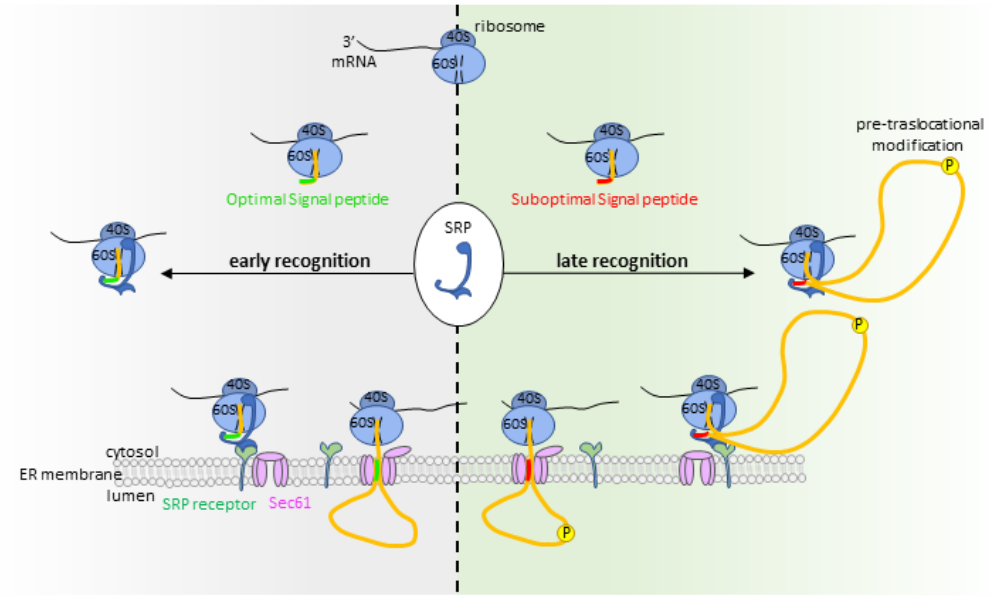
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXV CYCLE

Gianluca Scerra

A SUBOPTIMAL SIGNAL PEPTIDE DEFINES SPATIOTEMPORAL CONTROL OF PD-L1 EXPRESSION



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A SUBOPTIMAL SIGNAL PEPTIDE DEFINES SPATIOTEMPORAL CONTROL OF PD-L1 EXPRESSION

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1. List of abbreviations used

ADP	Adenosine DiPhosphate
AMP	Adenosine MonoPhosphate
AMPK	AMP-activated Protein Kinase
ATP	Adenosine TriPhosphate
BiP	Binding immunoglobulin Protein
CADA	CyclotriAzaDisulfonAmide
CHX	CycloHeXimide
CLX	Calnexin
CRT	Calreticulin
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
EGFP	Enhanced Green Fluorescent Protein
EndoH	Endoglycosidase H
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
GPI	glycophosphatidylinositol
GSK3β	Glycogen Synthase Kinase 3 β
GTP	Guanosine TriPhosphate
Hsp	Heat Shock Protein
IF	ImmunoFluorescence
IgV	Immunoglobulin Variable
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif
JAK	JAnus Kinase
MHC	Major HistoCompatibility
OST	OligoSaccharylTransferase
PD-1:	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
PD-L2	Programmed Death-Ligand 2
PTM	Post Translational Modification
RNA	RiboNucleic Acid
RNC	Ribosome Nascent Chain
RUSH	Retention Using Selective Hooks
SBP	Streptavidin Binding Peptide
Sec	Secretory
SP	Signal Peptide
SPn	native Signal Peptide
SPopt	optimized Signal Peptide
SR	SRP receptor
SRP	Signal Recognition Particle
TCR	T-Cell Receptor

2. Abstract

Malignant cells exploit the expression of the programmed death-1 (PD-1)-ligand 1 (PD-L1) to avoid T-cell-mediated immunosurveillance and promote tumor survival and expansion. Post-translational modifications have emerged as important regulatory mechanisms to modulate PD-L1 expression on the cell surface of cancer cells. However, spatiotemporal control of such modifications is still poorly understood. Here we show that amino-terminal signal peptide plays a major role in dictating the right temporal window for post-translational modifications. Particularly, we have developed the cytosolic version of the retention using selective hook assay (cytoRUSH) to demonstrate that the signal peptide of PD-L1 is suboptimal for the endoplasmic reticulum translocation and introduces a lagging time for pre-translocational modifications acquisition. Accordingly, PD-L1 equipped with an optimized signal peptide variant was insensitive to AMPK-dependent post-translational modifications relevant for driving PD-L1 down-modulation by the proteasome. Moreover, this variant renders PD-L1 defective in trafficking, maturation, and localization to the plasma membrane thereby confirming the physiological relevance of the lagging time introduced by its own signal peptide. In conclusion, these findings suggest that a less efficient kinetic of translocation into the endoplasmic reticulum represents the key step for PD-L1 protein modulation by post-translational modifications acquisition and open intriguing future perspectives for pharmacological applications based on PD-L1 signal peptide targeting in cancer therapy.

3. Background

3.1. The Compartmentalization of the Eukaryotic Cell

As early as the 19th Century, light microscopy had shown the existence of the nucleus, endoplasmic reticulum (ER), Golgi, mitochondria, and chloroplasts. Yet, the full extent of eukaryotic cellular complexity was only revealed with the advent of electron microscopy in the mid-20th Century. In parallel, biochemical fractionation led to the discovery of other compartments, such as the peroxisome and the lysosome[1,2]. Most importantly, biochemical approaches allowed the assignment of specific biochemical activities to distinct organelles, establishing the notion of biochemical or functional compartmentalization of the cell. The evolutionary emergence of organelles was a defining process in diversifying biochemical reactions within the cell and enabling multicellularity. Organelles are cellular structures that allow multiple biochemical environments to coexist in the cell and to be adapted independently. Extensive membrane-delimited compartments define the characteristic cellular architecture of eukaryotes. In animals, approximately half of the volume of a eukaryotic cell is membrane-enclosed, and in plants, the vacuole can make up to 95% of the cellular volume[3]. The eukaryotic endomembrane system comprises numerous organelles, such as the ER, the Golgi apparatus, lysosomes or vacuoles, and trafficking machinery that links these compartments via vesicular intermediates. The endomembrane system is functionally organized mainly along two major pathways: the secretory/exocytic pathway synthesizes, folds, modifies and targets proteins and lipids to the membranes and the lumens of several cellular organelles, the plasma membrane, or the extracellular space. On the other hand, the endocytic pathway operates in the opposite direction, taking proteins and material up from the surface or the extracellular space to be either recycled or degraded. Organelles coordinate their activity by exchanging lipids, proteins, and molecules across them, guaranteeing the maintenance of their proteome, morphology, and, collectively, their homeostasis.

However, compartmentalization also imposed a significant challenge: the need to import proteins synthesized in the cytosol into their respective sites of function by crossing a membrane. The two most prominent targeting destinations requiring a membrane cross are mitochondria and the ER. Mitochondrial proteins are synthesized in the cytosol and maintained in an unfolded translocation-ready conformation by cytosolic chaperones[4]. Their import into mitochondria is mediated by the translocase of the outer mitochondrial membrane (TOM) and the translocase of the inner mitochondrial membrane (TIM) complexes[4]. The ER is the entry site to the whole secretory/endomembrane system: hence, it is unsurprising that ~30% of all eukaryotic genes encode proteins that must target and translocate to the ER[4]. These genes encode membrane-spanning, membrane-anchored, secreted, organelle-residing proteins, which collectively play a role of paramount

importance in a broad set of functions, ranging from cardinal metabolic events to sophisticated, cell-type-specific pathways that define pluricellular life.

3.2. Membrane Translocation and Insertion Strategies Arisen throughout Evolution

The complex multicompartmental organization of eukaryotic cells entails the existence of topogenic signals able to direct a newly synthesized polypeptide to its correct physiological localization. Pioneering work by G. Palade demonstrated that secretory proteins cross the ER membrane before being transported in vesicles to the plasma membrane[5]. In the 70s, Günter Blobel and David Sabatini proposed the hypothesis that the topogenic information is intrinsic, thus encoded within the amino acid sequence of non-cytosolic proteins[6]. The laboratories of G. Blobel and C. Milstein then discovered that secretory proteins are directed to the ER membrane by signal sequences[6,7]. A little later, signal sequences were deciphered for proteins crossing the bacterial plasma membrane and the membranes delimiting mitochondria and chloroplasts[8,9]. Introducing what would become a staple in this research field - the incubation of microsomes with a mixture containing everything indispensable for translation - G. Blobel and B. Dobberstein observed how information encoded within the first amino acids of secretory proteins results in their segregation inside the lumen of microsomes, a process occurring in a co-translational fashion[10,11]. Their seminal work paved the way for the further characterization of topogenic sequences, or signal peptides (SPs), and the current understanding of protein sorting.

