A few good peptides: MHC class I-based cancer immunosurveillance and immunoevasion

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Abstract | The remarkable success of immune checkpoint inhibitors demonstrates the potential of tumour-specific CD8$^+$ T cells to prevent and treat cancer. Although the number of lives saved by immunotherapy mounts, only a relatively small fraction of patients are cured. Here, we review two of the factors that limit the application of CD8$^+$ T cell immunotherapies: difficulties in identifying tumour-specific peptides presented by MHC class I molecules and the ability of tumour cells to impair antigen presentation as they evolve under T cell selection. We describe recent advances in understanding how peptides are generated from non-canonical translation of defective ribosomal products, relate this to the dysregulated translation that is a feature of carcinogenesis and propose dysregulated translation as an important new source of tumour-specific peptides. We discuss how the synthesis and function of components of the antigen-processing and presentation pathway, including the recently described immunoribosome, are manipulated by tumours for immunoevasion and point to common druggable targets that may enhance immunotherapy.

Although our lifetime risk of cancer is approximately 40%, it is perhaps surprising that it is not higher. The 10^{13} nucleated cells in our body replicate approximately 3×10^9 base pairs per cell division with an intrinsic mutation rate of approximately $10^{-4.5}$ per base pair, with additional mutations generated from the daily barrage of chemical carcinogens and radiation. DNA quality control pathways repair much of the damage, but it is increasingly clear that the immune system plays an important role in limiting oncogenesis — the concept of immunosurveillance. Indeed, tumours evolve myriad mechanisms to evade immunity, a process termed immunoediting[1].

Boon et al.[2] were the first to define the molecular nature of cancer immunosurveillance by showing that CD8$^+$ T cell tolerance for self-peptides can be broken by cancer cell mutations that create amino acid substitutions, rendering peptides immunogenic. Over the next decade, work from a rapidly expanding number of laboratories established that cancer-specific peptides arise by numerous mechanisms and, further, that the immune system plays a vital role in controlling oncogenesis.

The discovery of immune checkpoint molecules such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) that limit T cell activation and function led to the development of immune checkpoint inhibitors, which have demonstrated the enormous potential of cellular immunity to eradicate human cancers[3]. However, the resistance of most cancers to checkpoint inhibitors and other immunotherapies underscores the need to better understand both the potential antigenic targets of cancer-specific T cells and the mechanisms that cancer cells exploit for immunoevasion. Defining how cancer-specific antigenic peptides are generated will improve predictive algorithms for peptide-based vaccines, provide insights leading to drugs that enhance the generation of cancer peptides and improve classifications of those patients who will benefit from immunotherapy. Better understanding of tumour immunoevasion will guide therapy choices and identify targets for enhancing T cell recognition of tumours.

Tumour immunosurveillance is an intricate symphony, composed of tumour immunogenicity and immunoevasion, immune cell infiltration, T cell checkpoints, productive T cell priming and the tumour microenvironment. Here, we focus on recent advances in the fields of peptide-processing and presentation and on how research in these areas has been applied to 'tumour cell-intrinsic' cancer immunity. Although we focus this Review on CD8$^+$ T cell-mediated recognition of cancer, it is important to recognize that CD4$^+$ T cells also have tremendous potential for immunotherapy[4–6]. Like CD8$^+$ T cells, CD4$^+$ T cells can directly kill tumours[7], even those derived from non-immune cells, as MHC class II molecules are readily induced by interferon-γ (IFNγ) and other cytokines released by CD8$^+$ T cells and natural killer cells. Moreover, tumours often require resident macrophages and other MHC class II-expressing cells to maintain the tumour microenvironment as stromal elements; their

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Law of mass action
The principle that processes are proportional to the concentrations of the participants.

elimination by the concerted efforts of $CD8^+$ and $CD4^+$ T cells can be decisive in tumour eradication[8].

Generating MHC class I peptides

Tumour immunosurveillance entails $CD8^+$ T cell recognition of tumour cells bearing MHC class I molecules with peptides from proteins synthesized by the malignant cell. The repertoire of peptides presented by MHC class I molecules in a given set of cells is termed the immunopeptidome[9] (BOX 1). MHC class I genes are often the most polymorphic genes in a jawed vertebrate species. As HLA-A, HLA-B and HLA-C molecules exhibit highly overlapping functions, the alleles at these loci are collectively referred to as allomorphs. Thousands of functionally distinct allomorphs are present in human populations at significant frequencies. Each allomorph binds a distinct repertoire of peptides, whose overlap with other allomorph repertoires varies considerably depending on the similarities of the peptide-binding grooves.

DRiPs, SLiPs and retirees. Peptides derive from the degradation of 'retirees' (proteins that have reached their natural lifespan), defective ribosomal products (DRiPs; defective forms of proteins that do not achieve functional integration into the proteome[10]) or short-lived proteins (SLiPs; which represent a grey-area subcategory of highly regulated retirees and DRiPs) (FIG. 1). Many SLiPs are intrinsically disordered proteins — representing ~30% of annotated proteins — that are degraded in the absence of a stabilizing binding partner. Whereas retirees have half-lives of ~46 h across the proteome, DRiPs and SLiPs (which together constitute up to 30% of nascent proteins[10–12]) are degraded with half-lives of minutes.

Box 1 | **There is no place like 'ome**

The immunopeptidome, defined as the repertoire of all peptides presented by MHC class I molecules in a given set of cells, can be determined empirically by mass spectrometry or computationally[161] by applying algorithms for peptide binding to the set of MHC allomorphs expressed by the cell. Peptide binding is based on its interaction with the binding groove in MHC class I molecules. Much of the free energy of binding comes from the interaction of peptide residues with pockets in the base of the groove and with the peptide N termini and C termini with the ends of the groove. This latter property limits the length of high-affinity peptides largely to 8–11 residues. Whereas longer peptides can bind and be presented on the cell surface, their lower affinity results in rapid elution from cells and under-representation in the immunopeptidome, although they still may be biologically relevant. For common human and mouse allomorphs, peptide binding predictions achieve ~90% accuracy and are constantly improving as ever-increasing empirical data of MHC class I-bound peptides and affinity measurement of individual peptides are fed into evolving machine learning algorithms[160,162].

Defining the immunopeptidome by mass spectrometry of peptides released from MHC class I molecules can be achieved by acid treatment of cells, which denatures cell surface MHC class I molecules, releasing bound peptides. Although simple, many irrelevant non-MHC class I-bound peptides are also released. This problem is avoided by purifying MHC class I molecules with a monoclonal antibody that binds nearly all MHC class I molecules (for HLA molecules, typically W6/32 antibody, which binds the vast majority of classical and non-classical MHC class I molecules). Owing to the vagaries of matching mass spectrometry-determined masses with genetically encoded peptides, the accuracy of peptide determination is greatly increased by minimizing the search pool of potential peptides of interest. Typically, data pipelines query the transcriptome as defined by mRNA or total RNA sequencing to generate the inferred translatome. In principle, the translatome defined by ribosome profiling (Ribo-Seq) provides the minimal relevant pool of translated peptides, and is therefore optimal.

