domain to pUL53 in fixed cells: pUL53Flag has been espressed with all the different domains of pUL50 fused to GFP. Fixed cells were analyzed by IIF using anti-Flag monoclonal antibody.



3.21 Analysis of pUL53 binding domain to pUL50 in fixed cells: pUL50V5 has been espressed with all the different domains of pUL53 fused to GFP. Fixed cells were analyzed by IIF using anti-V5 monoclonal antibody.

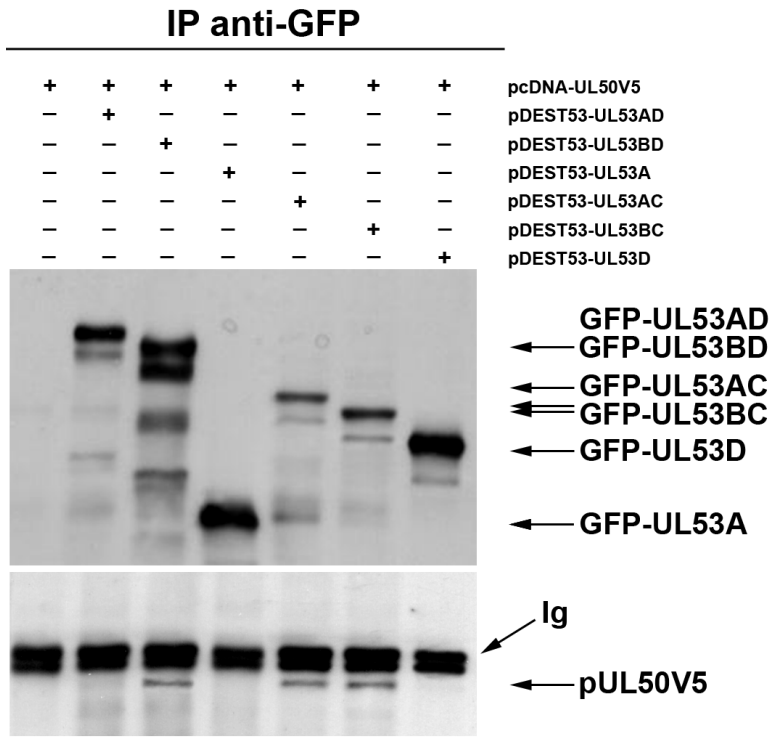


Fig. 3.22. Identification of the pUL53 domain of interaction with pUL50 by IP. (a) IP has been performed on lyates of cells expressing pUL50V5 in combination with the different pUL53 domains fused to GFP. The pUL53 domain interacting with pUL50 resides within the 200 central aminoacids.

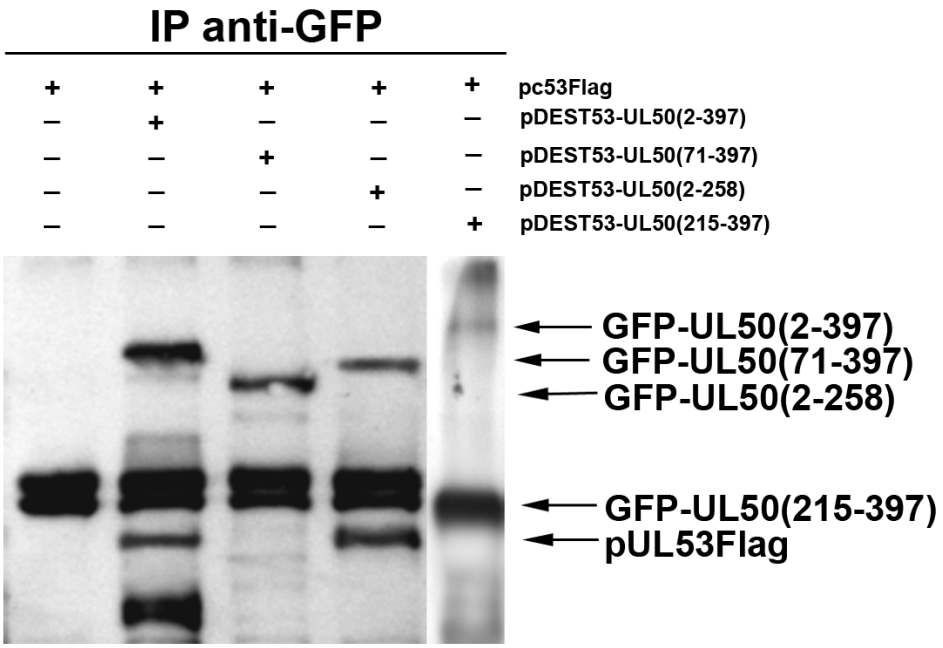


Fig. 3.23. Identification of the pUL50 domain of interaction with pUL53 by IP. (a) IP has been performed on lyates of cells expressing pUL53Flag in combination with the different pUL50 domains fused to GFP. The pUL50 domain interacting with pUL53 resides within the 70 first aminoacids.