Albeit different, the eukaryotic translocation systems share common features that determine their function. The protein that must translocate interacts with cytosolic factors that target the protein to the appropriate membrane thanks to the recognition of organelle-specific sequences. The concerted action of molecular chaperones also maintains the protein in a translocation-receptive state. Following the interaction of the cytosolic factor with its receptor, the amino acid chain interacts with a translocator, a hetero-multimeric protein forming a hydrophilic pore spanning the membrane through which the protein will be moved across. Proteins acting as translocation motors confer unidirectionality to the process, thus preventing the chain from sliding backwards while consuming energy in the form of nucleoside triphosphates. Finally, organelle-specific molecular chaperones and enzymes applying post-translational modifications (PTMs) aid the newly translocated polypeptide in reaching its native three-dimensional configuration[12]. The signal sequences of each organelle have shared motifs of polarity and structure but no sequence conservation. For example, ER import signals are basic at the N terminus, followed by a stretch of 8 to 14 apolar residues and a short cleavage motif that is recognized by a dedicated peptidase, while mitochondrial matrix preproteins have a distinct signal sequence, an amphipathic α-helical rod bearing a

hydrophobic and a cationic face[13,14]. Translocation within mitochondria occurs following the release of cytosolic precursors from free ribosomes, therefore in a post-translational fashion. Cytosolic proteins belonging to the Hsp70 and the Hsp90 families of molecular chaperones preserve the precursors in an unfolded, translocation-receptive state, preventing them from reaching a non-native configuration that could favor aggregation or engagement with the ubiquitin-proteasome system[15,16]. Additionally, these chaperones interact with the cytosolic domains of precursor protein receptors of the TOM complex, consisting of three receptor subunits - Tom20, Tom22, and Tom70 - and four pore subunits - Tom5, Tom6, Tom7, and Tom40[17,18]. Preproteins translocate across the outer membrane and access to the intermembrane space in an unfolded state and in an N-to-C direction[13]. The membrane proton-motive force drives the initial transport stage across the inner mitochondrial membrane through the TIM complex[13]. As proteins enter the matrix, they are captured by mHsp70, a TIM-associated ATP-driven chaperone that binds each segment of the chain as it enters, thereby restricting net movement to import[19].

3.3. The Function and the Amino Acid Composition of Endoplasmic Reticulum-targeting Signal Sequences

Non-cytosolic proteins bear signal sequences that may be found at the amino-terminus - a property of SPs -, at the carboxy-terminus, or within the protein sequence. These proteins may also expose signal patches once they have achieved their native configuration[20,21]. The length of a classical SP is highly variable, ranging from the extremely short SP of flagellin in methanogens to the almost one hundred residues long SP of the mouse mammary tumor virus Rem protein[22,23]. Excluding these extreme topogenic sequences, the common average is between 15 and 30 amino acid residues[24]. Sequencing of multiple preproteins bearing an amino-terminal SP - the most common sorting signal - has also highlighted the lack of a consensus sequence shifting the focus from the search for a specific succession of amino acids informing cellular localization to the broader properties of the peptide. Its physicochemical characteristics could hold the key to a greater understanding of the interaction between a nascent protein and the cytosolic factors involved in its docking near one of the various translocation machineries[25]. Sequence comparison experiments allowed the identification of recurrent characteristics confined to specific segments of the SP, enabling the description of a common tripartite structure[21]. It consists of an amino-terminal region rich in positively charged amino acids - the n-region - a central hydrophobic stretch - the h-region - and a carboxy-terminal region rich in polar amino acids bearing a consensus sequence recognized by signal peptidases, the c-region (**Figure 1**)[21]. The idea that SP-mediated localization relies on a consensus sequence was further disproven with the observation that a wide range of mutations does not abrogate the function of SPs and that their translocation-directing action is interchangeable even between evolutionarily distant species[26]. Regardless, it has emerged in the last two decades how the

extreme variability in terms of amino acid composition and length underlies additional functions extending from the modulation of the efficiency of translocation to the regulation of downstream events occurring after membrane crossing and/or insertion[27]. After analysing the hydrophobicity of SPs of GPI-anchored versus non-GPI-anchored proteins, it was suggested that GPI-anchored membrane proteins are routed to the ER in a post-translational fashion due to their less hydrophobic SPs[28–30]. This is reminiscent of the situation in yeast[30,31]. The long, highly conserved SP of the envelope glycoprotein of arenaviruses, such as the lymphocytic choriomeningitis mammarenavirus and the Lassa mammarenavirus, remains associated with the transmembrane protein following the action of the host signal peptidase to mediate a key role in plasma membrane targeting and proteolytic processing[32,33]. Conversely, in PVL+ Staphylococcus aureus strains, the SP of the Panton-Valentine leukocidin S component remains on the cell surface after cleavage enhancing the interaction with the host extracellular matrix[34]. Moreover, multiple disease-causing SP-single nucleotide polymorphisms exerting downstream effects on protein processing have been identified[35]. The parathyroid hormone C18R variant triggers the activation of the unfolded protein response due to the accumulation of the hormone precursor in the ER leading to apoptosis and the onset of the autosomal dominant form of familial isolated hypoparathyroidism[36]. Similarly, the CTLA-4 T17A polymorphism causes a defect in the glycosylation pattern, thereby impairing its immunosuppressive function and increasing susceptibility toward autoimmune disorders[37].

The properties of each segment of an archetypal tripartite SP fulfil a specific role in translocation dynamics. The basic character of the n-region, with a length oscillating on average between 1 and 5 residues, enables the establishment of electrostatic interactions with the negatively charged heads of the lipid bilayer, thereby facilitating insertion, and with the phosphate backbone of the ribonucleic acid associated with the signal recognition particle (SRP), a ribonucleoprotein complex involved in the docking of a nascent polypeptide chain to the membrane of the ER and associated to the Sec translocation system[21,38–40]. Regardless, in vitro translocation models using artificial SPs with no basic amino acids and an elongated h-region have illustrated how the presence of histidine, arginine, or lysine within the n-region is dispensable for efficient translocation[41].

The h-region, on average 7 to 15 residues long, features mostly nonpolar amino acids, among which leucine is the most common in eukaryotes and prokaryotes[21]. Nonetheless, its amino acid composition does not consist of random hydrophobic residues as conserved species-specific motifs have been identified, highlighting the abundance of leucine, the presence of phenylalanine, and the absence of polar residues in most human h-regions when compared to prokaryotic and eukaryotic species[21,28]. Moreover, helix breaker amino acids, such as proline, serine, and glycine, have been identified within the h-region

contributing to its acquisition of a hairpin-like conformation, thus facilitating membrane insertion[21]. The hydrophobic character of the core region has often been linked to the determination of the three-dimensional conformation of the SP and its helical propensity affecting membrane insertion, the efficiency of events downstream of initial insertion, including glycosylation and cleavage of the SP, and the specification of the translocation pathway[21,26,31]. In Escherichia coli, SPs specifying for the Tat export pathway are less hydrophobic than SPs specifying for the Sec pathway[42]. Nevertheless, hydrophobicity is not the sole factor determining whether a signal sequence will engage the SRP as not all SPs containing a highly hydrophobic h-region interact with the recognition protein, therefore implying the involvement of additional factors such as the length of the final product and the conformation of the SP after emerging from the ribosome[43,44].

The carboxy-terminal portion of a classical SP, the c-region, contains a stretch of 3 to 7 residues where it is possible to recognize a more variable region and a less variable region near the cleavage site[21,45]. Remarkably, the less variable region bears a conserved motif, the AXA motif, starting from three amino acids before the cleavage site and consisting of alternated alanine residues[46]. While substitutions affecting the second alanine are less tolerated, with threonine infrequently substituting the amino acid, replacement of the first alanine with nonpolar amino acids, such as leucine, valine, and isoleucine, does not alter the cleavage site. Additionally, serine, glycine, and cysteine have also been identified in place of either of the two alanine residues[21]. Even though aromatic or negatively charged amino acids replacing the first or the second alanine abolishes the processing of the SP, their presence between the two is not disruptive. Interestingly, helix breaker amino acids are often found at the border between the c- and the h-region[47]. Lastly, among the overall properties of the SP, the charge difference between the regions surrounding the hydrophobic core establishes whether the orientation of the SP will promote or oppose translocation. If the positive charge of the n-region is greater than that of the c-region, translocation will be favoured, otherwise, it will be impaired[48,49].

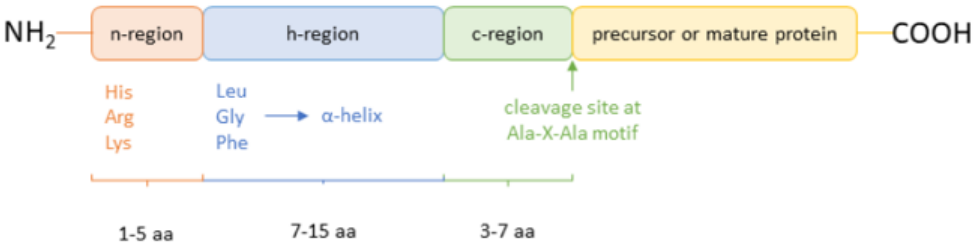

Figure 1. Structure of a canonical signal peptide.