The DRiP hypothesis was advanced to explain the extremely rapid generation of viral peptides from metabolically stable viral proteins[13]. The close relationship between the synthesis of viral proteins and antigenic peptide generation has been confirmed in numerous studies[14]. Most definitively, mass spectrometry reveals that for dozens of either poxvirus or influenza A virus MHC class I-presented peptides, nearly all are generated co-translationally with the synthesis of their ostensible source proteins, indicative of their DRiP origins[15,16]. SILAC (stable isotope labelling with amino acids in cell culture) mass spectrometry kinetic analysis of peptide generation in several different tumour cell lines is also consistent with a substantial contribution of DRiPs to the immunopeptidome[17,18], with many DRiP-derived peptides originating from rapidly degraded subunits of multisubunit complexes[17] generated in super-stoichiometric amounts[19]. Applying this approach to additional normal and tumour cells in vitro and even in vivo (which will require technological advances) is critical to better understand the contributions of DRiPs, SLiPs and retirees to the healthy and disease state immunopeptidome.

The mysteries of generating antigenic peptides. Contrary to typical textbook descriptions, a substantial fraction of peptides are generated independently of ubiquitylation[20] or even proteasomal cleavage[21], due perhaps to their synthesis as short translation products that can be processed by non-proteasomal proteases. Aminopeptidases are highly active in the cytoplasm of most cells, but carboxypeptidase activity is low to non-detectable, raising the question of how C termini are generated independently of proteasomes. Although alternative endopeptidases have been proposed to substitute for the proteasome, the available evidence does not support their routine participation in peptide generation[22]. For largely unknown reasons, the requirement for active proteasomes[23,24] and ubiquitylation[20] in generating MHC class I peptides varies widely among different MHC class I allomorphs, as do the contributions of transporter associated with antigen processing (TAP) and tapasin (and likely TAP-binding protein related (TAPBPR) as well[25]) in sculpting the peptide repertoire[26,27]. Thus, in addition to broadening the immunopeptidome, MHC class I allomorphs diverge to exploit differences in peptide generation and MHC class I biogenesis, presumably to optimize peptide presentation for immunosurveillance within the individual and across the species. Locus-specific functional differences in allomorphs must contribute to the unbalanced expression of HLA-A and HLA-B molecules among different tissues and tumours.

MHC class I molecules that predominantly bind hydrophobic peptides (for example, HLA-A2) are adept at presenting peptides derived from endoplasmic reticulum-targeting leader sequences. Such peptides are selectively presented in cells with compromised TAP function, which is relatively common in cancer cells, and are promising targets for $CD8^+$ T cell immunotherapy[28].

Another mysterious feature of MHC class I peptide generation is its frequently apparent failure to adhere to the law of mass action, apparent from numerous findings[29–35].

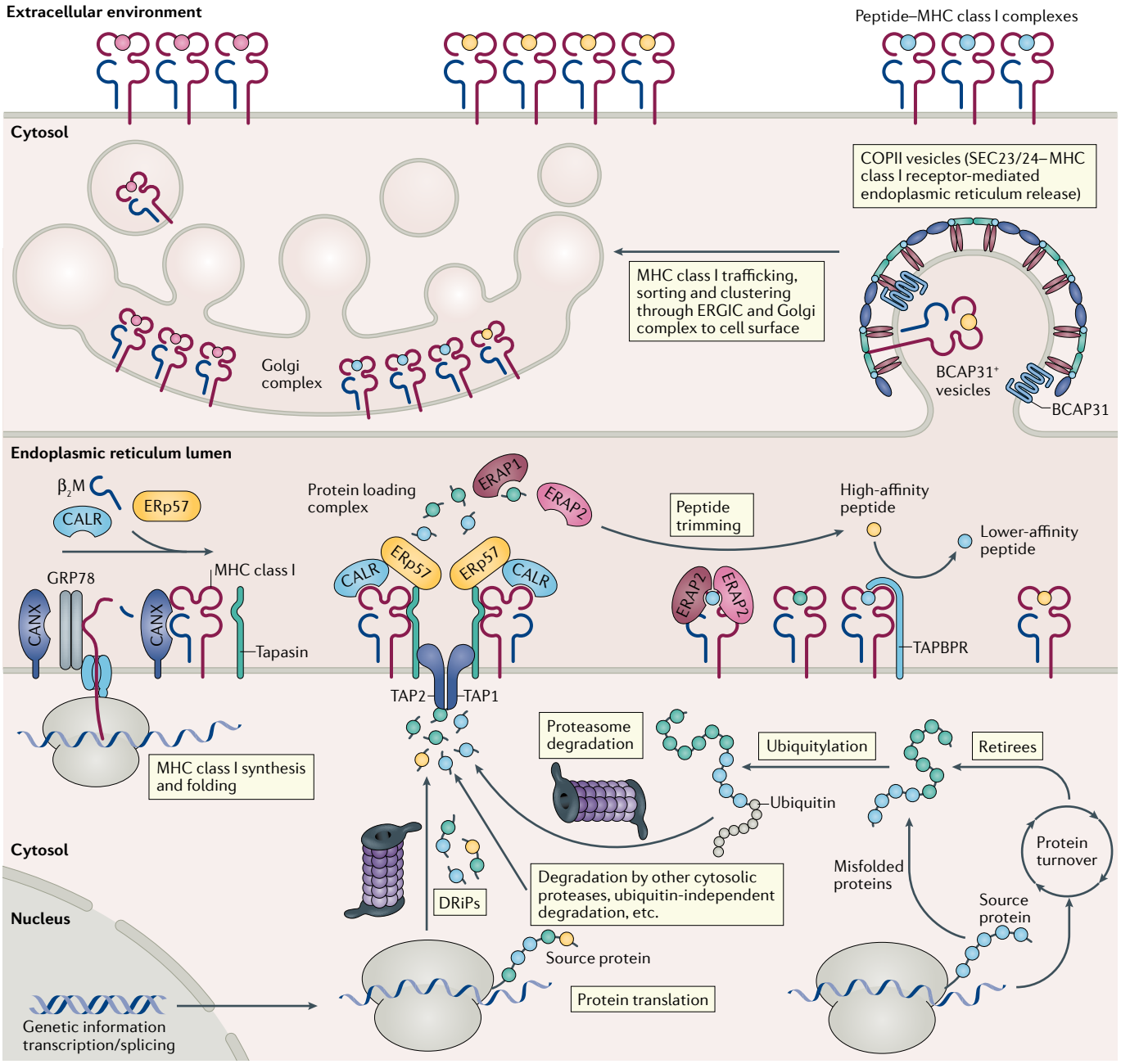


Fig. 1 | **Overview of MHC class I biogenesis antigen-processing and presentation machinery.** Peptides are generated from the degradation of source proteins, which can be categorized as 'retirees' (natural turnover of functional proteins) or defective ribosomal products (DRiPs; rapidly degraded polypeptides not integrated into the proteome). Degradation is mediated by proteasomes via ubiquitin-dependent and ubiquitin-independent targeting and by non-proteasomal proteases. Peptides that escape destruction in the cytosol can be imported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Trimming of peptide N termini occurs via the activities of endoplasmic reticulum aminopeptidase 1 (ERAP1) and ERAP2, and the peptide loading complex (comprising ERp57 and calreticulin (CALR)) assists in the loading and folding of MHC class I molecules with both peptide and β_2-microglobulin (β_2M). Folded MHC class I complexed with a high-affinity peptide is trafficked from the endoplasmic reticulum with the chaperone B cell receptor-associated protein 31 (BCAP31)[157,158] as an additional quality control step[159]. There is evidence that MHC class I molecules are clustered[160] based on their bound peptides[32,35], perhaps owing to localized peptide generation from individual mRNAs. Such clustering, which is detectable in the Golgi complex and cell surface, increases T cell sensitivity. CANX, calnexin; COPII, component of the coat protein complex II; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; TAPBPR, TAP-binding protein related.

