SUPPLEMENTARY NOTE

In silico simulation of tumours

Here we provide an overview of the simulation steps implementing tumour growth and mutations accumulation under negative selection, as described in the Online Methods and illustrated on Fig. 1a. First, a single progenitor cell is defined that already carries a set of mutations providing it with sufficient growth/survival advantage to outgrow a normal cell population. All of the cell's mutations have a unique identifier, and that cell has an intrinsic immunogenicity value determined by its mutations (represented by the colour of a schematic cell in Fig. 1a). Starting from this single-cell tumour, in each simulation step a cell in the population is selected, and that cell undergoes one of three possible life events:

– *Proliferation*: The cell divides and gives birth to two daughter cells. These cells carry all mutations and information contained in the mother cell, but also acquire new mutations. For each newly generated mutation, it is randomly decided whether the mutation is antigenic and then an antigenicity value is assigned to it from an exponential prior distribution. An example is highlighted in the middle panel of Fig. 1a, where two surviving offspring are created, which carry all the mutations of the mother cell, and accumulate new unique neutral (grey stars) and antigenic (denoted by red 'n's) mutations in the process, the latter increasing their immunogenicity by value A_j.

– *Death*: The cell dies and is removed from the population.

– *Waiting:* No proliferation or death event happens; the cell is not altered in any way.

The probability of each event is defined by the cell's proliferation and death rate (b and d_i) as $b/(b + d_{max})$, $d_i/(b + d_{max})$ and $1 – (b+d_i)/(b + d_{max})$, respectively.

In proliferation events, each daughter cell gains N_m new, independent mutations, where N_m is

sampled from a Poisson distribution with parameter μ, the cell's mutation rate. Antigenicity is randomly assigned to newly generated mutations according to the antigen production rate, p_a – the probability that a newly generated mutation has immunogenic properties –, and the prior distribution of mutation antigenicities (shown in the rightmost panel of Fig. 1a).

The above step of randomly selecting a cell and one of the three possible events is repeated until the tumour reaches a predefined population size (representing the tumour reaching a clinically detectable size, for simplification we set it to 10^5 cells) or sufficiently long time elapsed without tumour establishment (corresponding to no cancer formation in the patient's lifetime, set to 300 time units).

Constant population size model

To compare with our growing tumour model, we also simulated a fixed-size tumour population using a death-birth Moran process using the following steps. First, 10^4 cells (matching the final population in Extended Data Fig. 2a) are initiated with no mutations. In each iteration of the simulation, two steps are executed:

- Death: A cell is selected based on a probability weighted by its death rate (antigenic cells are more likely selected) and is removed from the population.

- Proliferation: A cell is chosen randomly (all cells with equal chances) to divide and replace the dead cell. During division, both daughter cells accumulate mutations and antigenicity in the same manner as in the expanding population model.

Birth and death rates are defined as in the growing tumour model: all cells have the same birth rate, while their death rate is computed from the summed antigenicity of their mutations and the

selection coefficient, s. Tumours are simulated up to 50,000 iterations to achieve a similar number of cell divisions as in the main model, and analysed analogously to growing tumours (Extended Data Fig. 2d-f).

Choice of simulation parameters

Parameter values of the model were chosen to be easily interpretable in terms of growth dynamics and to re-establish experimentally observed quantities, such as mutation burden (Extended Data Fig. 7). All rate values in the simulation are scaled to the rate of proliferation/birth events (which is 1), consequently the death rate of 0.9 or 1.2 correspond to overall population growth or decline, respectively, and the mutation rate, μ, represents the number of new mutations gained per cell division.