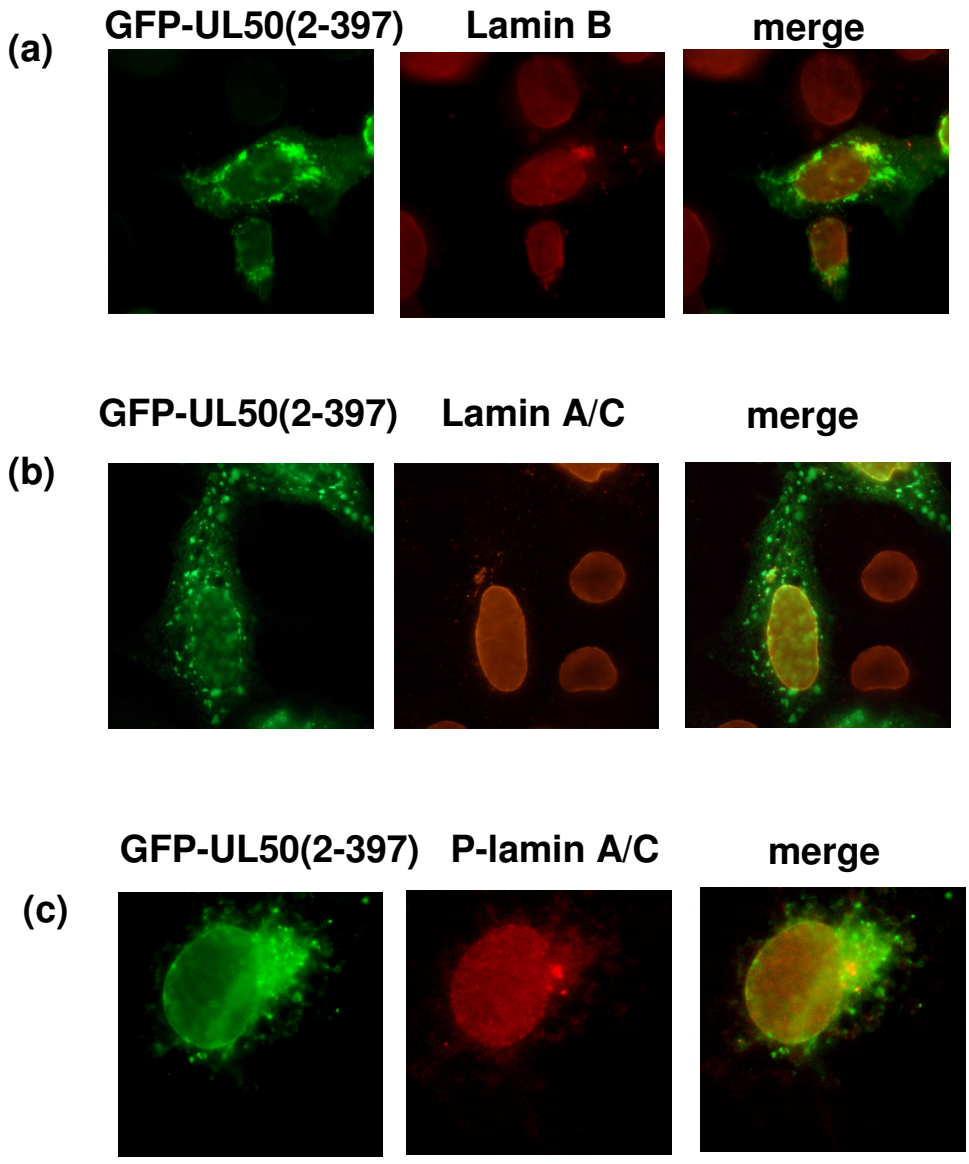


Fig. 3.24. Analysis by IIF of lamins in cells expressing GFP-UL50(2-397). pUL50 does not determine any significant alterations on lamin B or A/C, a part from a partial and occasional co-localization with lamin A/C Both in its phosphorylated and phosphorylated form in the cytoplasmic compartment.