Phase separation
The creation of distinct compartments from a homogeneous mixture.

For example, why is there preferential presentation of peptides from genetic hotspots despite high-affinity peptides existing randomly throughout the proteome[36]? 'Breaking' the law of mass action can only result from physically separating seemingly interacting components. Channelling, the physical confinement of enzyme substrates and products, is known to increase the efficiency of many cellular processes, including translation[37] and proteasome-mediated degradation[38,39]. One of the most exciting areas in cell biology is the discovery of numerous self-organizing, highly dynamic membrane-free domains in the cell based on bulk phase separation of

the seeding structural molecules[40]. The concept of phase separation has recently been extended to the ubiquitin–proteasome system[41] and has led to the hypothesis that MHC class I antigen processing may exploit channelling to optimize $CD8^+$ T cell immunosurveillance[42].

Non-canonical translation: a major source of DRiPs. Channelling may contribute to the preference in presenting peptides generated from non-canonically translated DRiPs (FIG. 2d). DRiPs can arise from translation initiation at CUG and other near-cognate start codons (codons with a single nucleotide change from AUG) using eukaryotic translation initiation factor 2A (EIF2A) in place of the canonical EIF2α–GTP–Met-$tRNAi_{Met}$ complex[43]. Such translation preferentially generates peptides from 3′ and 5′ untranslated regions (UTRs) of mRNA as well as alternative reading frames in annotated coding regions[44,45]. Non-canonical initiation is enhanced by viral infections and other stressors[44–46]. Peptides are also derived from intranuclear translation of introns and exons in pre-spliced mRNA[47–50] and even from 'forbidden' translation of negative strand influenza virus RNA[51].

Although non-canonical translation appears to be a major source of antigenic peptides, it constitutes a small fraction of total cellular translation. This highlights a key feature of immunosurveillance; the immunopeptidome poorly reflects either the transcriptome or the proteome[36,42,52,53]. This selectivity is an essential feature of antigen presentation; if all things were equal, peptides from a small fraction of gene products would overwhelm the antigen-processing and presentation (APP) capacity of cells, as a few dozen highly abundant proteins (histones, cytoskeletal elements, translation components and molecular chaperones) account for one-third of the proteome[54,55].

The skewing of the immunopeptidome away from abundant gene products is almost certainly underestimated by the current practice of assigning peptide masses detected by mass spectrometry to only genomic sequences present in annotated proteome or exome databases. A substantial fraction of peptides that cannot be assigned to annotated sequences[56] probably derive from introns, 3′ and 5′ UTRs, long and short non-coding RNAs, non-annotated splice variants of annotated mRNAs, frameshifts of annotated genes and proteasome-mediated peptide splicing (discussed further below). Many of these translation products are likely to be short, unstructured, non-functional DRiPs that are efficient sources of antigenic peptides and expand the genetic 'surveillance space' of the immune system.

Ribosome profiling defines the translatome. A key method for relating the translatome to the immunopeptidome is ribosome profiling (Ribo-Seq)[57,58]. Ribo-Seq is based on the deep sequencing of fragments of mRNA protected from ribonuclease digestion by their location within the ribosome decoding site. Protected fragments are contextualized using one or more sequence databases (transcriptome, exome or genome). Combined with the use of translation initiation inhibitors to identify initiation codons and measure ribosome transit times, Ribo-Seq provides quantitative information on how much a given mRNA is translated, which reading frames are translated, the start and termination codons used and the speed of translation over the length of the mRNA.

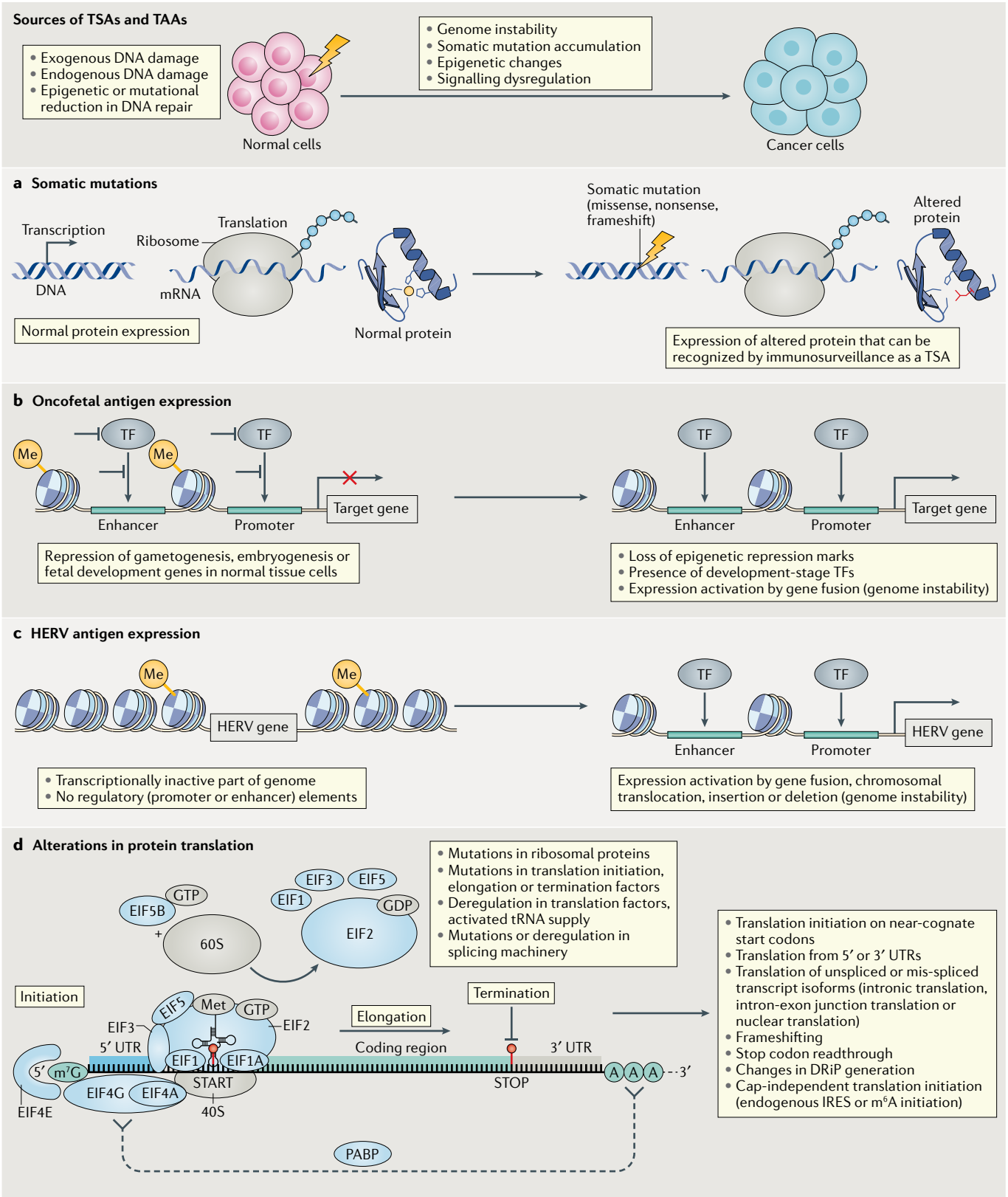
Ribo-Seq has rewritten the rules of translation[58]. Ribosome transit speed along a strand of mRNA is modulated by codon usage and mRNA structural elements to facilitate the folding of nascent protein chains as they emerge from the ribosome[59]. For any given mRNA, ~50% of translation initiation occurs at CUG or other near-cognate codons[60,61]. Highly translated mRNAs use AUG initiation codons more frequently, but the fraction of non-AUG-initiated translation is still ~25%. Non-AUG initiation reaches ~75% for the many 5′ UTRs that are, in fact, translated[61,62].