The probability of proliferation, death and waiting events (see steps above and Fig. 1a) for a cell with birth rate b and death rate d_i were $b/(b + d_{max})$, $d_i/(b + d_{max})$ and $1 – (b+d_i)/(b + d_{max})$, respectively. Due to the linear connection between the cumulated antigenicity of neoantigen harboured by a cell, and its death rate, d_{max} was computed from the maximum summed antigenicity of simultaneous antigens in any cell in the population. Non-immunogenic basal death rate, d_b=0.1, was chosen to represent a tumour population that would rapidly expand in the absence of immune surveillance. Together with the fixed birth rate, b=1, this rate summarises how well the first founding cell of the tumour is adopted to the environment. Consequently, a higher basal death means a less optimised founding cell, potentially including numerous previously acquired immunogenic mutations, whose off-spring are more sensitive to immune-induced fitness decrease. Therefore, we expect an increase in d_b to affect results similarly as increased selection strength, s. We explored this dependency on the basal death

rate in Extended Data Fig. 8. Increased non-immunogenic death rate led to an increased proportion of detectable tumours developing immune-escape (Extended Data Fig. 8a), and also increased antigen burden, due to a combined effect of increased effective mutation rate (μ/ß, dependent on *d*) and immune-escape counter-acting antigen depletion. The VAF distribution of tumours showed increased depletion of neoantigens as a function of d_b, but this signal disappeared at d_b = 0.9 as detectable tumours were exclusively immune-escaped and neutrally evolving. In summary, we confirmed that increased basal death rate at a constant selection effectively showed the same non-linear change in immunological phenotype as increased selection strength at constant d_b.

Mutation rate values (μ and p_a) were fixed for the entire population in the beginning of each simulation. To correspond to tumour samples that were sequenced using whole-exome sequencing, we set the mutation rate relatively low, $\mu = 1$, with the exception of hyper-mutated cancer, which had a higher mutation rate, $\mu = 10$. We used TCGA CRC samples to establish these parameter values (as our secondary dataset was also of CRC samples). Low and high mutation rates were chosen to match the amount of detected subclonal mutations in MSS and MMR cases, respectively (Extended Data Fig. 7a&b). The choice of $\mu = 1$ effectively meant that not all cell divisions introduced new (exonic) mutation in the daughter cells.

To measure a baseline value for p_a, we generated a synthetic tumour mutation datasets matching the mutation numbers and composition (trinucleotide composition and mutational signatures) and HLA haplotypes of TCGA MSS colon tumours (100 tumours per patient, the arising distribution is shown in Extended Data Fig. 7c). The value of neoantigen probability, p_a, was set to 0.075 based on the experimental observation that about 7.5% of missense mutations resulted in new peptides identified as neoantigens in this cohort. We used a similar cohort generated based on a combination of all CRC, UCEC and STAD samples, to evaluate subclonal and total proportional burden (in comparison to Fig. 5c), where we found that the synthetic

tumours maintained an average of 7.5% burden regardless of immune escape status and clonality (Extended Data Fig. 6f).

To confirm that the choice of p_a did not alter our qualitative predictions on VAF distribution, we also simulated tumours with neoantigen probability of 0.025 and 0.15, two extreme values of the distribution (Extended Data Fig. 7c). We found that the average signal of negative selection (Extended Data Fig. 9) – as measured in the neoantigen-VAF distribution – was highly similar to that obtained with different mutation-antigenicity thresholds (Extended Data Fig. 4), as decreased/increased antigen production rate effectively meant less/more neoantigens that would have high antigenicity and negative selection would efficiently act on. Additionally, as expected, we observed a more consistent (less deviation from the mean) distribution amongst tumours with the same parameters at high p_a, due to the higher number of negatively selected allowing for a high-confidence evaluation of the VAF distribution. Overall, the signal of negative selection is in good agreement with that obtained using p_a=0.075, but highlights that it is problematic to quantitatively measure selection (beyond a binary 'selection/no selection' choice) from the VAF distribution unless other parameters are known precisely.

Cell-antigenicity threshold (deciding which cells are labelled antigenic), T_c, was chosen to stratify cells that harbour a single neoantigen that falls in the top 10% of antigenicity values, or a combination of neoantigens that together account for the same immunogenicity. Note that T_c was only used for defining cell-immunogenicity during analysis (Fig. 1a) – during growth, the cells were subject to immune pressure directly computed from the sum of their neoantigens. The choice of cell-antigenicity threshold (T_c) shifted the observed composition of tumours similarly to selection strength, but did not alter the nature of the distribution (Extended Data Fig. 1a).