The structure of a classical SP consists of an amino-terminal region - the n-region - a central hydrophobic stretch - the h-region - and a carboxy-terminal region rich in polar amino acids, the c-region. The n-region, on average 1 to 5 residues long, is rich in positively charged amino acids such as histidine (His), arginine (Arg), and lysine (Lys), conferring to this tract a basic character required for the establishment of electrostatic interactions with the negatively charged heads of the lipid bilayer and with the sugar-phosphate backbone of the RNA associated with the SRP. The h-region, on average 7 to 15 residues long, often contains repeats of hydrophobic amino acids, such as leucine (Leu) interrupted by helix-breaker residues such as glycine (Gly). Phenylalanine (Phe) is more frequently featured within the h-regions of human SPs than within those of prokaryotic or other eukaryotic species. The hydrophobic character of the core region has been often linked to the determination of the SP three-dimensional conformation and its helical propensity. The c-region, on average 3 to 7 residues long, bears a conserved motif featuring alanine (Ala), the Ala-X-Ala motif, indispensable for the cleavage of the SP by signal peptidases (Adapted from Owji et al., 2018[21]).

3.4. Protein Translocation across the Endoplasmic Reticulum Membrane

The biogenesis of secreted and integral, peripheral, and luminal proteins residing in the intracellular compartments, except for peroxisomes and mitochondria, relies on the secretory pathway responsible for the correct folding, processing, and sorting of amino acid chains transiting from the ER and through the Golgi apparatus to their destination[50]. As the biosynthesis of nucleus-encoded proteins is confined to the cytosol, proteins entering the secretory pathway must translocate across the membrane of the ER. Protein translocation is a co-translational event for most amino acid chains beginning shortly after synthesising an appropriate SP and its protrusion from the ribosome exit site. The SRP, a cytosolic ribonucleoprotein, interacts with the nascent chain before targeting the ribosome to the ER membrane where its receptor, the SRP receptor (SR), resides. The docking of the ribosome-nascent chain complex (RNC) occurs in the proximity of the core protein of the Sec translocation system, known as SecY in prokaryotes and Sec61 in eukaryotes. Afterwards, the SRP is released from both the RNC and the SR so that SecY/Sec61 can interact with the former to mediate the insertion of the nascent chain into the lipid bilayer[51]. During co-translational targeting, the limited exposure of the nascent chain to conditions not characteristic of its destination reduces the risk of misfolding, which could impair localization and promote aggregation (**Figure 2**).

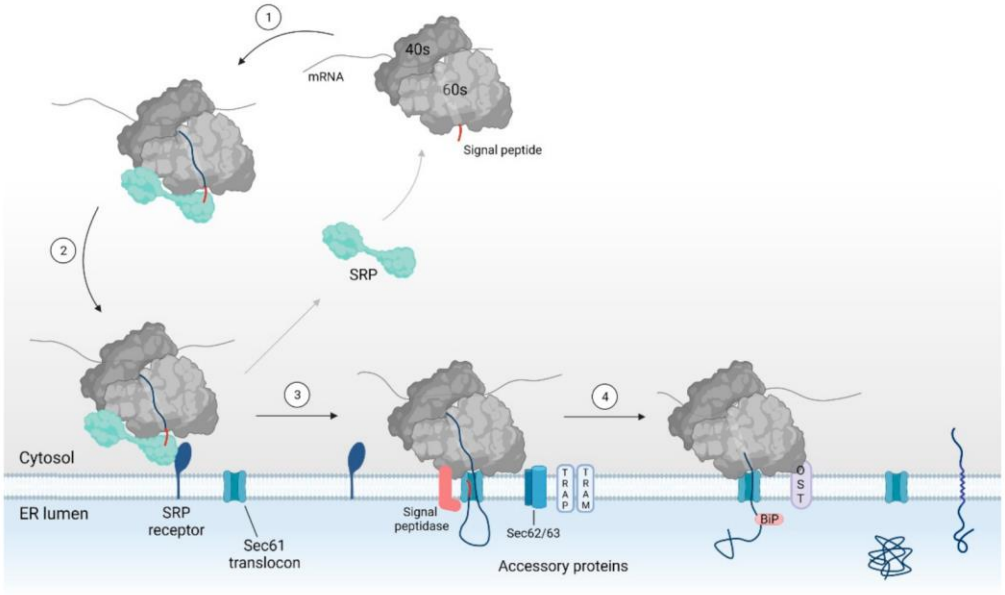

Figure 2. Overview of the co-translational translocation pathway.

A secretory or integral membrane protein is targeted toward the ER membrane by means of SRP binding to the SP (**steps 1-2**). SRP binding stalls protein translation to keep the nascent chain in a translocation competent state. At the ER membrane, SRP interacts with the SRP receptor. the RNC complex is then transferred to the Sec61 translocon (**step 3**). Interaction of the ribosome with the translocon reinitiates translation and induces conformational changes within Sec61 translocon, eventually leading to protein translocation. In the case of a weak hydrophobic SP, the protein requires help from accessory proteins such as TRAP, TRAM, Sec62/63 complex for successful translocation. In the ER lumen, the SP is cleaved by the signal peptidase complex and the protein is glycosylated by the OST complex (**step 4**) (Adapted from Pauwels et al., 2021[52]) .

3.4.1. Targeting the Membrane: The Signal Recognition Particle and its Receptor

Originally discovered in mammalian cells, the SRP and its interactors have been identified both in prokaryotes and in non-mammalian eukaryotes highlighting how the initial sequence of events characterizing the Sec translocation system is highly conserved in all domains of life[53–57]. Notwithstanding the high variability in terms of dimensions and overall constitution, the bacterial SRP can successfully restore efficient translocation in mammalian systems where the homolog has been knocked out[58–61]. While the bacterial SRP - known as Ffh - is much smaller due to a 4.5S RNA interacting with a single protein, the mammalian SRP consists of a longer RNA, the 7S RNA, and multiple associated proteins, including SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72[51]. The 7S RNA acts as a scaffold coordinating the protein subunits in space but also plays an active role by undergoing fundamental conformational alterations that reorient the SRP to promote the docking of the RNC to the SR following the interaction with appropriate cargo[51,62–65]. The synthesis of the 7S RNA occurs in the nucleus, and all protein subunits minus SRP54 are endowed with nuclear localization sequences that direct import to the compartment where assembly with the 7S RNA happens. The complex is exported back to the cytoplasm. The combined association of SRP19, SRP68, and SRP72 with the 7S RNA triggers the reorganization of the latter's internal loops resulting in the acquisition of a configuration that enhances the affinity of the complex for SRP54 that would otherwise weakly interact with the RNA[66–68]. The complex composed of SRP19, SRP54, SRP68, SRP72, and the 7S RNA minus its domain I is referred to as the S domain of SRP, while SRP9 and SRP14, in combination with the remaining portion of the RNA, constitutes the Alu domain of SRP[51]. In both eukaryotic and prokaryotic systems, SRP54 bears a methionine-rich region, known as the M domain, and a portion with GTPase activity, known as the NG domain[69]. The methionine residues line one face of the α-helices forming the groove where the SP is accommodated. The hydrophobic character of the amino acid, paired with the flexibility of its side chain projecting into the groove, enables the interaction with the extensive variety of SPs with picomolar affinity, an observation initially articulated in the form of the methionine bristle hypothesis[59]. However, these properties are not sufficient to facilitate SP recognition. Even without a bona fide SP, the SRP can interact with nanomolar affinity with RNCs, while isolated topogenic peptides show a micromolar affinity for the SRP[70,71]. Therefore, the presence of the ribosome is of paramount importance to guarantee high affinity in the interaction with an emerging nascent chain equipped with an appropriate SP. Notably, SRP molecules do not exist in equimolar amounts to ribosomes. Hence, to find its substrates, SRP quickly scans many nascent polypeptides and dissociates from translating ribosomes if it does not encounter a targeting signal[4,72]. Particularly, the association between SRP54 and the ribosome occurs near the ribosomal exit site thanks to the establishment of contacts between the amino-terminal basic