Translation of 3′ UTRs is less common than 5′ UTR translation, as 3′ UTR translation likely entails stop codon read-through (whereas 5′ UTRs are in the path of scanning 40S subunits recruited by capped mRNA), amounting to <1% of total translation[63,64]. This can, however, be considerably higher for viral transcripts[65] or stop codons generated by mutations in open reading frames (ORFs)[66]. Despite the low frequency, translation of 3′ UTRs may still represent a significant source of antigenic peptides, particularly because such translation may largely result from DRiP-generating errors[67]. In principle, Ribo-Seq can also determine the frequency of frameshift translation of standard genes, but this is difficult in practice. Still, this is estimated to account for 20% of the translation of any given mRNA[57].

Although Ribo-Seq is expensive and technically challenging, its power is evident from several recent studies. Ribo-Seq of tissue from healthy and diseased human hearts (as well as livers and kidneys) demonstrated the critical contribution of translational control to myocardial gene expression, revealed numerous instances of read-through of what would otherwise be disease-causing premature stop codons and revealed translation of hundreds of novel microproteins, including a number from long (ostensibly) non-coding RNAs[68]. Ribo-Seq has also been used to annotate thousands of non-canonical ORFs for genome-wide CRISPR screening that revealed the existence and essential roles for hundreds of novel ORFs in cell viability, with unique localizations and binding partners clearly identified[69]. Finally, Ribo-Seq was used to generate a translatome data set for assigning masses to MHC class I-derived peptides[70]. This revealed a substantial fraction of peptides derived from non-canonical translation of genes that are not present in standard databases used in most mass spectrometry studies.

The cancer immunopeptidome

The major challenge in creating therapeutic $CD8^+$ T cell-based cancer vaccines and adoptive T cell therapies is identifying MHC class I-binding tumour-specific antigens (TSAs) that escape T cell self-tolerance and generate T cells that are cancer specific. Mutations are common in most tumours, and current approaches are focused on predicting in silico 'neoantigens' generated by mutations in annotated genes that create tolerance-breaking TSAs with increased affinity for



either MHC class I molecules or T cell receptors (FIG. 2). The limited success of this approach has spawned the search for TSAs derived from mRNA splice variants, genetic fusion events and expression of non-tolerized retroelements (reviewed in REF.[71]).

Non-canonically translated tumour peptides. Given the importance of non-canonical translation in generating MHC class I binding peptides, the dysregulated translation that is a central feature of carcinogenesis[72–75] provides a potentially rich source of TSAs. Cancers cells

◀ Fig. 2 | **Potential sources of tumour antigens as targets for immunosurveillance and targeted cell immunotherapy.** Tumour-specific T cells recognize peptides exclusively presented by tumour cells (tumour-specific antigens (TSAs)) or peptides expressed by tumour cells and a limited number of normal cell types that escape tolerance mechanisms (tumour-associated antigens (TAAs)). These peptides arise from different sources and by different mechanisms during carcinogenesis. As cells age, they can acquire somatic mutations or chromosomal aberrations leading to changes in signalling, gene expression and epigenetics and, ultimately, to either cell death (by apoptosis or immunosurveillance) or transformation (immune escape). All of these processes can give rise to TSAs and/or TAAs. **a** | Somatic mutations alone can cause significant changes in the encoded protein to become presented and recognized as a neoantigen by immune cells. **b** | Changes in the signalling and expression of unusual transcription factors (TFs) can lead to the activation of genes involved in gametogenesis, embryogenesis and fetal tissue development, which are normally strictly repressed and can act as oncofetal TAAs. Similarly, genes that are normally expressed in immune-privileged organs or tissues can be activated and targeted by immunosurveillance (such as cancer/testis TAAs). **c** | Chromosomal aberrations (translocations, deletions and insertions) can lead to gene fusions with normally inactive parts of the genome (for example, various human endogenous retroviral (HERV) sequences (SINE, LINE, other HERVs and transposable elements)) and give rise to novel untolerized translation products. **d** | Recently, in the wake of ribosome profiling data, alternative and aberrant translational products (such as defective ribosomal products (DRiPs)) are proposed to be a substantial source of TSAs and TAAs. Changes in ribosomal proteins, translation factors and related signalling pathways can impact the fundamental processes of translation initiation, elongation and termination, leading to proteome-wide changes. Along with changes in RNA processing (such as splicing), novel translation products (translation from 5′ or 3′ untranslated regions (UTRs), intronic and intergenic regions, initiation on alternative start codons, frameshifting and stop codon read-through) make major contributions to the pool of TSAs and TAAs. EIF, eukaryotic translation initiation factor; IRES, internal ribosome entry site; m^6A, N^6-methyladenosine; m^7G, 7-methylguanosine; Me, methylation; PABP, poly(A)-binding protein.

are inherently stressed owing to their unprogrammed proliferation, resulting in inadequate blood supply and compromised lymphatic draining, which leads to hypoxia, nutrient deprivation and chronic toxin exposure. Under these conditions, normal cells inhibit cap-dependent translation and block cell division to await more propitious conditions. Cancer cells, however, are selected to proliferate, which results in their accumulation of genetic and epigenetic alterations that override normal translational control, enhancing non-canonical translation pathways.