Alterations that induce immune escape are rare, because these can only arise from a very

limited set of exonic loci. We therefore set p_e to 10^{-6} for all immune escape alterations. The decrease in immunogenicity in passive evasion was set to 0.9. This decrease in antigenicity indirectly affected death, as we assumed that only a fraction of the total antigenicity of neoantigens harboured in the cell remained effective, and updated the total antigenicity value, $\sum A_j$, accordingly.

Limitations of the model

It is important to note that our model is a simplified representation of the tumour-immune evolutionary interaction. First, the model only included negatively selected and neutral mutations. Positive selection is not considered here, but could be readily modelled as counter-acting the increase in death rate evoked by antigenicity. The two components defining negative selection (selection strength, *s*, and mutation antigenicity, *A*) both represent a net quantity arising from a combination of processes, e.g. T-cell activation, efficiency of antigen presentation, apoptosis signals. Therefore different conditions inside and outside of tumour cells can be implicitly modelled by changing the distribution of antigenicity effects and selection.

Furthermore, we considered a growing population, where complete eradication of the tumour is possible. The possibility of tumour extinction, and the resulting 'sampling bias' of measuring only successfully established tumours, effectively strengthens the signal of neutral evolution observed (Fig. 1 & Extended Data Fig. 2a-c). Different dynamics arise if the tumour population is maintained despite negative selection, such as in a constant size model; for example, hyper-mutated tumours without immune escape become common in a constant population size model, and therein selection forces are altered by the background antigen accumulation (Extended Data Fig. 2d-f).

We also note that fitness effects of founder mutations – including historically clonal neoantigens – are not simulated explicitly, but captured in the basal death rate, d_b; we explored how properties of the founding cells influence dynamics of clonal expansion in Extended Data Fig. 8.

We chose this level of abstraction in the model to enable us to investigate evolutionary paradigms on a general level, without having to rely on precise parameterisation of many sub-processes. Therefore, while the reactions and parameters included in the model might not correspond to a single biological event, the model can provide a qualitative description of a high range of tumour-immune environments with appropriate choice of parameters. Furthermore, the model can be easily extended to account for further biological processes, in the same manner in which immune escape was included, to provide predictions on therapeutic interventions.

Further simulations of hyper-mutated cancers under selection

We observed that negative selection led to a highly constrained landscape of tumours and further explored how the balance of selection strength and mutation rate determine the phenotype of arising tumours (Extended Data Fig. 2). In hyper-mutated tumours consistent with MMR mutator phenotype (case (iii): μ=10), growing subclones rapidly become neoantigen-hot and become eradicated by the immune system (as demonstrated in Fig. 1e). At lower, but still elevated mutation rate (μ=6), simulations clearly separated into two groups: tumours where subclones of low antigenicity were strongly differentially selected (viable (Extended Data Fig. 2b&c), and non-viable tumours where mutation accumulation increased antigenicity above the tolerated threshold.

In near-neutral conditions (case (ii): s=-0.1; μ=10) hyper-mutated tumours were successfully established, leading to exceptionally high antigenicity and many high-frequency neoantigens. However, at further increased mutation rate (μ=100) even very weak selection on an individual

neoantigen was enough to cause tumour extinction by immune-editing (Extended Data Fig. 2a), highlighting that the balance of selection and the mutation rate, rather than selection alone, determines tumour growth. Furthermore, we note that such conditions (very high mutation rate and very weak selection) are highly unlikely to occur in real tumours, as a high mutation burden would likely cause increased immune infiltration and so increase the efficacy of immuno-editing (stronger negative selection).

We would like to emphasise that extinction is a special property of an expanding size population. In contrast, when the population size was held constant (using a death-birth Moran process), we found that without extinction, higher mutation rates are lead to every lineage in the tumour becoming antigenic and so differential selection between lineages becomes much less stringent. Therefore a constant size population shows a very broad range of dynamics (Extended Data Fig. 2d-f) masked by the expanding population in our tumour model.