residues of the former and the subunits L23 and L35 of the latter[73–75]. Moreover, electrostatic attraction between the Alu domain and the ribosomal RNA occurs in the proximity of the latter's elongation factor binding site, thereby enhancing the affinity between the two proteins while interrupting translation. The interruption of translation - a striking feature of the mammalian SRP - generates a critical timeframe within which the RNC can be targeted to the translocon before the loss of translocation competence[51]. Conversely, the NG domain does not mediate direct interaction with a nascent chain but plays a key regulatory role in modulating the docking of the RNC to the membrane thanks to a mechanism of activation by nucleotide-dependent dimerization[76]. The dimerization partner is the NG domain found within the structure of both prokaryotic and eukaryotic SR. While the prokaryotic SR is a cytoplasmic protein known as FtsY, the eukaryotic SR is composed of a soluble subunit, SRα, and a transmembrane subunit, SRβ[77]. SRβ is a GTPase distantly related to the ADP ribosylation factor family[78]. The binding of GTP to SRβ strengthens the affinity for SRα, and de novo interaction between the two components is facilitated by the β subunit of the Sec61 complex, which acts as a nucleotide exchange factor[79]. Analogously, SRα can dimerize with SRP54 only when both proteins are active, resulting in mutual activation of the NG domain. Throughout dimerization, the two proteins undergo significant conformational shifts enabling the distinction between an early and partially open state, an intermediate and closed state, and a final, active state manifesting just before hydrolysis[51,80,81]. Specific environmental factors, including the recognition of an appropriate cargo by the SRP or the interaction with specific phospholipids in the ER membrane, regulate the equilibrium between the distinct conformations. The former stabilizes the early state, while the latter accelerates the achievement of the intermediate state[82,83]. The transition toward the active state weakens the stability of the SRP-RNC complex leading to its dismemberment once GTP is hydrolyzed[83]. Therefore, the translocon is free to interact with the detached ribosome, given that the binding occurs near the exit site where the SRP was previously attached[51,84]. The tight junction between the ribosome and the translocon is enough to exclude protease access, and nascent chains are discharged directly into the lumen[13]. Regardless, the exact mechanism of the cargo handover process has yet to be fully elucidated, even though the previously described interaction between the translocon and the SR could exert a regulatory function[51]. Moreover, multiple factors involved in protein biogenesis interact with the ribosomal protein L23 near the exit site, factors that could therefore modulate through competition the docking of the RNC to the membrane. The most relevant are the prokaryotic trigger factor and the eukaryotic nascent chain-associated complex[51].

3.4.2. Co-Translational and Post-Translational Membrane Targeting

Depending on the hydrophobicity and/or overall amino acid content of the precursor protein, transport can occur co- or post-translationally[30]. In Saccharomyces cerevisiae, SRP-dependent - co-translational - and Hsp70-

dependent - post-translational - pathways are equally important: however, the co-translational pathway is predominantly used by preproteins with more hydrophobic SPs[30]. The post-translational pathway seems to be used by a significant fraction of proteins in simpler organisms, such as bacteria and yeast, perhaps because in these fast-growing cells translocation does not always keep pace with translation. This pathway is used mostly by soluble proteins, such as secretory proteins, which possess only moderately hydrophobic SPs that cause them to escape recognition by the SRP during their synthesis[31,43].

Conversely, the co-translational pathway appears to be predominant in mammalian cells. Still, the mammalian ER has the capacity for post-translational protein transport[85–87]. The SRP-independent pathway is the only choice for precursors of GPI-anchored membrane proteins, and GPI-anchored membrane proteins are routed to the SRP-independent pathway because of their less hydrophobic SPs[29]. At present, the unifying feature of the post-translationally transported precursor polypeptides seems to be that they contain fewer than 100 amino acid residues: that is, they are below the minimal size of a nascent polypeptide chain to allow co-translational interaction of the SP with SRP[85–91]. The two mechanisms merge at the level of the ER membrane, specifically at the heterotrimeric Sec61 complex present in the membrane. The Sec61 complex provides a SP recognition site and forms a polypeptide conducting channel[30]. Cytosolic chaperones intervene to preserve the synthesized chain in an unfolded, translocation-receptive state before interacting with membrane partners mediating the transfer of the protein to the translocon[43,92]. Mainly in yeast, the cytosolic chaperone Hsp70 interacts with Sec72, a component - alongside Sec71 - of the Sec62/Sec63 complex, while in bacteria the chaperone SecB passes its substrate onto the peripheral membrane ATPase SecA, a protein associated with SecY[93,94]. Regardless, both mechanisms converge on the same channel, the SecY/Sec61 translocon. In 2004, the first structure of a Sec61 complex family member, the SecY complex from archae Methanococcus jannaschii, was solved by crystallization and subsequent X-ray analysis[95].

3.4.3. Crossing the Membrane: Structure and Function of the Sec61 Channel

The channel's core is a deca-spanning subunit - SecY in prokaryotes and Sec61α in eukaryotes - associated with two smaller integral subunits known as SecG and SecE and as Sec61β and Sec61γ in the respective domains of life. The ten α-helical transmembrane domains are evenly split into two asymmetrical segments constituting an hourglass-shaped pore with a maximal internal diameter of up to 0.8 nm: a polypeptide in the pore could therefore form an a-helix, but no tertiary structure, in agreement with experimental data[30,95]. The pore presents a constriction around the center of the lipid bilayer. The side chains of six aliphatic amino acids protrude toward the core of the constriction forming a ring. The access to the luminal side of the channel is impaired by the presence

of a short α-helical domain known as the plug. The combined action of the plug and the ring seals the channel when in an idle state blocking passage for small hydrophilic molecules that would otherwise access the ER. The channel also presents a lateral gate arising at the interface between the second and the third transmembrane domains on one side and the seventh and the eighth transmembrane domains on the other side. Of the two smaller subunits, only SecE/Sec61γ is indispensable for translocation considering that its interaction with the α subunit is required for preserving the functional conformation of the channel while SecG/Sec61β faintly interacts with SecY/Sec61α[95,96]. There is a second SEC61-gene present in mammals, termed SEC61A2. Thus, a second Sec61 complex may exist in the mammalian ER, termed as the alternative Sec61 complex (comprising Sec61α2, Sec61ß, and Sec61γ). However, no information on this complex is available[30].

In eukaryotic co-translational translocation, the channel must be primed for translocation. The interaction between the ribosome, SecA, or Sec62/63 with SecY/Sec61 triggers significant structural shifts that allow the channel to achieve a more open conformation[97]. Mainly, a slight opening of the lateral gate is present[96]. The aperture is more prominent upon the interaction of SecA with the channel as the ATPase binds to the cytosolic loops connecting the transmembrane domains of both halves of the pore, while the ribosome only binds to the carboxy-terminal half[98,99]. From the achieved conformation, the channel transits toward a translocation-receptive state characterized by the complete opening of the lateral gate and the displacement of the plug so that the luminal side is accessible[96]. The insertion dynamics of the signal sequence has been mostly clarified for secretory proteins whose SP inserts as a hairpin with the amino-terminal and the carboxy-terminal segments facing the cytoplasm. The positively charged n-region cannot breach through the anionic phospholipid head groups; therefore, by remaining in the cytosol, it forces the remaining portions to enter as a loop. Upon insertion, the h-region shifts across the lateral gate and into the hydrophobic core of the bilayer, remaining anchored to a groove on the side of the channel. The recognition of the topogenic sequence does not exclusively involve affinity for the phospholipid hydrocarbon tails but also the establishment of non-covalent interactions with the side chains of hydrophobic amino acids found on the exterior of the second transmembrane segment. The SP cleavage site remains inside the channel near its luminal side, thus inaccessible to the signal peptidase until ulterior conformational changes occur. The cleaved SP is then wholly released into the bilayer[96,100,101]. Contemporarily, the protein enters the channel contacting the translocator exclusively at the level of the pore ring thanks to the hourglass shape of the channel. Moreover, the absence of significant contacts facilitates translocation of even bulky side chains around which the pore ring can easily adapt but also allows the polypeptide to slide across the pore in either direction[96,101,102]. Directionality must be therefore provided to the system, a requirement fulfilled by distinct mechanisms according to the modality of translocation. In co-

translational translocation, the translating ribosome provides the driving force that impairs backwards sliding by occupying the cytosolic mouth of the channel[96]. Recent structural data cast doubt on the view that the intimate contacts between ribosomes and the Sec61 complex are sufficient to prevent ion efflux from the ER lumen during co-translational protein translocation. Indeed, there is a large gap between the ribosomal surface near the tunnel exit and the cytosolic surface of the Sec61 complex[30]. In addition, cytosolic Ca^{2+}-calmodulin was shown to contribute to Sec61 channel closing via an unrelated mechanism once Ca^{2+} has started to leak out of the ER[103]. This mechanism involves an IQ motif near the cytosolic amino-terminus of Sec61α, which is unique to vertebrates[91,103]. Moreover, according to the most recent cryo-electron microscopy data, some nascent precursor polypeptide chains can apparently form a loop on the cytosolic surface of the channel rather than directly entering the channel, thereby generating a cytosolic domain[104].