To date, only a handful of studies have used Ribo-Seq to characterize tumour cell translation[75,76]. In a mouse carcinogenesis model, EIF2A-dependent initiation greatly enhanced the translation of 5′ UTRs (a rich source of neopeptides) and was shown to play a central role in cancer progression[75]. Ribo-Seq of ten human hepatocellular carcinomas with paired normal adjacent hepatic tissue revealed that of 8,736 proteins translated fully 28% were tumour specific, with more than 50% arising from non-canonical translation[76]. Such non-canonical translation products, although much shorter, on average, than canonical proteins, are potentially a rich source of TSAs, given that DRiPs have preferential access to the MHC class I pathway. For hepatocellular carcinomas, this is not an academic point. A recent study failed to identify any TSAs among 100,000 peptides isolated from 20 human hepatocellular carcinomas[77]. As with nearly all published tumour immunopeptidome studies, peptides were identified against a database of only standard translation products. It is likely that potential TSAs were missed by not accounting for non-canonical translation.

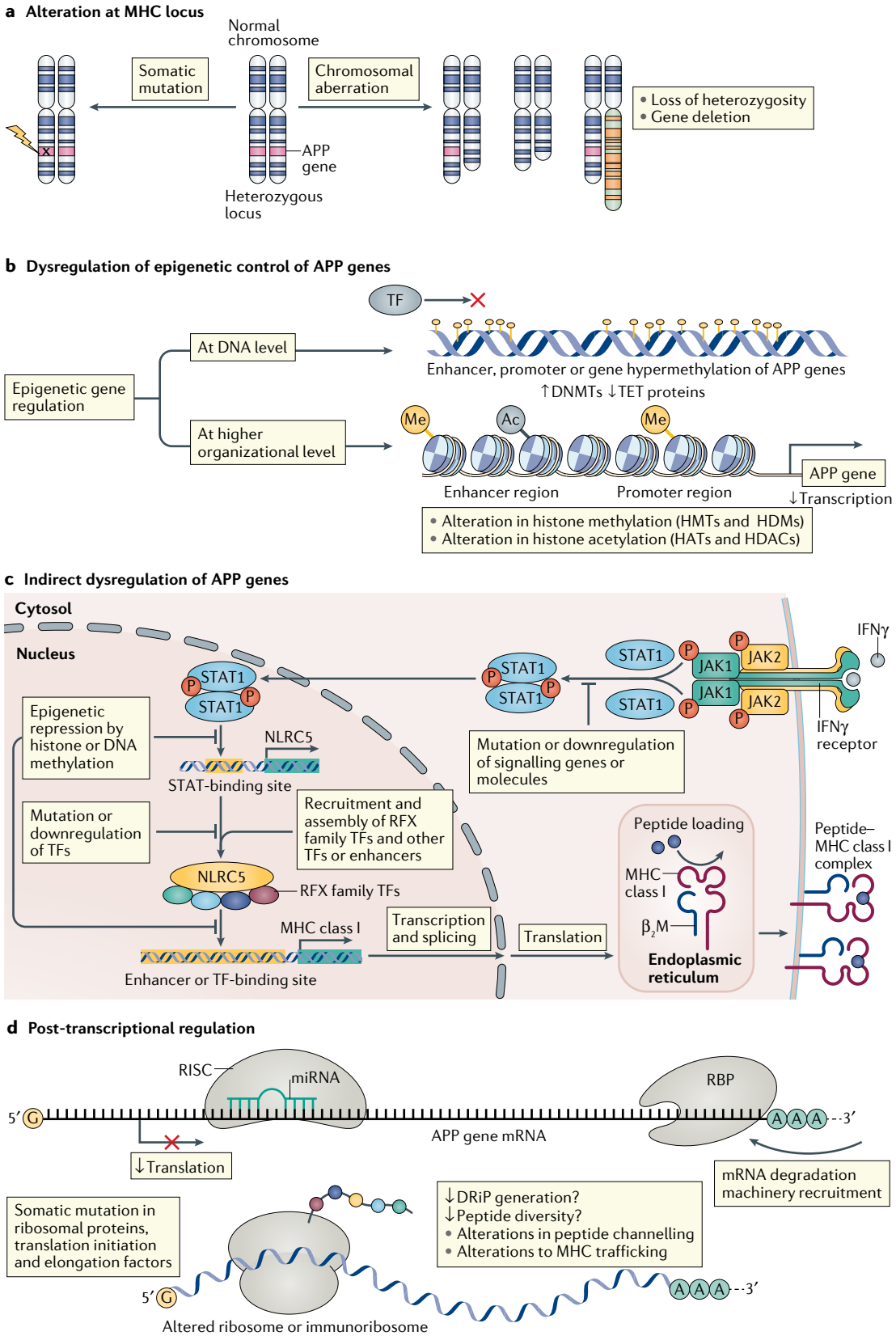
Negative selection
Also known as clonal deletion. The process by which developing lymphocytes expressing potentially autoreactive antigen-specific receptors are induced to undergo apoptosis in the thymus.

Mining translatomes and transcriptomes. The size of the database used to match mass spectrometry-detected peptides is a critical aspect of immunopeptidome studies. Larger databases, although able to identify more peptides, strain the available software and significantly increase the false discovery rate. The optimal database includes all possible tumour translation products that are not expressed by tolerizing normal cells. This strategy was used by a recent study that created a potential translatome for each tumour based on its transcriptome[78]. Generating a custom transcriptome for each tumour is important as alternative splicing is widespread in cancer cells[79]. Each library was narrowed by deleting mRNA sequences present in medullary thymic epithelial cells, which express the most diverse array of mRNAs of any known mammalian cell to enable negative selection of self-reactive T cells[80]. The remaining cancer-specific transcripts were divided into overlapping 33-mer nucleotide sequences translated in each of three potential ORFs. The most promising potential peptides were identified by the absence of both the peptide from the annotated mouse or human proteome and the corresponding mRNA from the normal tissue transcriptome.

For two mouse tumours, this approach identified ten and seven TSAs. Notably, only six of these peptides were tumour specific based on non-synonymous mutations, and only two peptides were present in standard translation products. The 15 other peptides were derived from various non-conventional sources: introns, intergenic sequences, UTR–exon junctions and, particularly, endogenous retroviral elements, which accounted for 9 out of 17 tumour-specific target peptides.

Extending this approach to four human leukaemias and three lung carcinomas revealed 22 TSAs, 19 of which derived from non-standard translation products. Of clinical relevance, leukaemias typically have a low mutational burden, and indeed none of the tumour-specific peptides were mutated, but each tumour had at least two peptides from aberrantly expressed genes. The alternatively translated peptides derive from introns, 3′ and 5′ UTRs, alternative reading frames, intergenic regions and endogenous retroviral elements. The latter are highly attractive, potentially conserved targets for immunotherapy as these sequences can be shared between individuals and their expression is often induced in cancer cells.