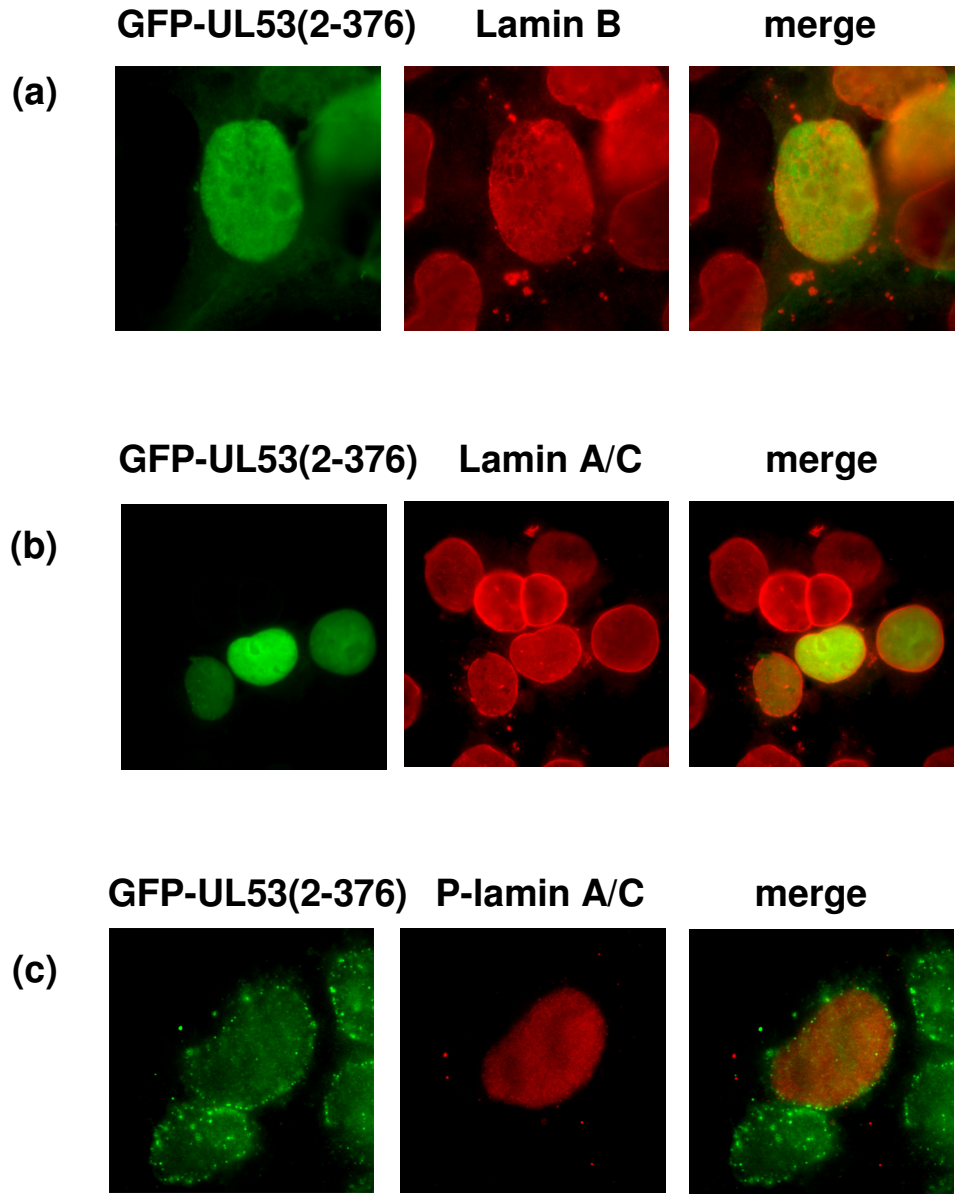


Fig. 3.25. Analysis by IIF of lamins in cells expressing GFP-UL53(2-376). GFP-UL53(2-376) shows occasionally to cause a dissolution of both lamin B (**a**)and lamin A/C(**b**) . Moreover, phospho-lamin A/C shows a partial decreased staining (**c**)