In eukaryotic post-translational translocation, fully synthesized precursor polypeptides are transported to the ER membrane with the help of cytosolic molecular chaperones, belonging to the Hsp70 and Hsp40 chaperone families[105–109]. By cycling on and off, the chaperones keep the precursor polypeptides soluble and competent for interaction with the transport components in the ER membrane. In the lumen of the ER, the member of the Hsp70 family BiP acts as a molecular ratchet by remaining tightly associated with the emerging polypeptide chain. Binding of BiP to the incoming polypeptide contributes to the efficiency and unidirectionality of transport[30]. BiP cycles between an open peptide-binding pocket state to a closed peptide-binding pocket state according to the bound adenosine nucleotide: ATP and ADP, respectively. The Sec62/63 complex recruits BiP in an ATP-bound state. The interaction between the chaperone and the luminal tail of Sec63 bearing the J domain triggers ATP hydrolysis, inducing a conformational change that forces BiP to close the binding pocket around the emerging polypeptide. Further exposure of the advancing chain enables the complex to recruit additional chaperones that, together, prevent backward sliding. Finally, BiP releases the bound ADP to interact again with ATP losing affinity for the substrate due to the reopening of the binding pocket. The process occurs slowly, often ending after the entire chain has been translocated[110]. In addition, BiP may be involved in the opening of the Sec61 complex by triggering the conformational changes in the heterotrimeric Sec61 complex that lead to plug displacement and allow the insertion of a precursor polypeptide into the complex[30].

However, co-translational and post-translational translocation are highly intertwined processes in eukaryotic cells, since when translation is terminated, there are still about 70 amino acid residues of the nascent polypeptide chain outside of the ER, buried in the ribosomal tunnel and the heterotrimeric Sec61 complex. Since the movement of polypeptides through the Sec61 complex is reversible, complete translocation of this carboxy-terminal peptide may require

help from BiP[30,111]. In either translocation mechanism, the polypeptide interacts with ER proteins that further aid its biogenesis.

3.4.4. Post-Translational Modifications and Protein Quality Control in the Endoplasmic Reticulum

Proteins translocating across the ER are either secretory proteins destined to the extracellular space or the plasma membrane or residents of the compartments laying along the secretory pathway. Regardless, all polypeptides transiting through the organelle receive PTMs required for their biogenesis and the achievement of their functional native configuration[112]. Proteins failing to fold activate the ER-associated degradation (ERAD) response, ensuring that misfolded chains do not aggregate or accumulate in the lumen. Mainly, luminal factors recognizing structural aberrations target the defective polypeptide to a transmembrane translocator complex consisting of multiple subunits that remove eventual PTMs while translocating the chain into the cytosol. Contemporarily, the protein is ubiquitinated on the cytosolic side to ensure downstream processing by the proteasome, a highly processive proteolytic machinery. The coordinated action of molecular chaperones and the enzymes applying PTMs prevents translocated proteins from activating the degradative response[113]. Glycosylation is one of the first events occurring upon exposure of a translocating polypeptide to the luminal side since the oligosaccharyltransferase (OST) complex is stably associated with the translocon. The enzyme transfers a dolichol-anchored oligosaccharide consisting of fourteen sugars, Glc3Man9GlcNAc2, to the side chain of most asparagine residues belonging to the consensus Asn-X-Ser/Thr (N-X-S/T). The phenomenon is known as asparagine-linked glycosylation or as N-glycosylation[114]. Furthermore, other proteins are also glycosylated on the hydroxyl groups of serine, threonine, hydroxylysine or hydroxyproline residues: an event known, instead, as O-linked glycosylation[115]. The consecutive actions of glucosidase I and glucosidase II rapidly trim the three glucose residues of asparagine-linked oligosaccharides producing the sugar Man9GlcNAc2. If the modified protein has yet to achieve its native conformation, the UDP-glucose glycoprotein glucosyltransferase adds again the terminal glucose enhancing the affinity of the unfolded polypeptide for the transmembrane lectin calnexin (CLX) and its soluble homolog calreticulin (CRT). CLX and CRT are calcium-dependent molecular chaperones that cooperate with multiple factors to prevent aggregation of the recognized chain, impair its export from the organelle, and facilitate its folding. While folding is incomplete, the protein shuttles between high and low-affinity states for the two lectins due to the continuous addition and removal of the terminal glucose. The cycle continues until the native configuration is achieved[116,117]. The action of the luminal chaperone BiP and its accessory proteins, such as the co-chaperone ERdj4 and the nucleotide exchange factor SIL1, also aids in protein folding[118–120]. Moreover, the slightly acidic environment of the ER favors the oxidation of adjacent thiolic groups on cysteines triggering the formation of disulfide bridges,

a phenomenon catalyzed by the thiol-disulfide oxidoreductases of the protein disulfide isomerase family bearing a thioredoxin-like domain[121]. Lastly, the precursor oligosaccharide can also be processed by two ER mannosidases. While the activity of mannosidase I is limited to the terminal mannose residues of the oligosaccharide B branch, the action of mannosidase II is confined to the residues of the C branch. This modification of the precursor oligosaccharide is applied to all glycoproteins exiting the ER but only glycoproteins persisting in the organelle for an excessive amount of time due to folding failure are significantly trimmed up until few mannose residues remain. The extensive trimming of mannose is one of the events leading to the activation of the ERAD response[113,122]. When accessory factors and modifying enzymes such as the SR, the signal peptidase complex, the OST, sugar-remodeling enzymes, and luminal chaperones are considered, the broader translocon holoenzyme is remarkably complex and contains over twenty integral membrane proteins[123,124].

3.5. The PD-1–PD-L1/PD-L2 Axis

The activity of the immune system relies on a fine balance between stimuli that promote the exertion of its protective functions against critical situations - including infections, inflammation, and tumorigenesis - and stimuli that reduce the intensity of the elicited response to prevent unnecessary damage and promote tolerance toward nontargets. Particularly, regulation of either the activation or the activity of T lymphocytes is of paramount importance to control how intense the adaptive response is and to reduce the risk of autoimmune reactions. Throughout the course of the previous decade, numerous costimulatory and coinhibitory pathways have been described, all intervening in the fundamental step driving the activation and action of T lymphocytes: the interaction between the T cell receptor (TCR) with major histocompatibility complex-restricted (MHC) peptides. The equilibrium between stimulatory and inhibitory signals is at the core of the molecular “decisions” influencing the fate of these lymphocytes. Diseases such as infections or tumors actively alter this equilibrium to guarantee prolonged persistence within the body[125]. Specifically, cancers progressively adapt to the local activation of the immune response, changing their phenotype to evade cytotoxicity and adverse reactions, a phenomenon described as adaptive immune resistance[126]. Targeting the upregulated proteins engaged in immunosuppression - also known as immune checkpoints - has risen as the standard treatment in multiple forms of cancer[127]. Interestingly, most immune checkpoint inhibitors approved by competent regulatory authorities target the PD-1–PD-L1/PD-L2 axis.

3.5.1. Overview of the Structure and Intracellular Signaling Mediated by PD-1

PD-1 - also known as programmed cell death protein 1 and CD279 - is a type I integral membrane receptor upregulated on the surface of T lymphocytes

upon activation, therefore following the stimulation of the TCR or ensuing the stimulation of cytokine receptors such as the tumor necrosis factor receptor[128]. Its expression has also been detected on the surface of double-negative lymphocytic precursors in the thymus, immature pancreatic macrophages, B lymphocytes, monocytes, and natural killer T cells[125]. PD-1 belongs to the CD28 superfamily, a subgroup of the larger immunoglobulin superfamily, featuring members that contain an immunoglobulin variable domain-like (IgV-like) domain[129]. Although PD-1 bears on the extracellular side an IgV-like domain connected to the transmembrane segment through a short chain, the receptor significantly differs from the other superfamily members. While both SH2- and SH3-binding motifs constitute the cytosolic tails of CD28 and CTLA-4, PD-1 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM)[125]. Phosphorylation of the ITSM enables the recruitment of the phosphatase SHP2, which exerts its action against the phosphorylated tyrosine residues of the first downstream mediators of TCR signaling: ZAP-70, Lck, and the CD3 ζ chain. Proximity to the TCR is required for the phosphatase to be effective since the phosphorylation of the ITSM is performed by the members of the Src tyrosine kinase family recruited to the cell membrane following the activation of the TCR[130–133]. Moreover, PD-1 impairs the phosphorylation of the phosphatidylinositol-3,4,5-triphosphate 3-phosphatase PTEN by inhibiting the kinase CK2, normally operative during lymphocytic activation[134,135]. Albeit less stable than the phosphorylated form, unphosphorylated PTEN is much more active, significantly weakening the activation of the PI3K/Akt pathway[136]. Lastly, PD-1 shuts down the MAPK/ERK pathway by interfering with the activity of both RasGRP1 and its upstream activator phospholipase C γ1[134,137](**Figure 3**). Collectively, the inhibition of these pathways impairs T cell activation, maturation, and proliferation in the thymus, limits the synthesis of pro-inflammatory cytokines including interleukins, tumor necrosis factor, interferon-γ, and blocks cytotoxicity[137,138]. The last function is of especially paramount importance for the prevention of autoimmune reactions, the promotion of self-tolerance, the homeostasis of immune-privileged organs such as the eye or the placenta, and fetal-maternal tolerance[128].