Ribo-Seq to the rescue. Combining total translation events from Ribo-Seq with detailed mass spectrometry-based immunopeptidomics shows that the immunopeptidome better correlates with Ribo-Seq than with RNA-sequencing data[81,82]. Ribo-Seq enabled the identification of more than 6,000 peptides encoded by 'novel unannotated ORFs' that were missed by standard proteome-based or transcriptome-based analyses. Ribo-Seq and immunopeptidomics analysis of 29 malignant and healthy samples enabled the identification of peptides requiring no processing (that is, the ORF was the exact MHC-bound peptide), as well as non-canonical ORF-encoded peptides with cancer-specific mutations[81]. It was estimated that 2% of the repertoire of peptides presented by cancer cells are encoded by these novel ORFs. As many of the ORFs are translated in a cancer-specific



manner, this should provide a rich source of potential targets for immunosurveillance, as it represents hundreds to thousands of peptides, given typical levels of HLA expression and the ability of many CD8+ T cell clones to kill cells expressing fewer than ten copies of a target peptide.

Future studies correlating the Ribo-Seq-determined translatome with the cancer-specific immunopeptidome

◀ Fig. 3 | **Mechanisms of cancer cell immunoevasion.** As normal cells accumulate somatic mutations and chromosomal aberrations during transformation, they are subject to immunosurveillance. Stress and selective pressure from the immune system can drive evolution and evasion of cancer cells. Immunoevasion is a major step that tumour cells must undergo to avoid immune-mediated death. This is frequently achieved by modulating the antigen-processing and presentation (APP) pathway. **a** | Somatic mutation and chromosomal aberrations — that is, genome instability itself — are often a major cause of diminished or fully abrogated APP in cancer cells. Somatic mutations (missense or nonsense mutations), loss of heterozygosity, frameshifting and other types of genetic alterations are commonly observed, especially in and around the MHC locus on chromosome 6. **b** | Tumour cell dysregulation of epigenetic control can also greatly affect APP machinery. This can be mediated by direct modification of DNA, for example by altered DNA methylation. Additionally, genes involved in APP can be regulated at the level of chromosome organization by altering the histone methylation (Me) and acetylation (Ac) status. **c** | Immunoevasion need not affect APP genes directly; in fact, upstream regulatory and signalling pathways are often disrupted instead. Somatic mutation and other genetic events often affect key genes in signalling pathways, which normally promote APP; for example, the interferon-γ (IFNγ)–Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling axis. DNA regulatory elements or histones engaged in regulating transcription factors (TFs) that control expression of the APP machinery are also often targeted by aberrant methylation, resulting in wholescale downregulation of APP. **d** | Post-transcriptional regulation is a common immunoevasion strategy. APP can be affected at the level of mRNA stability by microRNA (miRNA)-mediated silencing or by RNA binding proteins (RBPs) facilitating the degradation of specific mRNAs. Altered translation may also be responsible: somatic mutations in ribosomal proteins and translation factors are commonly found in many types of malignancies. The importance of these changes to translation machinery is not yet fully understood; however, even slight changes to the complex translation machinery can have far-reaching effects on the proteome and, more importantly, on the cell's immunopeptidome, for example through generating defective ribosomal products (DRiPs), peptide diversity and peptide channelling. β_2M, β_2-microglobulin; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase; NLRC5, NLR family, CARD-containing protein 5; P, phosphorylation; RISC, RNA-induced silencing complex; TET, ten–eleven translocation proteins.

should provide unique insight into the relationship between peptide expression and various aspects of translation, including start codon usage, ribosome pausing and the efficiency of peptide generation per translation event. Translation dysregulation in cancer cells may also impact ribosome quality control pathways, which are an important source of antigenic peptides[83]. We note that transcriptomes and translatomes are typically generated from cytoplasmic ribosomes and mRNA (often only polyadenylated RNA). This potentially misses cytoplasmic translation of microRNAs and all nuclear translation[48], both of which also may be important TSA sources.

Impact of radiation and chemotherapy. It is also important to examine how radiation and chemotherapy – often standard treatments prior to immunotherapies – alter the translatome and the immunopeptidome. Radiation induces multiple waves of enhanced peptide expression, including peptides arising from rapid degradation of damaged proteins (early retirees) and then DRiP-derived peptides (including novel peptides) as cells upregulate translation in the recovery phase[84]. Sub-toxic doses of radiation can also induce expression of APP machinery, perhaps increasing the peptide repertoire[85]. Doxorubicin was recently shown to alter the immunopeptidome of both mouse and human cells[86]. Notably, there was poor correlation between abundance changes in peptides and their corresponding source proteins, consistent with a major contribution from DRiPs. Moreover, many doxorubicin-induced peptides could not be matched to the cellular proteome, emphasizing the importance of using the translatome to identify potential tumour peptides.

Proteasome-mediated peptide splicing. Investigating how widely spaced mutations in a gene encoding a TSA could abrogate its antigenicity led to the discovery that antigenic peptides can be generated by post-translational splicing[87]. Soon thereafter, peptide splicing was shown to occur in proteasomes as a transpeptidation event catalysed by the proteasome[88].

Initial studies reported that up to 30% of MHC class I-associated peptides by diversity and 25% by mass were created by splicing[89], with a similar fraction independently reported by another group[90], who also identified a large number of peptides derived from splicing between different gene products ('*trans*-splicing'), which on a molar basis seems nearly impossible except for viral infections in which a large fraction of translation (and DRiPs) is devoted to a very limited number of viral proteins. However, these numbers are highly controversial, as several studies have concluded that 90% or more of the purported spliced peptidome results from misidentification[91–93]. Identifying spliced peptides is easier and more precise when using a smaller potential peptidome. Indeed, a study that identified spliced viral peptides estimated that the overall splicing rate of viral peptides was around 1% (REF.[94]). Spliced peptides could be relevant for immunotherapy even at low frequencies, if splicing generates MHC class I binding peptides from non-binding peptides with tumour-specific mutations or if splicing occurs in a tumour-specific manner[95].

The great escape: immunoevasion

Whereas TSA identification is critical for vaccination and adoptive T cell therapies, immune checkpoint inhibitor therapy aims to unleash the entire T cell repertoire that can destroy malignant cells. Typically, the relevant T cells and cognate peptides remain undefined. However, it is increasingly evident that successful therapy with immune checkpoint inhibitors depends on continued tumour expression of MHC class I molecules[96,97]. Indeed, perhaps the most compelling historical evidence for the existence of human tumour immunosurveillance is the high frequency of tumours with diminished MHC class I expression[98]. Viruses, particularly persistent viruses, also inhibit MHC class I expression for immunoevasion. But whereas viruses can gradually evolve to optimize genes that specifically target various components of the MHC class I processing system, tumours are on a much tighter schedule.

Consequently, tumour cells evade immunity by modulating existing cellular pathways and mutating genes that influence APP. Clear themes have emerged in the past two decades regarding cancer immunoevasion strategies (FIG. 3). Tumours can functionally or physically inactivate genes encoding antigen-processing components ('hard' mutations or lesions) or regulate

their expression epigenetically or through other cellular processes ('soft' mutations or lesions).