Evaluating selection and mutation rate from sequencing data

We have previously shown that neutral subclone evolution leads to a characteristic distribution of subclonal mutations, whereby the number of mutations at frequency f ($M(f)$) is proportional to $1/f^2$ (or $1/f$ in the cumulative distribution)[9]. In this work, we explored separately how negative selection alters this pattern found in the VAF distribution of (i) all mutations found in a tumour ('overall' VAF distribution); and (ii) antigenic mutations as compared to the baseline of all mutations.

Under positive selection, an expanding clone carries passenger mutations within the clone to higher frequency than expected from the neutral expectation, and hence positive selection is evident from these passenger mutations at high frequency in the overall distribution[23]. Under

negative selection, a contracting clone has reduced opportunity to acquire passenger mutations and the frequency of the passengers inside the negatively-selected clone remains small (and difficult to detect), and so the VAF distribution is dominated by passengers that accrue in neutrally evolving clones. Correspondingly, in growing tumours under immune surveillance the VAF distribution for all mutations (grey lines in Fig. 4a&b) is dominated by neutral non-antigenic mutations and is not discernibly different from the neutral expectation. Furthermore, as this depletion is increased in more selective environments, the stronger the strength of negative selection, the more neutral-like the evolutionary dynamics become. This is in contrast to constant-size tumour populations, where the selection acting on passenger mutations co-occurring with neoantigens can lead to more diverse mutation spectra (Extended Data Fig. 2c&f).

On the other hand, the VAF distribution computed of antigen-associated mutations (red lines in Fig. 4a&b) shows a deviation from both the neutral expectation and the overall VAF distribution (which overlap). We want to reiterate though that, as demonstrated in Fig. 4 and S5, negative selection means very few neoantigens persist in the tumour and most of the antigens that persist are at *very low* VAF. For instance, in the tumour in Fig. 4b, neoantigens make up <0.5% of the total detectable mutations (~9/3000 mutations), despite almost 5% of all new mutations in the simulation having above-threshold antigenicity. Therefore, in practice, detecting negative selection on neoantigens in tumour sequencing data is problematic, since (i) there are expected to be too few subclonal neoantigens to resolve the distribution, and (ii) most antigens are at low VAF where the power to detect variants and accuracy of VAF measurement are both lowest. It is also important to note that typical neoantigen prediction methods might suffer from a high false positive rate (which we tried to counteract in evaluating real tumours by using the method by Luksza et al.[19]). False positive neoantigen calls, unlike true positives, will be equally distributed along the frequency spectrum, attenuating the detectable effect of negative selection.

We therefore included the effect of false positive calls on the detection power, as detailed in Methods and shown in Fig. 4c.

Previous work demonstrated that in the case of effectively-neutral subclonal dynamics, the effective mutation rate (μ/ß), defined as the ratio of the per-cell division mutation rate (μ) divided by the per-cell division proliferation rate (ß), is specified by the steepness of the cumulatively plotted 1/f VAF distribution that arises under neutral dynamics. Since we established that negative selection leads to effectively-neutral overall VAF distribution, this method can be validly applied to tumours experiencing negative selection. We therefore evaluated the slope of the cumulative VAF distribution as a function of inverse frequency (grey line of Fig. 4a&b) in antigen-hot (carrying high frequency neoantigens) tumours, since in these tumours all cells are continuously disadvantaged by selection. We found in Fig. 5b that stronger negative selection increased the effective mutation rate through an increase in the per-division death rate and a decrease in proliferation (ß) of all cells in clonally antigenic populations, leading to an increase of the ratio (μ/ß). We therefore argued that an increased baseline mutation rate in tumours (i.e. hyper-mutated phenotype) can give rise to positive feedback loop further increasing the perceived mutation rate: more mutations lead to more neoantigens, resulting in more selection-induced cell death that raises the effective mutation rate.