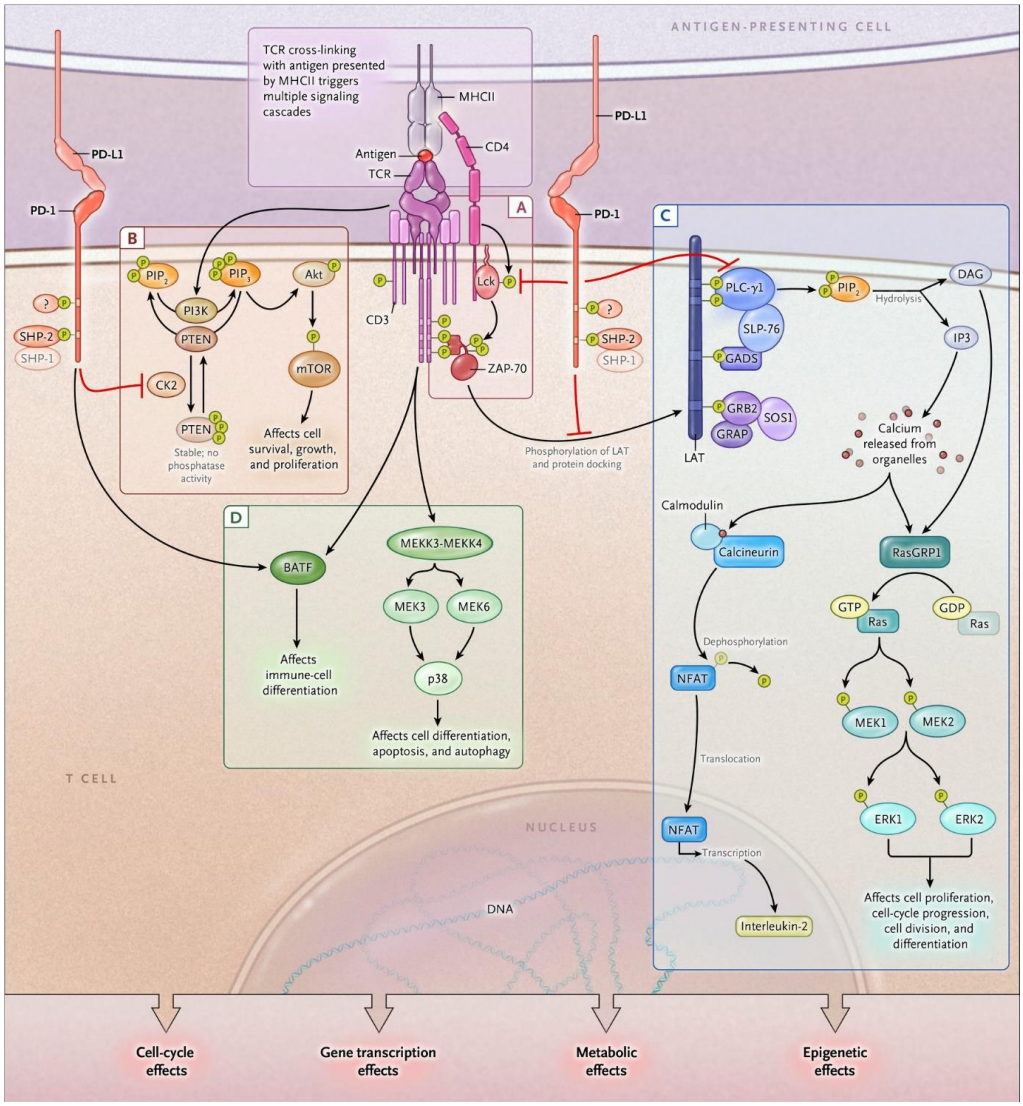

Figure 3. Role of the PD-1/PD-L1 Axis on T-Cell Receptor Signaling.

Contemporary activation of PD-1 and the TCR following its interaction with an antigen-loaded MHC triggers phosphorylation of the ITSM and the ITIM found on the cytoplasmic tail of PD-1. While the generated phosphotyrosine on the ITIM acts as a docking site for an unknown protein, phosphorylation of the ITSM leads to the recruitment of the phosphatase SHP2. SHP2 exerts its action against the phosphotyrosine residues of the first downstream mediators of TCR signaling: ZAP-70 and Lck (**Panel A**). The event results in absence of activation of the PI3K/Akt/mTOR pathway (**Panel B**) and of the Ras/MAPK pathway (**Panel C**). Regardless, other downstream signaling pathways, such as the p38 pathway, are not altered (**Panel D**)(from Boussiotis, V. A., 2016)[125].

3.5.2 Overview of the Structure, Function, and Expression of PD-L1 in Cancer

The abovementioned functions are exclusively exerted following the interaction of PD-1 with its ligands: PD-L1 and PD-L2. PD-L1 - also known as CD274 - is a type I integral membrane glycoprotein belonging to the B7 family, a subdivision of the immunoglobulin superfamily. PD-L1 bears two immunoglobulin-like domains on its extensive extracellular side: a domain approximating the variable portion of immunoglobulins in the portion further from the membrane and a domain approximating the constant portion of immunoglobulins in the portion closest to the membrane. The variable region-like domain is responsible for interacting with PD-1 thanks to a complementary determining region-resembling segment. Conversely, its cytosolic tail is short even though it contains two motifs - RMLDVEKC and DTSSK - that have been implicated in recent years in mediating pro-survival signaling[139,140]. PD-L1 has been detected in hematopoietic and nonhematopoietic cell types, including antigen-presenting cells, T lymphocytes, B lymphocytes, macrophages, and mastocytes, among the former, and epithelial cells, myocytes, astrocytes, and hepatocytes, among the latter[125,128,139]. On the contrary, the expression of PD-L2 - also known as CD273 - is restricted to macrophages and dendritic cells[128]. Moreover, multiple pathways activated by extrinsic or intrinsic stimuli converge on the modulation of the expression of PD-L1, which can be also induced in cell types distinct from the previously mentioned. Pathways that regulate the expression of PD-L1 include the MAPK/ERK, the PI3K/Akt, and the receptor tyrosine kinase pathways, which are often strengthened in cancer due to their role in proliferation, growth, and survival[125,141–146]. The tumor microenvironment rich in pro-inflammatory cytokines such as interleukins, TNF, and GM-CSF also favors the expression of the ligand not only on the surface of the cancer cells but also on the surface of the surrounding population including the infiltrated cells of the immune system[125,147]. Interleukin-6 and interferon-γ upregulate the ligand, for instance, by mediating the activation of the Janus kinase(JAK)/STAT pathway while expression of TGFβ in the tumor microenvironment has been associated with a decrease in patients' responsiveness toward PD-1/PD-L1 target therapy[142,148–150]. Finally, metabolic cues also instruct the cell to promote the expression of PD-L1. The most relevant are hypoxia through stabilization of the hypoxia-inducible factor, the accumulation of cyclic AMP, and high concentrations of oxidized nicotinamide adenine dinucleotide[133,151–153]. The upregulation of PD-L1 weakens the efficiency of the cytotoxic activities of the immune system fostering immunosuppression in the tumor microenvironment while allowing the cancer cells to thrive[125,126].

3.5.3 Post-Transcriptional Modulation of PD-L1 Expression

The mechanisms through which the abovementioned pathways upregulate the expression of PD-L1 on the surface of cancer cells do not exclusively involve transcriptional events such as the interaction of a transcriptional activator with its promoter or positive epigenetic modifications. PD-L1 is finely regulated at multiple levels, with both proteins and microRNAs modulating the stability of its messenger RNA and numerous PTMs applied to the protein. Both the 5' untranslated region and the 3' untranslated region of the PD-L1 messenger RNA are rich in elements regulating the balance between the stability of the transcript and its degradation[133]. The 5' untranslated region bears an upstream open reading frame which is preferentially translated under conditions that do not compromise the efficiency of protein synthesis[154]. The activation of stress-induced mechanisms, such as the unfolded protein response and the heat shock response, triggers the phosphorylation of the eukaryotic translation initiation factor subunit 1, reducing the global rate of protein synthesis. The chance that successful translation will begin at the level of the primary open reading frame is much higher under these conditions since recruitment of the required initiation proteins in an active form will fail at the level of the upstream open reading frame[155,156]. Meanwhile, the 3' untranslated region contains multiple elements recognized by either microRNAs or RNA-binding proteins. Among the former, members of the miR-200 family promote the degradation of PD-L1 messenger RNA and have been identified as downregulated in distinct forms of cancer[157–159]. Notably, long non-coding RNAs such as MALAT1 function as competing endogenous RNAs, subtracting microRNAs targeting PD-L1 transcript from their interaction with its 3' untranslated region[160–163]. Conversely, RNA-binding proteins can either stabilize or destabilize the messenger RNA, with most competing for the binding to AU-rich elements found within the untranslated region[164,165].