Epigenetic dysregulation. Antigen processing begins with the transcription of pathway-relevant genes, in which DNA methylation can control gene expression. As an example of soft alterations, DNA hypermethylation-induced suppression of APP gene transcription has been characterized in several tumours, including oesophageal squamous cell carcinoma[99,100], gastric cancer[101] and colorectal cancer[102]. More generally, a comprehensive omics study[103] of 22 tumour types revealed that a wide variety of tumours with chromosomal instability are paradoxically less immunogenic despite their high mutational load. This is in part due to hypermethylation of APP genes and, consequently, diminished transcription, even in the face of increased gene copy number. DNA methyltransferase inhibitors may, therefore, be a useful adjunct to enhance MHC class I expression during immunotherapy[101,104].

Histone acetylation also plays a key role in APP gene transcription. The balance of histone acetyltransferases and histone deacetylases (HDACs) is typically altered in cancer cells, in part to enable immunoevasion. HDAC-mediated MHC class I downregulation, originally described in adenovirus type 12 transformed cells, is now well characterized, even in the presence of MHC class I transcriptional activators such as nuclear factor-κB (NF-κB)[105]. HDAC inhibitors are reported to restore MHC class I surface expression in various cancer cell types[106–108]. Modulating HDAC activity changes the expression of many non-APP genes, but this can potentially enhance immunotherapy. For example, in melanoma cells, blocking HDAC6 enhanced the transcription of not only APP genes but also TSA genes, such as gp100 and MART1, whose antigenic peptides are defined immunotherapy targets. Initial clinical trials suggest that HDAC inhibitors can induce MHC class I expression in tumours[109]. HDAC inhibitors can also induce the expression of PDL1, so the interplay between these small molecules and immune checkpoint inhibitors is under active investigation[110–112].

Epigenetic dysregulation within cancer cells is a double-edged sword. The abnormal activation of genes (such as cancer/testis antigens and endogenous retroelements) can provide critical TSAs for T cell control and immunotherapy[78,113] (FIG. 2). Conversely, tumours can aberrantly express epigenetic regulators that downregulate APP gene expression. For example, the early embryonic transcription factor DUX4, typically absent in adult tissues, is re-expressed by numerous solid tumours[114], in which it represses MHC class I surface expression by interfering with IFNγ-mediated immune signalling. Presumably, this contributes to the association of DUX4 expression with resistance to immune checkpoint inhibitor therapy.

Similarly, the broadly acting histone methyltransferase Polycomb repressive complex 2 (PCR2) was recently reported to repress APP gene expression in various cancer types, including small cell lung cancer and neuroendocrine tumours[115]. Inhibitors targeting EZH2, the catalytic core of PCR2, restored surface MHC class I levels, suggesting potential synergy with immune checkpoint inhibitors. Mutations in EZH2 that enhance enzymatic activity are highly enriched in a subset of patients with B cell lymphoma[116]. A recent genetic/phenotypic comparative analysis of 347 primary diffuse large B cell tumours[117] revealed a highly significant correlation between these EZH2 activating mutations and low levels of MHC class I and II surface expression, suggesting their potential synergy with immune checkpoint inhibitor treatment for these lymphomas[118].

Manipulating signalling and transcription of APP genes. As might be expected, tumours also suppress APP gene expression by physically or functionally downregulating MHC class I transcription factors and upstream signalling pathways. APP genes are induced acutely via IFNγ–Janus kinase (JAK)–signal transducer and activator of transcription (STAT) activation and constitutively via RFX family proteins working in conjunction with the scaffolding protein NLRC5 (see REF.[119] for a review). Each pathway component can be manipulated by tumours for immunoevasion. Several elegant genome-wide CRISPR screens independently confirmed the importance of these pathways. An in vivo screen identified loss of *STAT1*, *JAK1*, *JAK2*, *IFNGR2* and *IFNGR1* for promoting outgrowth of B16 melanoma tumours and identified tyrosine-protein phosphatase non-receptor type 2 (PTPN2) as an important regulator of IFNγ-mediated signalling, antigen presentation and success from immune checkpoint blockade[120]. A tumour cell and T cell co-culture screen found that loss of *APLNR* enables melanoma resistance to $CD8^+$ T cell killing[121]. APLNR directly associates with JAK1 to enhance IFNγ signalling. Inactivating *APLNR* mutations were identified in patients resistant to immune checkpoint inhibitors, supporting its clinical relevance. Such co-culture screens also revealed that loss of the SWI/SNF chromatin remodelling complex enhances T cell-mediated clearance of tumour cells by improving IFNγ pathway signalling[122]. Indeed, mutations in JAK and STAT genes themselves are common in primary tumours and tumours resistant to immune checkpoint therapy[97,123,124], highlighting the importance of upstream signalling on APP gene repression in cancer immunoevasion.

At the transcriptional level, NLRC5 scaffolds the RFX family of transcription factors at promoters for MHC class I genes and other APP genes in nearly all nucleated cells. Accordingly, tumour cells frequently display aberrant hypermethylation, copy number loss and mutation of *NLRC5*, which correlates with poor outcomes in multiple cancers[125]. *NLRC5* inactivation may be a common initiating event in cancer immune escape as multiple stem cell types use mechanisms to reduce NLRC5 expression to enable immunoevasion[126].

Targeting APP genes directly. Tumour cells also exploit brute-force mutations to impair dedicated APP gene product expression or function (hard alterations). Classical HLA class I and II genes themselves, owing to extreme polymorphism, are notoriously difficult to analyse from a bioinformatics perspective, although sequencing-based analyses suggest direct mutations are

fairly low when analysed across cancers; mutations have been identified in ~3% of patients for HLA-A, HLA-B and HLA-C genes[127] and in ~1.8% for β_2-microglobulin (β_2M)[128]. This varies considerably across cancer types. For example, 90% of cervical carcinomas have loss-of-function mutations in HLA-A, HLA-B, HLA-C or β_2M genes[129], whereas 92% of colorectal carcinomas with microsatellite instability exhibit genetic alterations in APP genes[130]. Mining sequencing data for more than 10,000 tumours revealed that mutations in HLA-A, HLA-B, HLA-C and β_2M are associated with increased predicted neoantigen burdens[131], consistent with immunoevasion. As immunotherapy advances, it will be important to consider heterogeneity within cancers. For example, whereas the overall mutation rate for HLA-B in diffuse large B cell lymphoma is 21.6% (REF.[132]), it reaches 62% in a subset of tumours with aberrant B cell receptor signalling and NF-κB dependence.

Loss of MHC class I need not be biallelic; up to ~40% of non-small cell lung cancers were identified as displaying loss of heterozygosity in classical MHC class I alleles[133]. On a similar note, patients with more diverse HLA allomorphs respond better to immune checkpoint inhibitors, which is consistent with presenting a greater variety of TSAs[134,135]. Immune evasion can even be achieved via selection against oncogenic mutations that create immunogenic neopeptides[136].