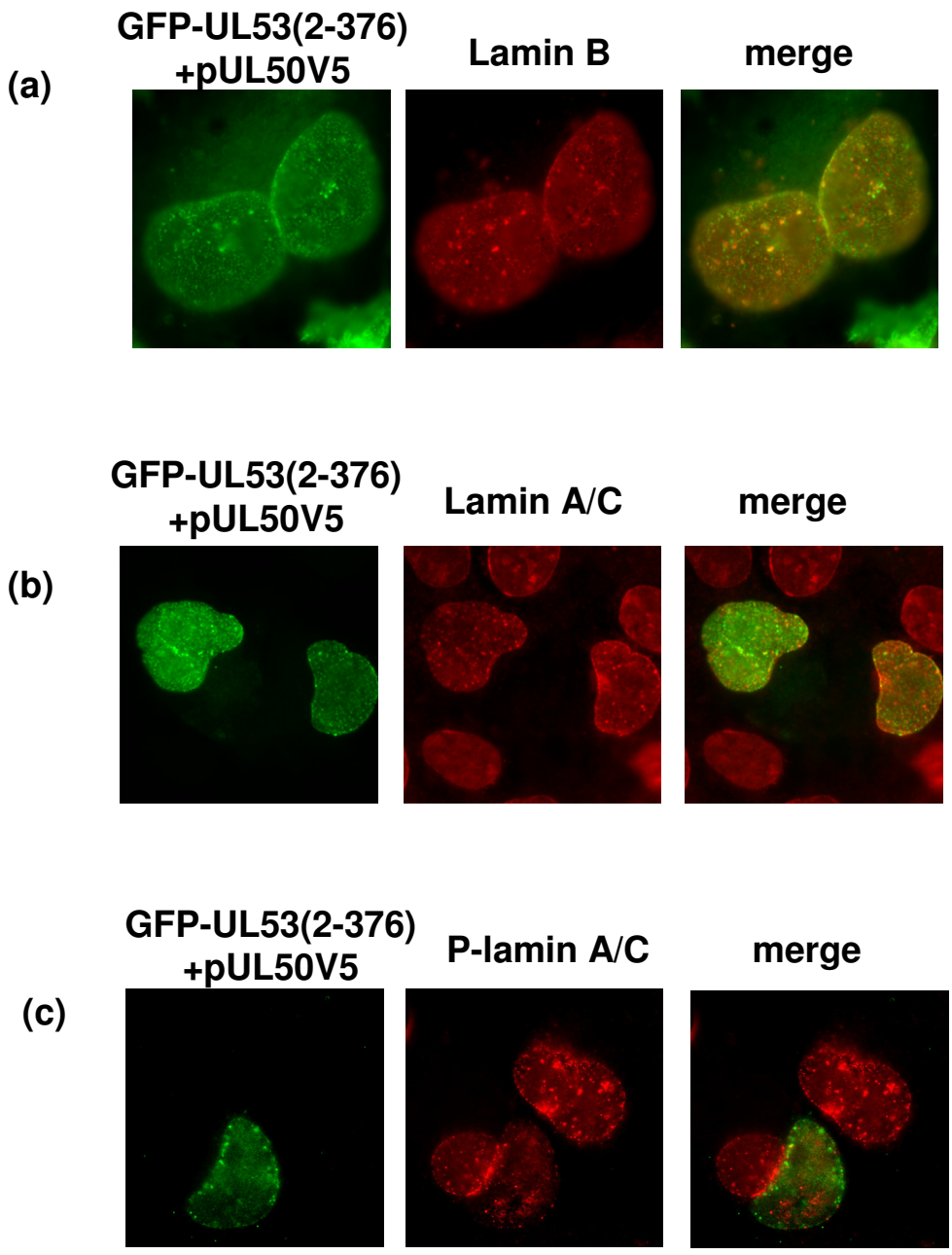


Fig. 3.26. Analysis by IIF of lamins in cells expressing GFP-UL53(2-376) and pUL50V5. The interaction between pUL50 and pUL53 is indicated by the formation of aggregates of GFP-UL53(2-376) at the nuclear rim. In pUL50and pUL53 co-expressing cells, both lamin B and lamin A/C show an irregular staining, with formation of invaginations corresponding to the aggregates of GFP-UL53(2-376) (**a**) and (**b**); phospo-lamin A/C displays a decrease in overall signal, but the aggregates rarely co-localize with GFP-UL53(2-376) punctuate distribution (**c**).