3.5.4. Post-Translational Modulation of PD-L1 Expression

PD-L1 is a target of multiple PTMs, including glycosylation, phosphorylation, ubiquitylation, acetylation, and palmitoylation, each affecting its stability, localization, and function[133]. The amino acid sequence of PD-L1 contains four glycosylation consensus sequences enabling its asparagine-linked glycosylation at four residues: N35, N192, N200, and N219[166]. The precursor oligosaccharide attached to the asparagine residues in positions 192 and 200 is modified in the Golgi apparatus with the addition of poly-N-acetyllactosamine (poly-LacNAc) by the enzyme β-1,3-N-acetylglucosaminyltransferase 3. The addition of poly-LacNAc is required for the function of the transmembrane protein since its removal prevents the interaction with PD-1[167]. Moreover, glycosylation at these sites impairs the phosphorylation of T180 and S184 by the β isoform of glycogen synthase kinase 3 (GSK3β), a modification required for the ubiquitylation and subsequent proteasomal degradation of PD-L1.

Glycosylation at N219 has the same protective effect. Phosphorylation on the threonine and the serine is driven by a GSK3β phosphorylation motif - SXXXTXXXS - which begins at S176 and ends at S184. The event is propaedeutic for recruiting a ubiquitin ligase complex whose substrate-binding component is the β-transducin repeat-containing protein. The α isoform of GSK3 (GSK3α) also triggers a similar cascade of events with its phosphorylation of S279 and S283 promoting the recruitment of the E3 ubiquitin-protein ligase ariadne-1 homolog[168]. Phosphorylation of serine and/or threonine residues also occurs at S195/S283 and T194/T210, reactions performed by the AMP-activated protein kinase (AMPK) and by NEK2, respectively. The activity of AMPK results in the generation of a mannose-rich, aberrantly glycosylated protein. The resulting glycoprotein accumulates in the lumen of the ER, eventually activating the ERAD response. Phosphorylated S195 could induce conformational changes that enhance the affinity of PD-L1 for the components of the ERAD system, block the interaction with the export machinery, or promote association with the resident mannosidases reducing the time within which the protein must fold before being targeted for degradation[133,169]. Furthermore, phosphorylation of S283 by AMPK disrupts the interaction of PD-L1 with the ubiquitously expressed plasma membrane CMTM6 protein[170]. CMTM6 prolongs the half-life of the immune checkpoint molecule by favoring, following endocytosis, recycling to the plasma membrane[171]. Impairment of the interaction between PD-L1 and CMTM6 allows the E3 ubiquitin-protein ligase CHIP to polyubiquitinate the former, targeting it for lysosomal degradation[172]. Conversely, the action of NEK2 on T194 and T210 promotes glycosylation of PD-L1 at N194, N200, and N219, preventing proteasomal degradation[173]. Phosphorylation of the tyrosine residue Y122 by JAK1 also stimulates glycosylation by enhancing the affinity of PD-L1 for the catalytic subunit of the OST complex STT3A[174]. Glycosylation of PD-L1 also depends on the contribution of co-chaperones such as FKBP51s. FKBP51s is a splicing isoform of FKBP51, an immunophilin with peptidyl-prolyl cis/trans isomerase activity[172]. Lastly, acetylation and palmitoylation events have also been described for PD-L1, both with a protective and stabilizing role. While palmitoylation occurs at the cysteine residue C272 thanks to the activity of the palmitoyltransferase ZDHHC3, acetylation occurs at the lysine residue K263[175–177]. Histone acetyltransferase p300 is the enzyme responsible for the modification, while histone deacetylase 2 (HDAC2) mediates its removal. Acetylated K263 impairs the interaction of PD-L1 with HIP1R, resulting in more remarkable recycling of the immune checkpoint molecule to the plasma membrane. HIP1R would direct, otherwise, PD-L1 to the lysosome since the protein contains a lysosomal targeting signal(**Figure 4**)[177,178].

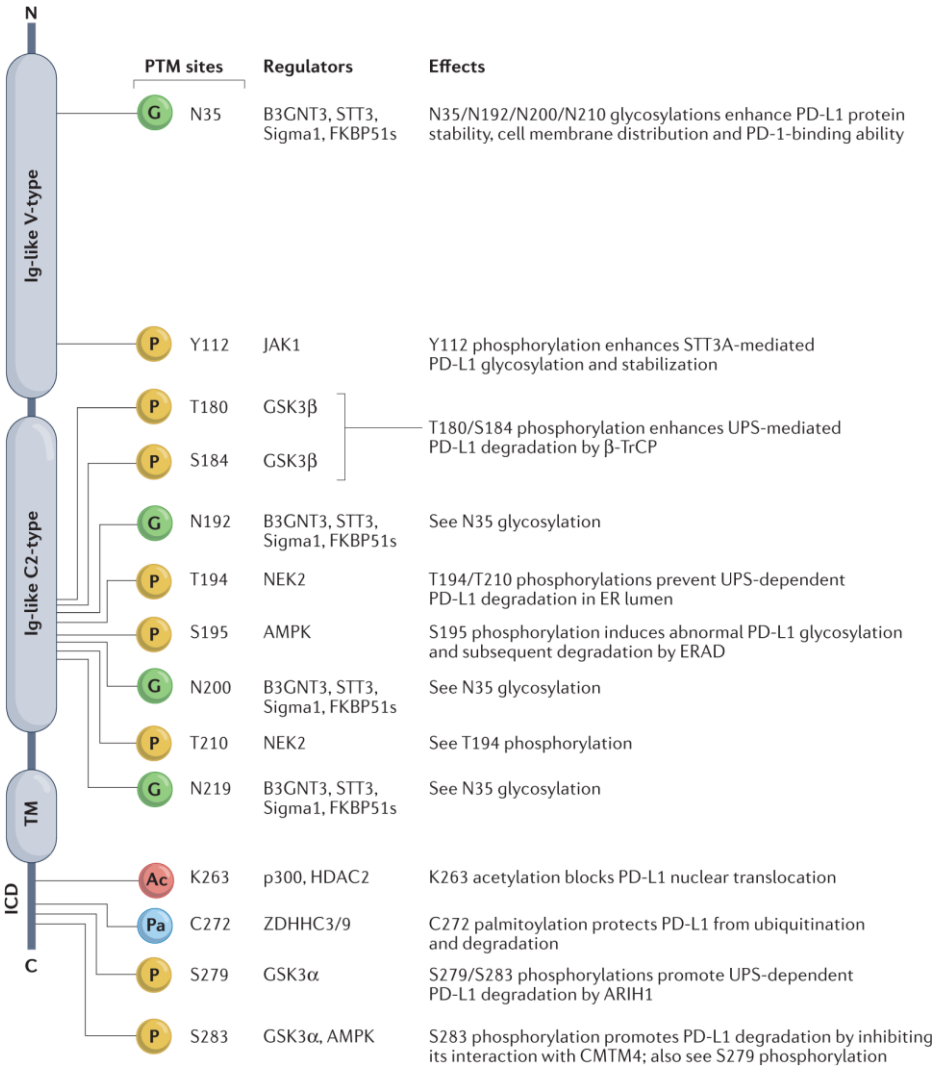

Figure 4. Post-Translational Modifications Modulating PD-L1.

Representation of the topology of PD-L1 and the PTMs modulating its intracellular fate. PD-L1 is a type I integral membrane protein bearing a short intracellular domain at its carboxy-terminus, a hydrophobic transmembrane segment, and an extensive extracellular region at its amino-terminus containing two immunoglobulin-like domains. Each PTM has been reported indicating the affected residue, proteins responsible for its application and/or removal, and effects exerted on the fate of PD-L1. Ac, acetylation; G, N-linked glycosylation; P, phosphorylation; Pa, palmitoylation (Extracted from Yamaguchi et al., 2022)[133].

4. Aims of the Thesis

Among the previously mentioned post-translational modifications, most phosphorylation events involve amino acids that reside in the extracellular portion of PD-L1 once its correct topology has been achieved. Notably, the research groups investigating the action of the kinases NEK2 and AMPK on PD-L1 suggest that phosphorylation of T194, T210 and S195 occurs inside the lumen of the endoplasmic reticulum. However, this statement contradicts the characterized localization of NEK2 and AMPK and the other kinases acting on the extracellular residues, such as JAK1 which, by lacking a classical amino-terminal translocation-specifying signal sequence, reside in the cytoplasm. As such, phosphorylation could occur before translocation is completed but when the target amino acids are still available for interaction with the various kinases. Therefore, a temporal gap should exist between the beginning of translation and the recognition of the emerging signal peptide by the SRP since the latter pauses protein synthesis. The gap should be long enough to synthesise the target residues, and their exposure to the cytosolic environment before interaction with the SRP occurs. Physiologically, a signal peptide with a lower affinity for the SRP than a model signal sequence - therefore less optimal in driving the initial recognition event due to slower interaction kinetics - could generate the temporary uncoupling between its exposure and its targeting to the endoplasmic reticulum membrane. Interestingly, PD-L1 signal peptide is slightly divergent from the properties of the tripartite structure discussed before. Its h-region contains amino acids with encumbering side chains - such as tyrosine, tryptophan, and phenylalanine - instead of valine and/or leucine repeats commonly patterning the core region of several classical signal peptides. Moreover, its c-region bears a net positive charge, significantly different from the either neutral or negatively charged c-region of a model signal peptide. Its divergent properties could result in lower efficiency in recognition of the SRP and, therefore, in the targeting of the emerging peptide to the membrane after the modifiable residues have emerged from the ribosome. Although numerous assays allow the researcher to analyse the kinetics driving the interaction between a signal peptide and the SRP, these assays do not allow the assessment of the proposed temporal gap. Here, we developed a novel derivative assay with the main aim of determining whether the suggested hypothesis stands and highlighting how the sequence and the properties of the signal peptide itself are the bearers of the temporal information.