Tumour cells also exploit microRNAs (miRNAs) to target APP gene transcripts. In colorectal carcinomas, miR-27a targets the peptide loading complex chaperone calreticulin to compromise MHC class I peptide presentation[137], although calreticulin downregulation has other effects that impact carcinogenesis, which complicates interpretation[138]. Many additional examples of miRNA-mediated regulation of APP genes are summarized in REF.[139]. RNA-binding proteins add an additional layer of control at the transcript level. In melanomas, RNA-binding protein MEX3B, which is a ubiquitin E3 ligase, binds the 3′ UTR of HLA-A mRNA promoting its degradation[140]. Previously, the related E3 ligase MEX3C was reported to degrade HLA-A2 mRNAs[141].

Immunoribosomes. As discussed above, translational dysregulation is a hallmark of cancer. Ribosomes possess 80 core integrated ribosomal proteins, and mutation of ribosomal proteins is remarkably frequent across malignancies[142,143], with 11.7% (1,272/10,845) of human tumours exhibiting a double deletion of at least one ribosomal protein gene and 8,910 tumours with at least a single deletion of a ribosomal protein gene. Knockdown of expression of each ribosomal protein individually revealed that 14 ribosomal proteins modulate the presentation of specific peptides or globally alter MHC class I expression in an allomorph-specific manner[144]. Detailed examination of several ribosomal protein knockdowns revealed surprisingly minor effects on overall gene expression, suggesting a more direct effect on peptide generation than might be expected. Depletion of 40S ribosomal protein S28 (RPS28) enhanced $CD8^+$ T cell killing of melanoma cells, establishing the relevance of ribosomal protein mutations to immunosurveillance. These findings dovetail with the genome-wide CRISPR screen-based identification of 60S ribosomal protein L23 (RPL23) as a negative regulator of $CD8^+$ T cell killing of melanoma cells[121]. Together, these findings support the long-standing immunoribosome hypothesis[145], which proposes that a subset of ribosomes are particularly efficient at generating MHC class I peptides[146] and suggests that immunoribosomes are exploited by tumours for immunoevasion.

Proteases. Proteasomes exist in multiple configurations, with various permutations of the three catalytic subunits, which each have both a constitutive and a cytokine-inducible form. Further, proteasomes can function without or with three different types of regulatory adaptor complexes at each end, with further heterogeneity of the regulators. Alterations in proteasome complex composition are well known to alter, both positively and negatively, the generation of given peptides. Changes in the gene expression of these components, as well as *ERAP1* (which trims the N termini of proteasome-generated peptides in the endoplasmic reticulum), are associated with alterations in immune-mediated outcomes in human cancers[147–149]. A detailed study of an immunodominant human melanoma peptide showed that coordinate induction of the proteasome β2 immunosubunit PA28 activator and ERAP1 combined to destroy the peptide[150]. Remarkably, in mouse tumours, expression of the REGγ proteasome regulator enables tumour immunoevasion by enhancing nuclear degradation of peptides synthesized in the nucleus from pre-mRNAs[151].

MHC class I mistrafficking. Relatively little attention has been paid to the contribution of post-translational regulation of MHC class I and other APP components to immunoevasion. Although some general mechanisms of MHC class I recycling and degradation have been established[152], much remains to be elucidated. Indeed, virtually nothing is known about how cell surface MHC class I is downregulated at this level in cancers. Cathepsin G, which can be secreted by neutrophils, can modulate MHC class I expression levels on tumour cells[153,154], although its direct relevance to cancer immunosurveillance and immunoevasion remains to be established. In melanoma cells, oncogenic $BRAF^{V600E}$ enhances HLA-A2 internalization and degradation by phosphorylating a highly conserved serine residue in the HLA-A2 cytoplasmic domain, reducing $CD8^+$ T cell recognition[155]. This mutation is present in nearly 25% of melanomas, raising the question of how broadly it acts on other HLA allomorphs and in other tumours. Importantly, a recent report details how MHC class I complexes are selectively routed to autophagy pathways in pancreatic ductal adenocarcinoma[156]. This process relies on the autophagy receptor NBR1 and can be pharmacologically targeted to enhance surface MHC class I levels and improve checkpoint blockade-mediated control over tumours. Similarly to virus-mediated immunoevasion strategies, there are clearly numerous pathways used to alter post-translational localization of MHC class I molecules; we have likely only begun to scratch the surface.

CRISPR-mediated forward genetic screens will be essential tools in fully elucidating MHC class I dynamics

beyond transcription and peptide loading. A recent study uncovered dozens of novel regulators of MHC class I through unbiased screening in diffuse large B cell lymphoma. Whereas some genes clearly affect transcription, quality control and folding in the endoplasmic reticulum, and as yet undescribed cellular processes, there are numerous candidates for trafficking factors as well[118]. More than ten genes involved in clathrin-mediated endocytosis and endolysosomal targeting were identified as negative regulators of cell-surface MHC class I levels, presumably by altering internalization, degradation or recycling. Further study of these types of genes and trafficking-based regulation of MHC class I is clearly warranted. Finally, we have little appreciation of how mechanisms of immune evasion synergize (for example, missense mutations in HLA genes in combination with altered cellular trafficking or protein folding capacity), which will require a more comprehensive understanding of MHC class I biogenesis, trafficking and degradation.

Future perspectives

Characterization of immunotherapeutic CD8$^+$ T cell responses and immunoevasion in humans has been performed, with few exceptions, in ignorance of the actual targeted tumour-specific peptides. There is a desperate need for a more detailed characterization of T cell responses at the level of target peptides. Technical advances in all aspects of omics now enable the correlation of the immunopeptidome with the relevant -omes — transcriptome, translatome or degradome (and sub-omes) — with the goal of a global understanding of immunopeptidome biogenesis, which will enable accurate prediction of potential tumour-specific peptides in individual patients.

We would be remiss in failing to mention that CD8$^+$ T cells function in immunosurveillance in conjunction with other immune cells. Natural killer cells constrain MHC class I-mediated escape by lysing cells with low MHC class I levels. Other immune cells (including CD4$^+$ T cells, B cells, dendritic cells and macrophages) have less direct, but still potentially potent, antitumour (and protumour) activity. Optimizing immunotherapy will require modulating multiple adaptive and innate immune cell populations.

Characterization of immunity at the level of individual tumour-specific peptides is crucial to both understanding and manipulating therapeutic T cell responses and immunoevasion. Indeed, oncogenesis-related changes to antigen presentation may affect specific peptides without globally altering MHC class I levels or even levels of the relevant presenting MHC class I allomorph. Thus, current understanding of immunoevasion may be limited to the most extreme cases of immunoediting, with far more subtle alterations in the generation of tumour-specific peptides falling under the radar. The pay-off for these efforts will not be limited to cancer immunotherapy; rather, the knowledge will apply to all aspects of MHC class I immunosurveillance, including infections, autoimmunity and tissue transplantation, and, more broadly, to central questions regarding protein synthesis, degradation and trafficking. So much remains to be discovered!

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