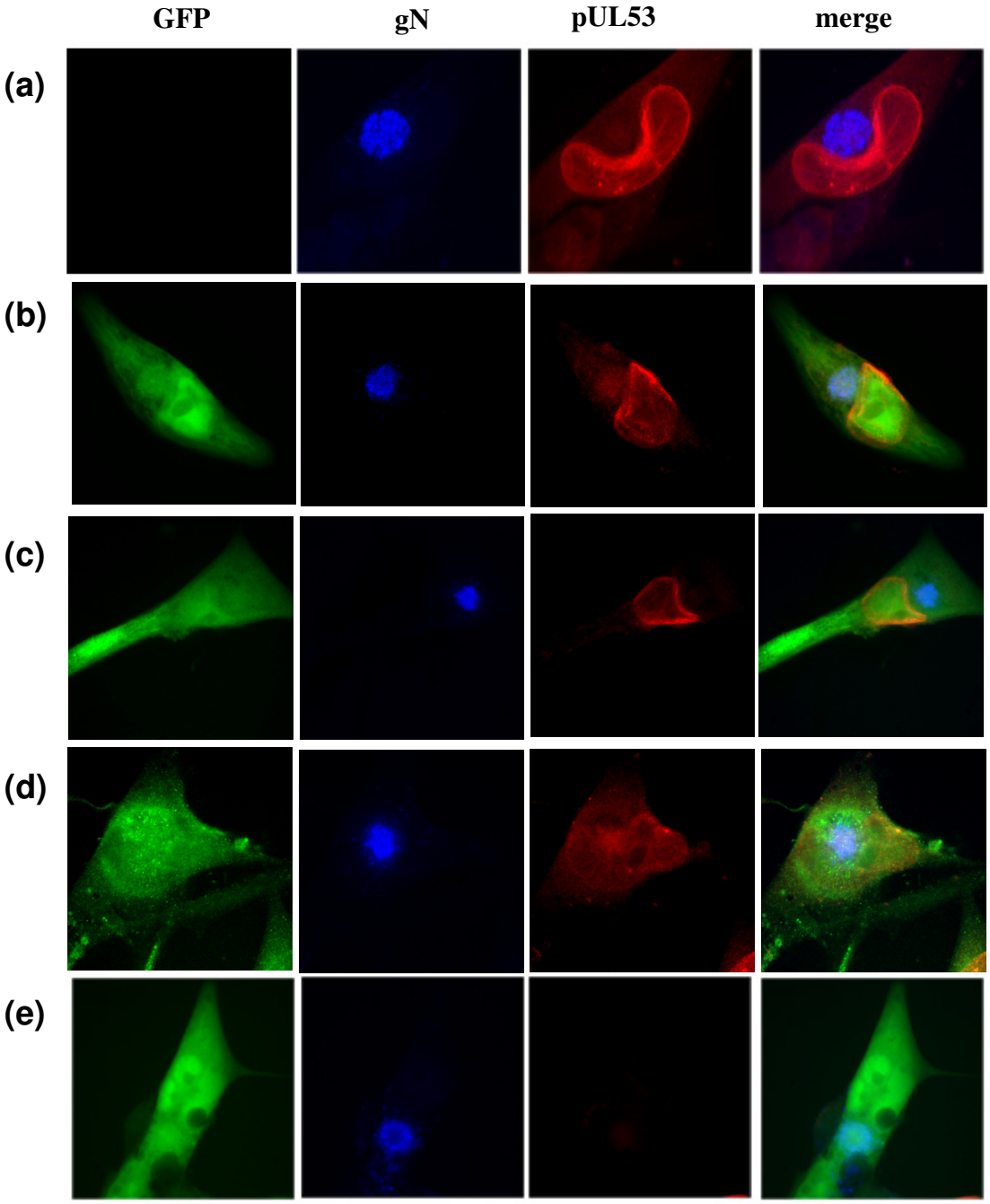


Fig. 3.27. RNA interference against HCMV UL53 transcript. HELFs have been transfected at day 0 with the construct pG-SUPERUL53-224 and infected at day 1 with HCMV AD169. Cells were fixed 120h *post-infection* and revealed by IIF for pUL53 and gN. (a) Untransfetcted cells; (b) transfected with pG-SUPER; (c) transfected with pG-SUPER-UL53-757; (d) transfected with pG-SUPER-UL53-782; (e) transfected with pG-SUPER-UL53-224.

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