5. Materials and methods

Reagents
All the culture reagents were obtained from Sigma-Aldrich (Milan, Italy). The solid chemical and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochemica (Heidelberg, Germany), Delchimica (Naples, Italy) and BDH (Poole, United Kingdom). The enhanced chemiluminescence reagents were from Biorad (Hercules, California, USA). Metformin, Compound C, MG132 and Cycloheximide were purchased from Sigma-Aldrich, Milan, Italy.

Cell culture and transfection experiments
GB138 cells, established in 2011 from a resected adult GBM sample, were kindly provided by Rogister[179]. GB138 cells and HeLa cells were routinely grown at 37°C in Dulbecco's modified essential medium (DMEM), containing 10% fetal bovine serum (FBS), 100 U/ml Penicillin/Streptomycin, 2 mM L-Glutamine (L-Gln). GB138 cells stably expressing the SPn- and SPopt-PD-L1-HA protein were grown in the same medium, supplemented with 400 μg/ml G418 (Gibco). Cells were transfected by using X-tremeGENE HP DNA transfection reagent (Merck) according to the manufacturer's instructions, or calcium phosphate method: briefly, plasmidic expression vectors were resuspended in a solution containing Tris 1mM pH 8.0 and $CaCl_2$ 0.25M, mixed with Hepes-buffered saline solution (Thermofischer) and eventually dispensed to cells drop by drop.

Antibodies
The following antibodies were used: horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (ImmunoReagents Inc); alexa Fluor 488-conjugated anti-mouse and anti-rabbit IgG (Invitrogen), alexa fluor 546-conjugated anti-mouse and anti-rabbit IgG (Invitrogen); mouse monoclonal and rabbit polyclonal anti-HA antibody (Sigma-Aldrich); rabbit polyclonal anti-CD274/PD-L1 antibody (Novus Biological); rabbit polyclonal anti-GFP antibody (AbCam); Rabbit polyclonal anti-Calnexin (Sigma-Aldrich); mouse monoclonal anti-actin (Sigma-Aldrich); rabbit anti-GM130 (Cell Signaling); rabbit anti-KDEL receptor (Enzo Life Sciences); Human monoclonal anti-PD-L1 was kindly provided by Prof. Claudia De Lorenzo[184], rabbit anti-streptavidin was kindly provided by Dr. Franck Perez.

Constructs, cDNA cloning, and plasmid construction
PD-L1-turboGFP (Origene) was used as template for generating PD-L1-HA constructs. SPn-PD-L1-HA was generated by PCR using the following couple of oligos:

Fw (EcoRI): 5'-CGGAATTCCCACCATGAGGATATTTGCTGTCTTTATA
TTC-3'

Rv (XbaI): 5'-GCTCTAGATTAAGCGTAATCTGGAACATCGTATGGGT ATCCTCCTCCCCGTCTCCTCCAAATGTGTATCACTTTGC-3'.

SPopt-PD-L1-HA was generated by PCR using the following couple of oligos:

Fw (EcoRI): 5'-CGGAATTCATGAGGATATTTGCTGTCTTTATATTCAT GACCCTGTGGCTGTTGCTGAACGCATTTACTGTCACGGTTCCCAAGG -3'

Rv (XbaI): 5'-GCTCTAGATTAAGCGTAATCTGGAACATCGTATGGGTA TCCTCCTCCCCGTCTCCTCCAAATGTGTATCACTTTGC-3'.

SP and HA tag were included into the oligos sequences.

SPn-SBP-EGFP-PDL1, SPopt-SBP-EGFP-PDL1, cytosolic streptavidin, TurboID-V5 expression vectors were kindly provided by Dr. Franck Perez. SPn-SBP-EGFP-KDEL and SPopt-SBP-EGFP-KDEL expression vectors were purchased from Genescript.

CytoRUSH assay
CytoRUSH assay was developed from the original RUSH assay from Franck Perez lab. HeLa cells were grown in 60mm dishes for 24h and transfected with plasmidic expression vectors encoding cytosolic streptavidin and SBP-bearing reporter proteins, with a 1:1 ratio. After 6h, cells were trypsinized and equally distributed in 24-multiwell. After 16h, cells were treated with Biotin (Sigma-Aldrich) 40µM and then lysed and processed by Western Blot analysis.

Nascent chain-biotinylation assay
HeLa cells were grown in 35mm dishes and transiently transfected with plasmidic expression vectors encoding cytosolic TurboID-V5 and SPn- or SPopt- SBP-EGFP-KDEL, with 1:1 ratio. After 24h, cells were treated with biotin (Sigma-Aldrich) 150µM for 10h, lysed, and protein extracts were incubated with streptavidin-coated magnetic beads (Sigma-Aldrich) overnight. After that, magnetic beads were extensively washed with lysis buffer, resuspended in Laemmli buffer, boiled at 95°C for 5 minutes and samples were analysed by SDS-PAGE.

Immunofluorescence and confocal microscopy
Cells were grown on coverslip for 24 hours before fixation in formaldehyde 3,7% dissolved in phosphate buffer (PBS) for 30 minutes at room temperature. Fixation was blocked by incubating cells with ice-cold PBS containing glycine 0.1M for 5 minutes and then washed three times with only PBS buffer. Next, cells were permeabilized with a blocking buffer containing saponin as non-ionic detergent (PBS, BSA 1%, Saponin 0.05%, and sodium azide 0.01%) for 15

minutes at room temperature. After permeabilization cells were incubated with primary antibodies diluted in blocking buffer for 1 hour, washed in PBS for 5 minutes each, and incubated with secondary antibodies diluted in blocking buffer for 45 minutes. Finally, cells were washed three times with PBS and one time in deionized distilled water before mounting on slide. Confocal images were acquired at 63x magnification on a LSM700 (Carl Zeiss, Jena, Germany) and SP8 (Leica, Germany). Co-localization and total level of fluorescence were measured by using ImageJ Biophotonics and Fiji software by measuring 50 cells for each experimental point.

Preparation of cell extracts, immunoprecipitation SDS-PAGE and western immunoblotting

Total protein cell extracts were performed in a buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, and 1% Triton-X-100) supplemented with protease and phosphatase inhibitors. Protein concentration was measured by the Bradford assay. Proteins were separated on SDS gels and transferred to PVDF membranes. For EndoH assay, lysates were mixed with EndoH buffer and boiled at 95°C for 5 minutes, then incubated with EndoH for 1h at 37°C before separation by SDS-PAGE. EndoH buffer and EndoH were kindly provided by Prof. De Matteis. Membranes were then treated with a blocking buffer containing 5% non-fat powdered milk dissolved in PBS and incubated overnight with primary antibodies. Membranes were finally incubated with an HRP-conjugated secondary antibody, and chemiluminescence was determined using the ECL detection system. Densitometric analysis was performed using the Fiji software.

Quantification and Statistical Analysis

All the results are given as mean ± s.d. obtained by three independent experiments. Statistical analysis was performed by Student's t-test and one-way ANOVA. p-Values are shown as asterisks: *** for $p<0.001$, ** for $p<0.01$, * for $p<0.05$ and ns when data were not statistically significant,

9. List of publications

Scerra G, Serio MG, Bonavita R, Caporaso MG, Romano S, Romano MF, Renna M, Perez F, D'Agostino M. A suboptimal signal peptide defines spatiotemporal control of PD-L1 expression. Manuscript in preparation.

Bonavita R, **Scerra G**, Di Martino R, di Gennaro M, Caporaso MG, D'Agostino M, Renna M. A method for the analysis by isopycnic ultracentrifugation of the oligomerization profile of the HD-associated, aggregation-prone mutant huntingtin protein. Manuscript in preparation.

Bonavita R, **Scerra G**, Di Martino R, Di Gennaro M, Williams S, Caporaso MG, Caiazza C, D'Agostino M, Fleming A, Renna M. The HSPB1- p62/SQSTM1 functional complex regulates the unconventional secretion and transcellular spreading of the HD-associated mutant huntingtin protein. Manuscript submitted for publication.

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